CHARLES UNIVERSITY IN PRAGUE

FACULTY OF PHARMACY IN HRADEC KRÁLOVÉ

Department of Pharmaceutical Chemistry and Drug Control

Metabolomic analysis of urine samples using ultra performance liquid chromatography and high resolution mass spectrometry

MASTER'S THESIS

Hradec Králové, 2012 Andrea Mrvová

ČESTNÉ PROHLÁŠENÍ

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Datum: 8. 4. 2012 Andrea Mrvová

I would like to thank to Prof. Seppo Auriola and his team, especially to Marko Lehtonen, MSc. and Pekka Keski-Rahkonen, MSc. for their valuable advices, willing help and great experiences from Kuopio.

Thanks to RNDr. Milan Mokrý, CSc. for his comments on my work.

Also I would like to thank to my family for their support during my studies.

ABSTRAKT

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Název diplomové práce:

Metabolomická analýza vzorků moči s využitím ultra vysokoúčinné kapalinové chromatografie a hmotnostní spektrometrie

Metabolomika se zabývá analýzou nízkomolekulárních metabolitů uvnitř biologického systému. Tato disciplína slibuje velký pokrok nejen v diagnostice onemocnění. Jako nejvýhodnější materiál pro analýzu jsou tělní tekutiny (např. moč, krevní sérum, sliny) a běžně používanými analytickými technikami jsou NMR, GC/MS and LC/MS. Metabolomická analýza poskytuje značné množství dat a jejich vyhodnocení je problematickým úkolem. Je proto snahou vyvinout systém, který zefektivní identifikaci. Záměrem této práce je nalezení optimálních podmínek pro metabolomickou analýzu a testování spolehlivosti počítačem asistovaného postupu detekce a identifikace látek. Práce je předstupněm následné snahy o objevení biomárkrů a definování rozdílů v metabolomické analýze moči zdravého člověka, pacienta trpícího hypertenzí, diabetem a dalšími onemocněními.

ABSTRACT

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Title of Thesis:

Metabolomic analysis of urine samples using ultra performance liquid chromatography and high resolution mass spectrometry

Metabolomics is inquiring into the analysis of small-molecule metabolites within biologic system. This branch promises great progress not only as a diagnostic tool for disease. As suitable objects for the analysis are bio-fluids (e.g. urine, serum, saliva) and as common analytical techniques that are employed for the metabolomic analysis are NMR, GC/MS and LC/MS. Metabolomic analysis provides a great number of data and it is a problematic task to go through it. There is an effort to design system of effective identification. The aim of this work is to find optimal conditions for metabolomic analysis and to test reliability of computer-assisted workflow for the detection and identification of compounds. This should be as the first stage to following effort to discover biomarkers and to define differences between the results of metabolomic analysis of urine sample obtained from health person and patients with hypertension, patients with diabetes and other diseases.

CONTENTS

1 INTRODUCTION

Nowadays we have highly precise medical examinations and methods of disease diagnosis. Instrumental medical examinations such as ECG, endoscopy, spirometry and many others are the most important points of the diagnosis but these methods are usually able to find manifestation of already developed process of disease. With laboratory examination methods it is possible to find out minimal differences between the levels of defined compounds or changes of the sample composition.

Even if the medicine of these times is high quality and it is competent to cure majority of the patients still there are many examples of medicine helplessness and it is obvious that it is necessary to find new methods of diagnosis. Few of the reasons are incidence of new infectious agents, increased antibiotic resistance, increased incidence of cancer, age related neurodegenerative conditions, insulin resistance and obesity.

Profile of metabolomics has many examples of promising use as a diagnostic method. Successes achieved in studies of diabetes, Alzheimer's disease, osteoarthritis and various renal diseases are published in many articles and literature. Metabolomics as biomarkers pose a kind of prospect of early diagnosis and so better chance of curing.

2 OBJECTIVE OF THE THESIS

Analysis of urine sample using ultra performance liquid chromatography and high resolution mass spectrometry provides great number of data. Mass Hunter workstation software makes identification process more effective and facilitates processing of all data.

Before the beginning of looking for some differences between urine of healthy person and urine of the patient and attempt to find some biomarkers and metabolomics changes it is necessary to optimize settings of the system and to be sure automatic identification is reliable.

Objective of this work should be testing of the automatic system infallibility and to find out the best measuring condition for the detection of broad number of compounds. To do so two types of columns were used and results of the identification of urine samples were compared with amino acids standard.

3 TEORETICAL PART

3.1 METABOLOMICS

Metabolomics is a platform whose aim is the analysis of endogenous low-molecular weight metabolites as components of biological systems (lipids, amino acids, peptides, nucleic acids, organic acids, vitamins, thiols, carbohydrates). Analysis of these compounds is becoming important as a characterization of potential biomarkers for disease diagnosis and pharmaceutical areas. Because metabolite changes are the first indicator of disease and traditional markers of disease are not region-specific and they are increasing only when serious disease is present. More sensitive biomarkers and techniques to identify them can arrange earlier diagnosis of disease and better chance to cure patients. (1,2)

As the suitable object of the representative sample of organism are bio-fluids. One of the benefits of bio-fluids (serum, urine, saliva, cerebrospinal fluid) is these can be used for non-biopsy tests and also that bio-fluids associate the majority of the whole system of organism. But also tissues are used for metabolomic studies, in vivo, tissues can e.g. act as a model of liver function and their reaction on different stimulus or as a model of tumor behavior. (1)

Metabolomics has demonstrated promise in many areas such as toxicology, system biology, clinical diagnosis, drug development, diabetes, environmental stress, genetic manipulation, dietary habits, lifestyle effects (both benefit or adverse), and others. And because every kind of trustworthy help to predict potential disease or toxicity is generously supported there are still many other branches interested in and also many new methods of research are coming. Wide range of “omics” sciences are focused on the various levels of the organism from the studies of the structure, function and the processes of the cell to whole organism. (1)

As the main analytical techniques for studies of metabolomics are used nuclear magnetic resonance (NMR) spectroscopy and spectrometry techniques, mainly mass spectrometry (MS). These techniques of the analysis needs initial separation of the molecules of the sample. For this combination with gas

chromatography (GC) or liquid chromatography (LC) is used. Gas chromatography technique requires chemical derivation of the sample for analysis. As a liquid chromatography is nowadays used ultra-high-pressure LC increasingly. (2)

Thanks to these techniques we already have massive amounts of data and it is a great task to go through it and to find the effective way of data processing. The first point is to determinate what is normal biochemical process of the organism and to understand biochemical changes caused by disease. Next point is to identify the differences between the healthy organism, diseased organism and the organism with medical therapy. Aim is to find biomarkers of disease that is at the beginning and hidden, without any symptoms, and to aid human disease diagnosis because early treatment increases probability of recovery. (2)

In the future time is an effort to measure details of time-related fluctuation so any changes of the biochemical process of organism will be observed not only as a level but also its progress depending on time. (2)

3.2 MASS SPECTROMETRY

Mass spectrometry is powerful analytical technique that is served for generating ions from either inorganic or organic compounds, to differentiate these ions by their mass-to-charge ratio (m/z), and to detect them qualitatively by their m/z, abundance and subsequently to detect relative intensity of each ion. Mass spectrometry is highly sensitive method that is used to identify unknown molecules, to quantify molecules, to find out their structure and chemical properties. All of these functions with minimal sample consumption. (3)

First interpretation of mass spectrometry experiments started over a century ago. In the 19th century scientists wanted to gain some understanding of the nature of electricity. Joseph John Thomson measured the charge-to-mass ratio of electron and estimated electron‘s mass. He also asserted particles are construction components of all atoms. In 1906 Nobel Prize in Physics for the electron discovery was awarded to J. J. Thomson

and in 1907 Thomson published the book Corpuscular Theory of Matter. British chemist and physicist Francis William Aston worked as assistant of J. J. Thomson at the Cavendish Laboratory, Cambridge, on studies of positive rays. By his invention of the mass spectrograph he did the separation of the isotopes of neon and with this principle he discovered many naturally occurring isotopes and Whole Number Rule. In 1922 he received Nobel Prize in chemistry. (3,4)

In 1957 Holmes and Morell made the first connection of a gas chromatograph to a mass spectrometer (GC/MS). Chemical ionization (CI) was developed in 1966 by Munson and Field. In 1973 the first connection of mass spectrometer with high performance liquid chromatography was performed by Baldwin and McLafferty. MALDI (matrix-assisted laser desorption ionization) was developed by Hillenkamp and Karas in 1989. Koichi Tanaka and Kurt Wüthrich shared Nobel Prize in Chemistry for the development of methods for identification and structure analyses of biological macromolecules in 2002. Fenn developed electrospray ionization method. (4)

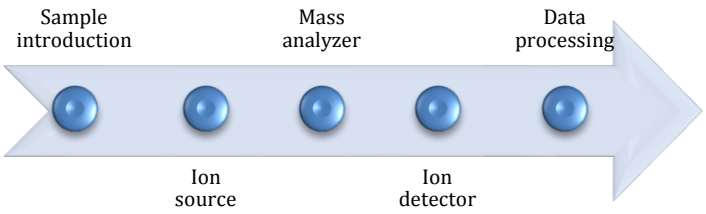
Nowadays mass spectrometry has multiple applications such as: the identification and structural analysis of the compounds, as GC or HPLC detector for quantitative analysis, analysis of very minute quantities of compounds or impurities, isotope representation (determination of age), in metabolomics, proteomics and lipidomics studies (identification of proteins, metabolites and lipids and their modifications), and mass spectrometric imaging. So there are many filed of research and industry where mass spectrometry is utilized in. Analysis of proteins, peptides, oligonucleotides and lipids, their identification de novo or confirm their sequence with the help of the database, post-translation modifications, macromolecular structure, number of disulphide bridges, enzyme formation and also reactions can be studied in biotechnology by MS. Also clinical application is significant, e.g. biomarkers discovery, various testing, and analysis of bio-fluids. Pharmaceutical application is mainly drug discovery, pharmacokinetics, and purity determination. Others are environmental application to detect industry pollution of nature and also application in food industry branch to study food contamination.

There are many other branches of mass spectrometry application and because of the continual development of the instrument and the implementation of new types of analyzers, e.g. triple-quadrupole or ion trap, introduction of new methods of ionization, e.g. electrospray or MALDI (matrix-assisted laser desorption ionization) and also financial availability more and more branches of research and industry start to use MS. (3)

3.2.1 FUNDAMENTALS OF MASS SPECTROMETRY

The basic components of all mass spectrometers are:

- Sample introduction device
- Ion source
- Mass analyzer
- Ion detector
- Computer for data processing



These parts are interrelated as a series of processes. (5)

3.2.1.1 Ion source

The main role of ion source is to convert molecules into ionized form. Because ions are formed with excess energy and also because of their charge, they can react with residual gas. To prevent this collision mass spectrometers are operated under high vacuum or at low pressure and the level of contaminants and atmospheric gases is minimized. (6,7)

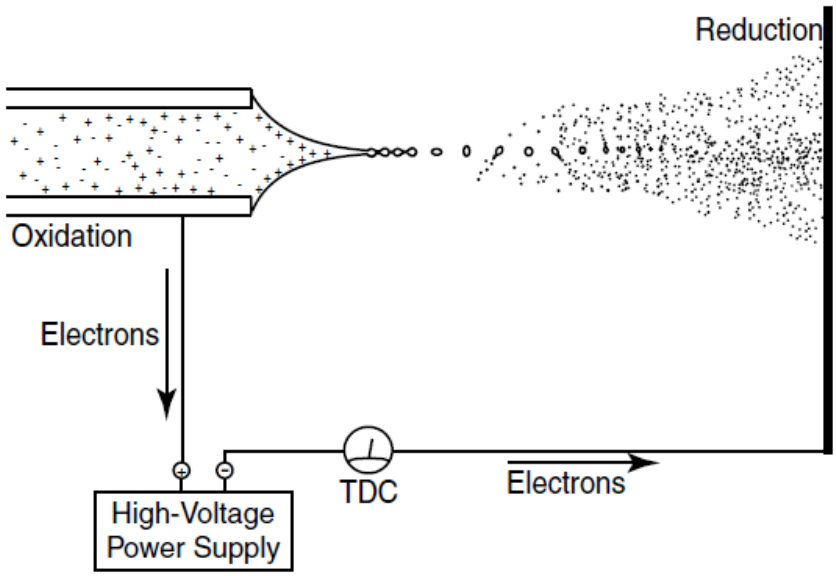
There are many ways of sample ionizations, e.g. electron ionization (EI, known as electron impact), electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionization (APPI), matrix-assisted laser desorption/ionization (MALDI), desorption electrospray ionization (DESI), direct analysis in real time (DART) or nanospray desorption electrospray ionization (nanoDESI). (7,8)

In further experiments electrospray ionization (ESI) was used. It is a kind of “soft” ionization technique by which molecules remain intact. ESI is a method of conversion of solution into gas-phase ions which can be detect by mass spectrometric means. Thanks to ESI it is possible to analyze also heat-labile compounds, high molecular weight compounds and many other samples that were previously not suitable for this type of analysis. (8)

Main steps of ESI are:

- Charged droplets production at ES capillary tip
- Solvent evaporation and so shrinkage of the droplets
- Disintegration of droplets and so producing of very small and highly charged droplets
- Mechanism of gas-phase ions production (8)

Figure 1: Scheme of electrospray ionization (ESI) describes formation of cone at the end point of capillary and evaporation and disintegration of charged droplets. (8)



Solution of the sample is leading from HPLC column into electrospray capillary which is ending in a very fine tip. High voltage is applied to the tip of capillary, both negative or positive charge can be applied (positive or negative ion mode) and this electric field causes formation of a dipolar layer at meniscus. Polarizability, dipole moments of the solvent molecules and rich presence of the same charges are the reasons of destabilization of meniscus.

A highly charged cone shaped (Taylor cone) liquid is formed and subsequently split into droplets enriched with positive (or negative) charge. Solvent of droplets is evaporated in evaporation chamber with assistance of nitrogen gas as nebulizing gas and because charges in the droplets are identical and they are getting closer together Columbic repulsion force causes disintegration of droplet into smaller. Evaporation and this process are repeated until each droplet is single charged molecule. Because proteins and peptides can be ionized at several points multiple charged ions can be present. (9-11)

3.2.1.2 Mass analyzer and detector

Mass analyzer separates ions under vacuum or at low pressure according to their mass-to-charge ratio (m/z) through magnetic or electric field. In this step there are also several methods and types of mass analyzers and each of these has some advantages and limitations. There are six basic types of mass analyzers: quadrupole, ion trap, time-of-flight (TOF), double focusing magnetic analyzer, orbitrap, ion cyclotron resonance (ICR). (6)

Table 1: Table shows various types of mass analyzers and principles of their separation method.

Type of analyzer	Symbol	Principle of separation
Electric sector	E or ESA	Kinetic energy
Magnetic sector	B	Momentum
Quadrupole	Q	m/z, trajectory stability
Ion trap	IT	m/z, resonance frequency
Time-of-flight	TOF	Velocity (flight time)
Fourier transform ion cyclotron resonance	FTICR	m/z, resonance frequency
Fourier transform orbitrap	FT-OT	m/z, resonance frequency

For this experiment time-of-flight (TOF) analyzer was used.

The flight time depends on mass-to-charge ratio and so it is unique for every type of ion. From the physical principle time-of-flight (TOF) may be the simplest way to perform mass spectrometric analysis. *Time-of-flight is measure of time that ions need to cross in a field free tube of about 1m length.*

(7) It begins when a high-voltage pulse is applied to the back plate of the ion pulser and ends when the ions of the interest strike the detector. *The flight time (t) is established by the energy (E) to which an ion is accelerated, the distance (d) it has to travel and its mass (strictly speaking its mass-to-charge ratio).* (9)
We can represent time-of-flight relationship by following formula:

$$m = \left(\frac{2E}{d^2}\right) t^2$$

This principle was described by combination of two relationships:

- Kinetic energy is the energy of an ion in motion:

$$E = \frac{1}{2} m v^2$$

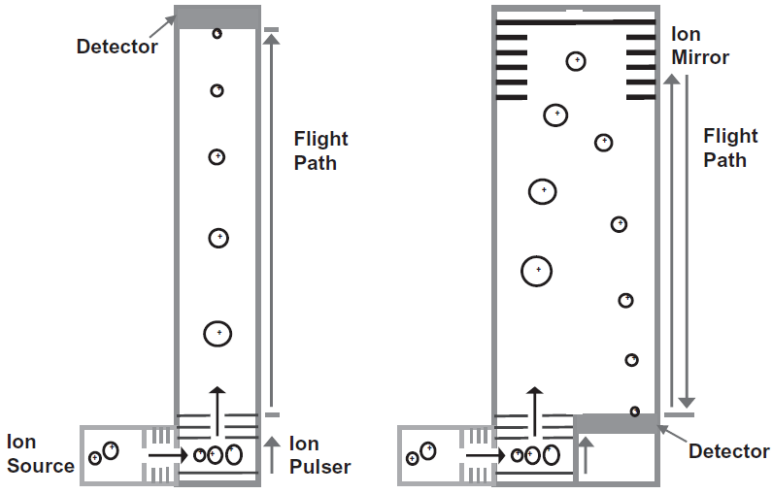
- Velocity of an ion:

$$v = \frac{d}{t}$$

(9)

Relationship describes that smaller masses will move faster and arrive earlier to the detector.

Figure 2: Two versions of time-of-flight analyzer, version on the left is linear geometry, on the right is version with ion mirror. Figure also indicate that smaller masses have larger velocity and so they early reach the detector. (9)



Ions are converted into signal which is transformed into mass spectrum. There are several types of detectors. As the first detector was used photographic plate. Another type of detector is Faraday cup where ions generate current caused by ions impact to the surface of dynode and following electron emission. Or typically electron multiplier type of detector is applied. (10)

As a detector also MS can be applied as it was done in this experiment.

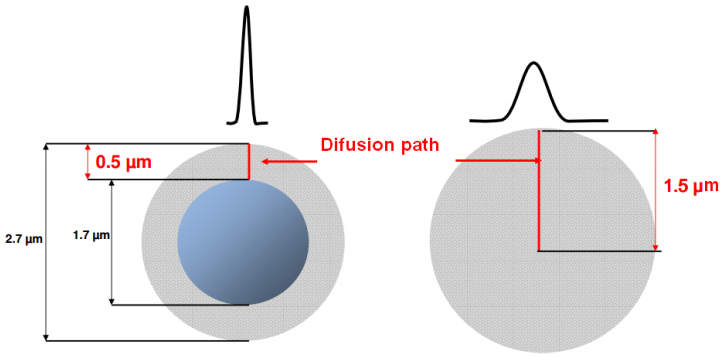
3.2.2 COLUMNS

3.2.2.1 F5 column

The pentafluorophenylpropyl stationary phase of so called F5 column has many advantages in comparison to C18 type of columns and therefore multifaceted application. Retention profile is similar to letter “U” so F5 stationary phase can be used for both reversed and normal phase composition of mobile phase. (11)

For further experiment Ascentis Express F5 column was used. This column is based on Fused-Core© particle technology, it means structure of particle is formed from *thin porous shell of high-purity silica surrounding a solid silica core. This particle design exhibits very high column efficiency* (12) thanks to extremely narrow particle size distributions, minimal analyte diffusion through the column provided by consistent path length, sharper peaks due to shorter diffusion path. (13)

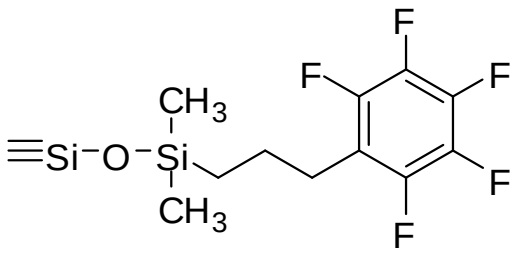
Figure 3: Comparison of Ascentis Express Fused-Core© particle and standard HPLC particle (13)



Thanks to the structure of fluorinated phases, pentafluorophenylpropyl stationary phase is capable to provide various types of interactions. It does work with both reversed phase mechanism of separation and also normal phase separation. Phenyl rings enable π-π interaction (π-acceptor) with other compounds containing aromatic circle, phenyl rings are electron-deficient due to the presence of electronegative Fluorines and this state causes ability of other

interactions as dipole-dipole interaction. There is also potential of hydrogen bonding interaction, charge-transfer interaction, and shape selectivity in size and spatial distribution. (14,15)

Figure 4: Structure of pentafluorophenylpropyl phase bonded to silica base



3.2.2.2 HILIC column

HILIC is abbreviation of hydrophilic interaction liquid chromatography. It is perspective technique for separation of polar molecules with polar stationary phase and aqueous-organic mobile phase. This technique facilitates separation of highly polar compounds as saccharides, amino acids and peptides. Separation of highly polar compounds is difficult because their retention with reversed phased system is low and retention with normal phase system of mobile phase is too high. (16)

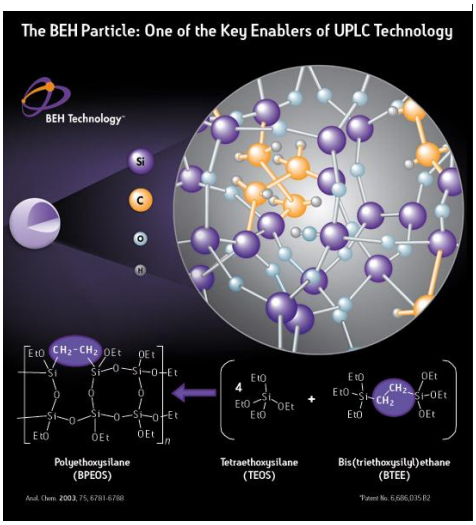
At first HILIC technique was used for separation of sacchcarides, amino acids and peptides. In last few years application of HILIC separation technique is increasing and there are many braches using HILIC such as environment, food industry and natural or synthetic drugs. (17)

Retention is getting higher with higher polarity of analyzed molecules and on the other side retention is getting smaller with higher polarity of mobile phase. Composition of mobile phase is similar as the composition of mobile phase that is used for reversed phase separation. (18)

Stationary phase is highly hydrophilic. For HILIC separation various types of polar columns can be used, e. g. bare silica gel stationary phase, silica based, amino-, amido-, cyano-, carbamate-, diol-, polyol-, zwitterionic sulfobetaine, or poly(2-sulphoethyl aspartamide) and other chemically bonded

polar stationary phases. Hydrophilic stationary phase attract water from mobile phase and so retention of analyzed molecules is higher. (16)

Figure 5: Description of the organization of the BEH particle (14)



High sensitivity of LC-MS is with HILIC separation technique thanks to better electrospray ionization and high concentration of Acetonitrile in mobile phase (range of Acetonitrile concentration is from 50% to 90%). (18)

For further experiment the 1.7 µm Ethylene Bridged Hybrid (BEH) particle was used (figure 5). This hybrid particle technology enables to use wider pH range (1 - 12) and multipurpose separation technology. (14)

3.3 REVERSED-PHASE CHROMATOGRAPHY

Reversed-phase chromatography is widely used method of liquid chromatography. It is more efficient, convenient, and robust in comparison to other methods of HPLC such as normal-phase chromatography. Reversed-phase chromatography is a type of separation method in which polar mobile phase is used and stationary phase is non-polar. Reversed-phase chromatography is ideally suitable for analysis of polar compounds, peptides and other biochemical substances. (19,20)

Stationary phase is constituted from non-polar particles represented mostly often by chemically modified silica (with C8 or C18 chains ending) or styrene-divinylbenzene copolymers. As a mobile phase is used aqueous-organic mixture of water or buffer with organic ingredient such as preferred Acetonitrile.

Figure 6: Preferred organic components of mobile phase (15)

Separation mechanism consists in different solubility of analyte in mobile phase and bonding to stationary phase and because polar compounds prefer interaction with polar mobile phase and less polar compounds interacts with non-polar stationary phase, polar compounds leave the column first and less polar compounds are retained and so leave column last. Compounds leave column in order of decreasing polarity.

With gradual elution the composition of mobile phase is changing mostly into more hydrophobic with increasing quantity of organic solvent. The purpose of the gradient is to move retained compounds faster. Several types of gradient elution can be used. One of these is stepped type and the better one is smooth one without flat regions. With smooth gradient we can improve column efficiency and so affect peak tail and its width. (15)

3.4 AGILENT MASS HUNTER WORKSTATION SOFTWARE

Mass Hunter workstation software is highly effective fully automated system of data analysis. It provides system controlling, data acquisition, qualitative and quantitative analysis, and reporting for Agilent time-of-flight (TOF), quadrupole time-of-flight (Q-TOF), inductively coupled plasma mass spectrometer (ICP-MS), and triple quadrupole systems. (21)

This software provides all required tasks; it shows all raw data about sample and also generates answers quickly by data processing. By molecular-feature-extraction list of compounds found in sample is generated with accurate masses, RTs, molecular features and m/z. Mass Hunter also enables to identify compounds against Agilent's METLIN database of metabolites.
For the identification it is used accurate mass figures, isotopes position, concentration, and optional RT. Score of identification signals assurance

of identification and also there is a list of all known possibilities that fit identification criteria. (21)

For user it is also very helpful Mass Profiler Pro option of comparing two or more samples or compounds, to identify compounds by comparison with standard, or to exclude impurities. Finding out differences between samples also can be way of unknown compounds detection. (22,23)

3.5 METLIN DATABASE

METLIN (METabolite LINk) is freely accessible web-based metabolite database containing over 42 000 structures. It is catalogue of known metabolites and their masses, chemical formulas, structures, ions fragmentation data and also direct link to information of outside resources such as the Kyoto Encyclopedia of Genes and Genomes (KEGG). It has been developed by the Scripps Research Institute to facilitate the identification of metabolites using accurate mass data. (24)

Its role is to archive and provide information from multiple sources.

1. Structural and physical data of known endogenous metabolites, drugs, and drug metabolites
2. High-accuracy FTMS (Fourier transform mass spectrometry) data from reference bio-fluid/tissue samples
3. Reference tandem MS data from known metabolites and metabolite derivatives
4. LC/MS profiles from primarily human and some model organisms (24)

METLIN also enables researches to populate database with own RT. (25)

It is very useful in a metabolite research and also metabolite identification to use MS/MS spectra and LC-MS profiles of human plasma and urine sample. Its function is based on combination of both physical properties (e.g. mass value, chemical formula) and sample specificity (e.g., bio-fluids from healthy and disease states). There are also multipurpose search parameters such as: accurate mass using, elementary composition, name or a part of the name, CAS (Chemical Abstract Service) number, KEGG number, fragment m/z,

multiple fragments, and MS/MS spectrum match so it is easy to use database via user specified parameters and also selected error of searching (ppm). (26-28)

4 EXPERIMENTAL PART

4.1 EQUIPMENT

4.1.1 MASS SPECTROMETER

- Agilent Technologies 6540 UHD Accurate – Mass Q-TOF LC/MS, Model G6540A
  Serial No. US 11068203

4.1.2 ULTRA PERFORMANCE LIQUID CHROMATOGRAPHY

- Agilent Technologies 1290 Infinity
- G4212A 1290 DAD
  Serial No. DEBAF00625
- G1316C 1290 TCC
  Serial No. DEBAC00969
- G4226A 1290 Sampler
  Serial No. DEBAI01074
- G1330B 1290 Thermostat
  Serial No. DEBAK02872
- G4220A 1290 Bin pump
  Serial No. DEBAA00829

4.1.3 SOFTWARE

4.1.3.1 Qualitative analysis

Agilent Technologies, Mass Hunter Qualitative Analysis, B.04.00

4.1.3.2 PCDL manager

Agilent Technologies, Mass Hunter PCDL Manager

4.1.3.3 Mass Profiler Pro

Agilent Technologies, Mass Profiler Pro

4.1.4 COLUMNS

4.1.4.1 F5 HPLC column

Ascentis Express F5 HPLC Column, 53567-U, particle size 2.7 µm, L x I.D. 5 cm x 2.1 mm, Col: 122514-05

4.1.4.2 HILIC HPLC column

Acquity UPLC® BEH Amide, particle size 1.7 µm; 2.1 x 100 mm column, Part No. 186004801, Lot No. 0116312451

4.1.5 PIPETS

- VoluMate LIQUISYSTEMS, Mettler Toledo
  - 0.5 - 10 µl, 10 - 100 µl, 20 - 200 µl,100 - 1000 µl

- Finnpipette, LABSYSTEMS
  - 1 - 5 ml, J38684

4.1.6 BALANCE

- Mettler Toledo AX205: Capacity: 220 g, Readability: 0.01 mg, calibrated: 8. 9. 2011, Certificate: K019-V23391 (Finas Lahti precision)

4.1.7 INTEGRAL WATER PURIFICATION SYSTEM

- MILLIPORE, Milli-Q®, Gradient , A10, PMC-LA-0034

  Cartridge filter: Quantum®EX, Ultrapure Organex Cartridge, LOT-FOPA84536, 18.2 (18.3) MΩ×cm

4.2 MATERIALS

Table 2: List of the chemicals that were used for preparation of the samples and mobile phases of HPLC

Acetonitrile	HiPerSolv CHROMANORM for HPLC gradient grade 20060.320
Methanol	J.T. Baker 8402
Formic acid	Fluka Analytical 06450, 98%
L-Tyrosine	Fluka Analytical 93830
Uracil	Aldrich 22405-041
L-Pyroglutamic acid	Fluka chemika 83160
L-Threonine	Sigma-Aldrich 128K00192
2-L-Glutamine	Fluka 96120
Nicotinic acid	Sigma-Aldrich N4126-100g
L-Glutamine	Merc 1114454 1250
L-Proline	Fluka Biochemika 81710
p-Coumaric acid	Fluka Biochemika 28200
2-Deoxy-D-ribosa	Aldrich-chemistry 121649-5g
L-Lysine	Sigma BCBC1966
Cystidine	Sigma C4654-1g
Glycine	Riedel-de Haën 33226
L-Cystine	Sigma C8755100g
L-Aspartic acid	Sigma A9256-100g
Nicotin amide	Fluka 72340
L-Alanine	Sigma A7627-100g
Malonic acid	Aldrich S09041-073
Hippuric acid	Aldrich 11020-114
Caffeine	Aldrich 11020-116
L-Methionine	Sigma 63H0802
L-Isoleucine	Sigma 46H14431
Tryptophan	Sigma 126H03791
Adenosine-5-monophosphate	Sigma 078K1492
L-Phenylalanine	Sigma 32H0521
D-Glucose-6-phosphate	Sigma 093K3789
L-Rhamnose	Sigma 57H0567
L-Arabinosa	Sigma 87H0721

D-Galactose	Sigma 67H0561
γ-aminobutyric acid	Sigma 026K07361
Guanidine	Sigma 68H5437
Leucin enkephalin	Sigma 38H5803

4.3 PREPRATION OF THE SAMPLES

4.3.1 PREPARATION OF THE SAMPLES FOR F5 COLUMN

Urine was taken from healthy persons therefore it should not contain proteins. The samples were prepared from male and female urine, both types of urines have the same procedure of preparation and each type of urine has two replicates. Urine samples were stored in plastic vials at -20 °C.
50 µl of the urine was given into plastic vials and diluted by adding 450 µl of pure (>18MΩ Millipore) water, then vortexed for 30 s and centrifuged for 15 min and 13200 rpm. The supernatant was taken by syringe (1 ml without needle, Termo Company) and filtrated into HPLC vials with inserts by using syringe filters (Millex®-HV, Syringe Driven Filter Unit, non-sterile, 0.45 µm, 13 mm, and cat. No. SLHVO13NK; Lot No. R9EN97841).

Exactly the same process was used for preparation of the blank, the pure (>18MΩ Millipore) water was applied instead of urine.

4.3.2 PREPARATION OF THE SAMPLES FOR HILIC COLUMN

For the HILIC column samples were also prepared from male and female urine and two replicates of both of these samples were prepared.
At the beginning the method of preparation was exactly the same as the preparation of the samples for F5 column.

The urine was diluted with the pure (>18MΩ Millipore) water in a ratio of 1 part of urine and 9 parts of water. After that it was mixed by Vortex for 30 s and centrifuged for 15 min and 13 200 rpm, the supernatant was taken and diluted with 300 µl of cold 90% Acetonitrile. This solution was vortexed for 30 s and thereafter it was kept in the fridge for 100 min. The sample was centrifuged again for 15 minutes and 13200 rpm; the supernatant was taken by syringe (1 ml without needle, Termo Company) and filtrated into the HPLC vials

with inserts by using syringe filters (Millex®-HV, Syringe Driven Filter Unit, non-sterile, 0.45 µm, 13 mm, cat. No. SLHVO13NK; Lot No. R9EN97841).

The same technique of preparation was repeated three times with each type of urine so 3 same samples of male urine and 3 same samples of female urine were created and for making blank 90% Acetonitrile was added instead of urine.

4.3.3 MIX OF AMINO ACIDS STANDARD PREPARATION

It was used the NuGO Standard Operating Procedure (SOP) number 43 produced by the University of Copenhagen for the preparation of the standard of amino acids. (29)

4.3.3.1 Chemicals

Table 3: List of the chemicals used for the preparation of the amino acids standard

	Name	Solvent	Information
1	L-Tyrosine	10% ACN	Fluka Analytical 93830 (100g)
2	Uracil	10% ACN	Aldrich 22405-041
3	L-Pyroglutamic acid	2% ACN	Fluka chemika 83160
4	L-Threonine	2% ACN	Sigma-Aldrich 128K00192
5	2-L-Glutamine	2% ACN	Fluka 96120
6	Nicotinic acid	2% ACN	Sigma-Aldrich N4126-100G
7	L-Glutamine	0,1% Formic acid	Merc 1114454 1250
8	L-Proline	2% ACN	Fluka Biochemika 81710
9	p-Coumaric acid	10% ACN	Fluka Biochemika 28200
10	2-Deoxy-D-ribosa	2% ACN	Aldrich-chemistry 121649-5G
11	L-Lysine	2% ACN	Sigma BCBC1966
12	Cystidine	2% ACN	Sigma C4654-1G
13	Glycine	2% ACN	Riedel-de Haen 33226
14	L-Cystine	2% ACN	Sigma C8755100G
15	L-Aspartic acid	2% ACN	Sigma A9256-100G

16	Nicotin amide	0.1% Formic acid	Fluka 72340
17	L-Alanine	2% ACN	Sigma A7627-100G
18	Malonic acid	2% ACN	Aldrich S09041-073
19	Hippuric acid	2% ACN	Aldrich 11020-114
20	Caffeine	2% ACN	Aldrich 11020-116
21	L-Methionine	2% ACN	Sigma 63H0802
22	L-Isoleucine	2% ACN	Sigma 46H14431
23	Tryptophan	10% ACN	Sigma 126H03791
24	Adenosine-5-monophosphate	10% ACN	Sigma 078K1492
25	L-Phenylalanine	2% ACN	Sigma 32H0521
26	D-Glucose-6-phosphate	2% ACN	Sigma 093K3789
27	L-Rhamnose	2% ACN	Sigma 57H0567
28	L-Arabinosa	2% ACN	Sigma 87H0721
29	D-Galactose	2% ACN	Sigma 67H0561
30	γ-aminobutyric acid	2% ACN	Sigma 026K07361
31	Guanidine	2% ACN	Sigma 68H5437
32	Leucin encephalin	2% ACN	Sigma 38H5803

4.3.3.2 Solvents

0.1% Formic acid, 2% Acetonitrile, 10% Acetonitrile, 70% Acetonitrile

4.3.3.3 Solutions

The way how solution of chemicals was made is that approximately 1 mg of each chemical was dissolved with exactly 1 ml of solvent per mg of chemical. From these solutions standard mix of chemicals was produced by mixing exactly 40 µl of each dissolved chemical into HLPC vial.

4.4 MEASURING CONDITIONS

4.4.1 MEASURING CONDITIONS FOR F5 HPLC COLUMN

Method name: F5 positive 1290

One micro liter of the sample solution was injected onto F5 HPLC column using a gradient mobile phase consisting of 0.2% Acetic acid in water as a solvent A and 0.2% Acetic acid in Methanol as a solvent B. The solvent's ratio starts with 2 % of solvent B, it increased to 98 % of solvent B at 13 min, continuing with 98 % of solvent B until 15 min and reduce ratio of solvent B to 2 % at the time 15.1 min, the same composition was held up to the end at 19th min. The mobile phase was delivered at 0.4 ml/min and column temperature was maintained at 40 °C.

Following ionization conditions were used: dual ESI positive ion mode, drying gas (nitrogen) temperature 325 °C, drying gas flow rate 10 l/min, nebulizer pressure 45 psig, capillary voltage 4000 V, fragmentor voltage 100 V.

Conditions for MS mode were 50 m/z as minimal range, 1600 m/z as maximal range and 1.50 spectra/sec scan rate. Values of reference masses were 121.05087300 and 922.00979800.

4.4.2 MEASURING CONDITIONS FOR HILIC COLUMN

Method name: Amide BEH

Injected volume of the sample was 1 µl analyzed with HILIC HPLC column and gradient composition of mobile phase. As a solvent A was used 50% Acetonitrile in 20mM Ammonium formate and 90% Acetonitrile in 20mM Ammonium formate as a solvent B. The starting conditions for gradient were 100 % of solvent B and the same composition was preserved till 2,5th min, at 10 min there was 0 % of solvent B and this solvent composition was held until 14.5 min, at 14.6 min returning to 100 % of solvent B and the 100 % was remained till stop time at 19th min. The flow rate of mobile phase was 0.6 ml/min; temperature of column was kept at 45 °C.

Ionization conditions were following: dual ESI positive ion mode, temperature of the drying gas (nitrogen) 325 °C, gas flow rate 10 l/min, nebulizer pressure 45 psig, capillary voltage 4000 V, fragmentor voltage 100 V.

Conditions for MS mode: minimal range 50 m/z, maximal range 1600 m/z, scan rate 2.40 spectra/sec. As reference masses were used 121.05087300 and 922.00979800.

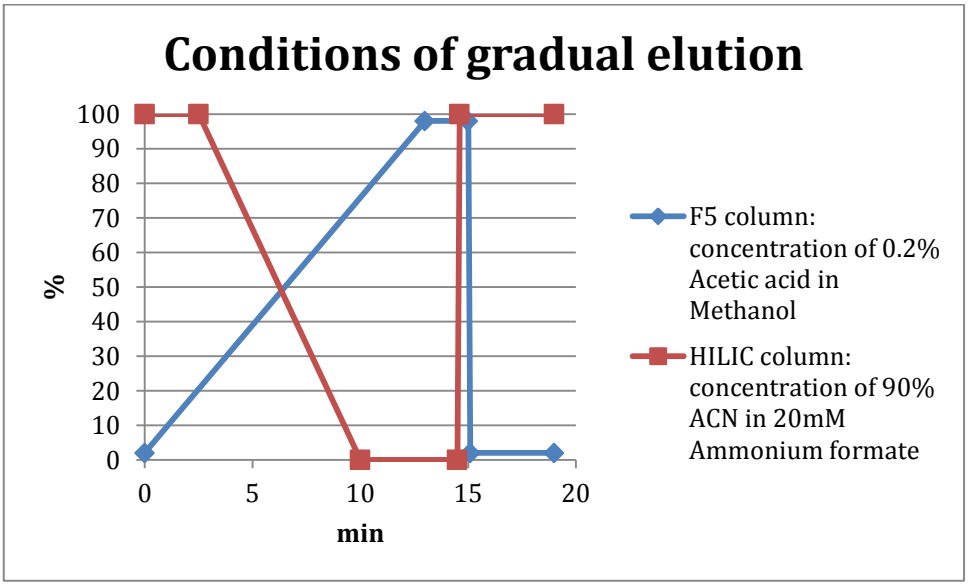
Table 4: Gradient elution used for F5 column

Time (min)	0.2% Acetic acid in water % (V/V)	0.2% Acetic acid in Methanol % (V/V)	
0 - 13	98 → 2	2 → 98	Concentration of 0.2% Acetic acid in Methanol is increasing
13 - 15	2	98	No change
15 - 15.1	2 → 98	98 → 2	Concentration of 0.2% Acetic acid in Methanol is decreasing
15.1 - 19	98	2	No change

Table 5: Gradient elution used for HILIC column

Time (min)	50% Acetonitrile in 20mM Ammonium formate % (V/V)	90% Acetonitrile in 20mM Ammonium formate % (V/V)	
0 - 2.5	0	100	Start
2.5 - 10	0 → 100	100 → 0	Concentration of 90% Acetonitrile in 20mM Ammonium formate is decreasing
10 - 14.5	100	0	No change
14.6 - 18	100 → 0	0 → 100	Concentration of 90% Acetonitrile in 20mM Ammonium formate is increasing

Figure 7: Gradient elution used for F5 and HILIC column



In the case of F5 column reversed-phase chromatography method was used, concentration of 0.2% Acetic acid in Methanol is increasing to 100 % for elution to extract remaining componds. Amino acids are polar and so in this case they are not retained well. Than we can see especially amino acids with small molecule at the beginning of chromatogram.

The similar principle was used for HILIC column but in this case it is not reversed-phase chromatography method so the lines are almost like the image in the mirror. Polar compounds as amino acids are retained by hydrophilic interaction with stationary phase and with higher proportion of 50% ACN in 20mM Ammonium formate they are elute.

4.5 IDENTIFICATION OF THE SAMPLES

Separation and identification of the urine samples was made by UPLC/MS.

4.5.1 IDENTIFICATION OF THE COMPOUNDS

For identification of the compounds was mostly used automatic searching of compounds and automatic identification but in some cases the automatic system was wrong or it found the same compound more than once. In the cases of incorrect identification manual process of compounds identification from chromatogram was used.

4.5.1.1 Automatic identification of the samples against METLIN and created database

Compounds were found by the option *Find by molecular feature* and then identified by the option *Searching database*, for the identification METLIN database was used at first after that created database.

Program: Mass Hunter Qualitative Molecular Feature Extraction

4.5.1.1.1 Finding compounds by molecular-feature-extraction, settings of these option in the program were following

- Extraction
    - Extraction algorithm: As targeted data type was selected small molecules (chromatographic)
    - Input data range: Restrict m/z to 60.0000 – 250.0000 m/z
    - Peak filters: to use peak with height (Profile and centroid spectra) ≥ 600 counts

- Ion Species
    - Allowed ion species:
        - Positive ions: H^+, Na^+
        - Negative ions: H^-, Cl^-
- Charge State
    - Isotope grouping: Peak spacing tolerance was allowed to 0.0025 m/z plus 5.0 ppm and as isotope model was chosen the option common organic molecules.
    - Charge state: Limited charge states was assigned to a maximum of 1
- Compound Filters
    - Height: absolute height was restricted as ≥ 5000 counts
- Mass Filters
    - Mass filters: Filter mass list was used with error 5.000 ppm and including only the masses in the METLIN database
- Mass Defect: not used
- Peak Filters (MS/MS): not used
- Results:
    - Chromatograms and spectra: ECC (extracted compound chromatograms) spectra were extracted

4.5.1.1.2 Identifying compounds by the option Searching database, settings of this option in the program were following:

- Searching Criteria: as a value to match was chosen mass with match tolerance 5.00 ppm
- Database: at first the METLIN database was used for identification than created database
- Peak Limits: Maximum number of peaks to search when peaks are not specified graphically was set to 5
- Positive Ions:
    - Charge carriers: H^+, Na^+ and charge state range 1
    - Neutral losses: H_2O
    - Aggregates: Dimmers e.g. $[2M+H]^+$

- Negative Ions:
    - Charge carriers: H^- and charge state range 1
- Search Results were limited to the best 10 hits

4.5.1.2 Manual identification of the samples against METLIN and created database

4.5.1.2.1 Define chromatogram

With the option *Define chromatogram* the specific accurate m/z value and allowed mass error 5; 10 or 20 ppm was selected.

4.5.1.2.2 Find compound by formula

With the option *Find compound by formula* the selected formula was assigned with allowed mass error 5; 10 or 20 ppm.

4.5.1.2.3 Comparison with standard

Complicated cases of identification were solved by comparison of chromatograms of samples with chromatogram of the amino acids standard.

4.5.2 CREATING OF DATABASE

Database was created by selection of desired compounds from the METLIN database and formation of subset PCDL (Personal Compound Database and Library). Components of created database were obtained from four sources:

1. List of Metabolites Detected in Human Urine Samples A and B that matched the synthetic urine standards in the METLIN database published in the article Molecular formula and METLIN personal metabolite database matching applied to the identification of compounds generated by LC/TOF-MS (25).
2. List of Amino acids contained in Calibration standard of EZ:faast™ User's manual (30)

3. Compounds contained in the amino acids standard (table 3)
4. Following list of polyamines (table 6)

Table 6: List of polyamines included in database

Systematic name	Chemical formula	Exact Mass
propane-1,3-diamine	$C_3H_{10}N_2$	74.08
butane-1,4-diamine	$C_4H_{12}N_2$	88.1
pentane-1,5-diamine	$C_5H_{14}N_2$	102.12
N1-(3-aminopropyl)butane-1,4-diamine	$C_7H_{19}N_3$	145.16
N1,N1-(butane-1,4-diyl)bis(propane-1,3-diamine)	$C_{10}H_{26}N_4$	202.22
N-(4-aminobutyl)acetamide	$C_6H_{14}N_2O$	130.11
N-(5-aminopentyl)acetamide	$C_7H_{16}N_2O$	144.13
N-(3-((4-aminobutyl)amino)propyl)acetamide	$C_9H_{21}N_3O$	187.17
N-(4-((3-aminopropyl)amino)butyl)acetamide	$C_9H_{21}N_30$	187.17
N-(3-((4-((3-aminopropyl)amino)butyl)amino)propyl)acetamide	$C_{12}H_{28}N_4O$	244.23
N,N'-(butane-1,4-diyl)diacetamide	$C_8H_{16}N_2O_2$	172.12
N,N'-(pentane-1,5-diyl)diacetamide	$C_9H_{18}N_2O_2$	186.14
N-(3-((4-acetamidobutyl)amino)propyl)acetamide	$C_{11}H_{23}N_3O_2$	229.18
N,N'-((butane-1,4-diylbis(azanediyl))bis(propane3,1-diyl))diacetamide	$C_{14}H_{30}N_4O_2$	286.24

5 RESULTS AND DISCUSION

5.1 COMPARISON OF THE RESULTS OBTAINED FROM METLIN AND CREATED DATABASE

Table 7: Comparison of the numbers of compounds found against created and METLIN database

Database	Created database				METLIN			
Column	F5 Column		HILIC Column		F5 Column		HILIC Column	
Urine	Male urine	Female urine	Male urine	Female urine	Male urine	Female urine	Male urine	Female urine
Total number of all compounds	53	56	56	69	305	275	312	337
Number of different kinds of compounds	41	36	33	36	232	212	212	197

The number of all compounds acquired from METLIN database was too high and many types of compounds were occurred several times in one sample. In the table 7 we can see differences between the numbers of compounds found in male urine sample and female urine sample and also evident difference between the numbers found by HILIC and F5 column.

Because the task is to test reliability of identification in an effort to simplify searching and identification database shortlist was created. With created database results were more obvious and it was possible to analyze all of the compounds properly.

Table 8: Table describes the contrast between these two databases with using few examples of amino acids.

Number of compounds in database	Formula	$C_3H_7NO_2$	$C_5H_9NO_2$	$C_5H_{11}NO_2$	$C_6H_{13}NO_2$	$C_9H_9NO_3$
	METLIN	5	3	8	5	7
	Created database	3	1	1	2	1

With created database testing should be more demonstrable and faster.

Table 9: Table explains differences between various errors tolerances used for identification.

Error (ppm)	2	5	7	10	12	15	20	30	50	100
Number of compounds found in F urine	28	46	48	54	57	63	64	66	66	83
Number of compounds found in M urine	23	39	43	47	48	50	54	56	60	75

(F urine means female urine sample; M urine means male urine sample)

For automatic analysis it was used error window 5 ppm but in some cases error tolerance was too narrow even if the system should be able to identify compounds with accuracy 5 ppm. That is the reason why for manual identification of some compounds error window 10 or 20 ppm was used. It is obvious that with wider error window more and more compounds are identified but reliability of the identification is lower.

5.2 IDENTIFICATION OF THE COMPOUNDS

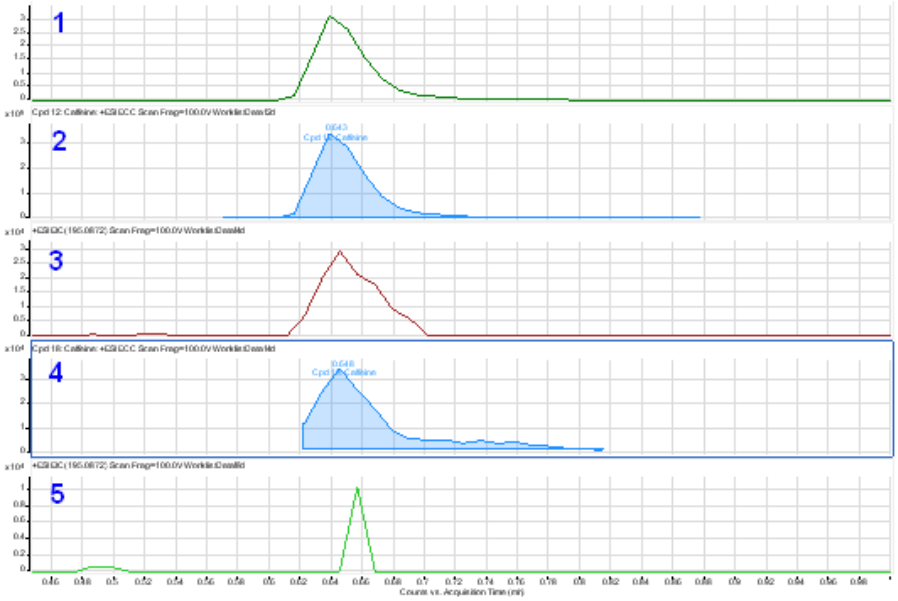
Identification of the compounds was made by comparison of the outcomes of the samples identification and the amino acids standard identification.

5.2.1 F5 COLUMN

Manual analysis is described on few representative compounds which describe main problems of automatic searching and identification.

5.2.1.1 Caffeine

Figure 8: Chromatogram results; Caffeine; amino acids standard; male and female urine samples



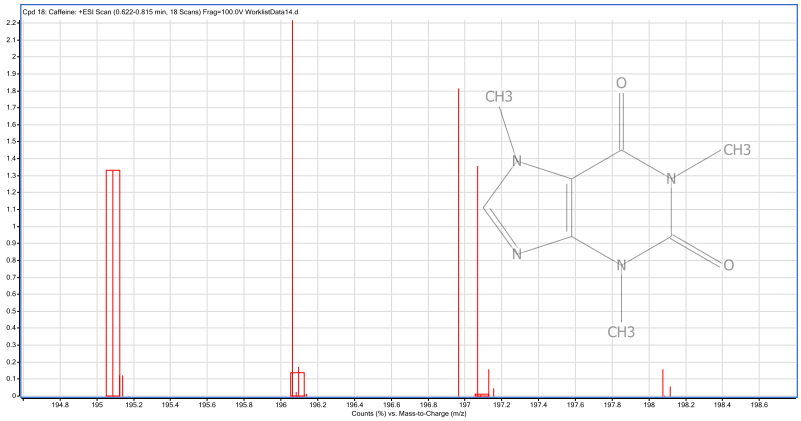
Legend:

1. Caffeine found manually in the amino acids standards; Define chromatogram with allowed error window 20 ppm
2. Caffeine found automatically in the amino acids standards; Find by molecular feature with allowed error window 5 ppm
3. Caffeine found manually in the male urine sample; Define chromatogram with allowed error window 20 ppm
4. Caffeine found automatically in the male urine sample; Find by molecular feature with allowed error window 5 ppm
5. Caffeine found manually in the female urine sample; Define chromatogram with allowed error window 20 ppm

We can compare results of female and male urine samples with results of the amino acids (AA) standards. Peak of AA standard has a good shape and it is not cut.

This example describes how allowed error tolerance impact identification. System should be able to find compounds with error 5 ppm. But in female urine sample wasn't found any Caffeine with using 5 ppm error. In male urine sample peak is cut at the beginning with 5 ppm error, m/z values differ. It was applied 20 ppm error window for both male and female urine samples and also for AA standard. With 20 ppm error window system is able to find peak also in female urine sample, but peak is not in a good shape and also the height of the peak in female urine sample is really small. With 20 ppm error window peak of male urine sample is not cut.

Figure 9: MS Spectrum Results; Caffeine; male urine sample

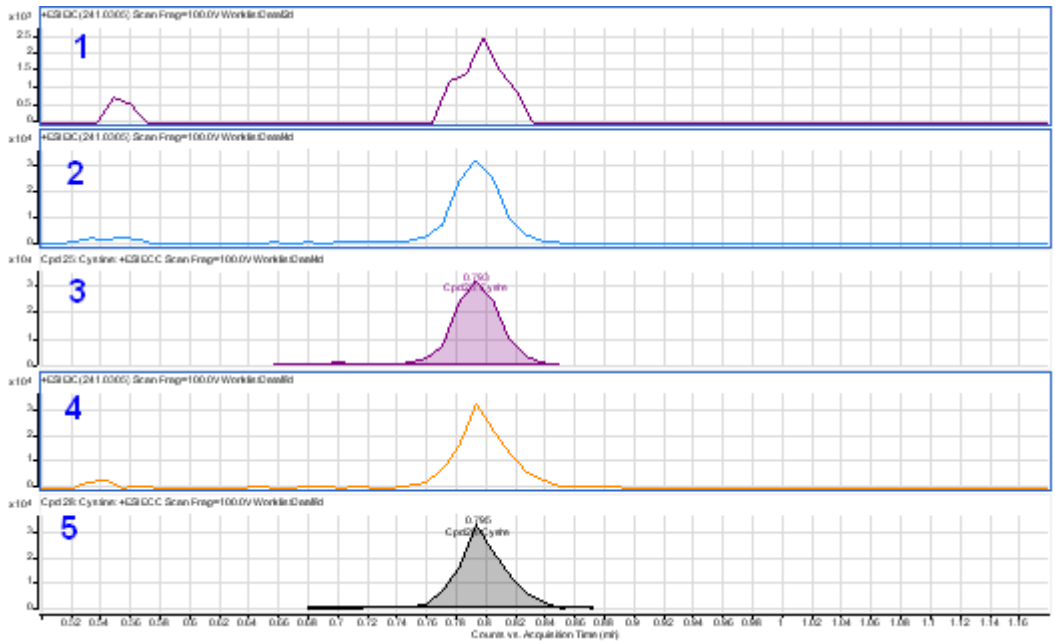


MS spectrum shows isotopes are not exactly situated in the frames. This step of identifications is not giving good results. Even if the first peak (m/z = 195.0876) match into the box, isotope of Carbon 13 is a bit out of the frame and there are also peaks of some other compounds such as peak with m/z value around 196.05 and also nearby the peak of the latest isotope (m/z = 197.08).

Score of Caffeine is only 69.46% which is relatively low.
So also automatic system indicates identification of Caffeine is one of the problematic cases. Solution is to make a standard containing only Caffeine and MS/MS spectra results.

5.2.1.2 Cystine

Figure 10: Chromatogram results; Cystine; amino acid standard; male and female urine samples



Legend:

1. Cystine found manually in the amino acids standard; Define chromatogram with allowed error window 20 ppm
2. Cystine found manually in the male urine sample; Define chromatogram with allowed error window 20 ppm
3. Cystine found automatically in the male urine sample; Find by molecular feature with allowed error window 5 ppm
4. Cystine found manually in the female urine sample; Define chromatogram with allowed mistake 20 ppm
5. Cystine found automatically in the female urine sample; Find by molecular feature with allowed error window 5 ppm

Cystine wasn't found automatically in the amino acids standard, the signal intensity is small and allowed error with automatic searching 5 ppm is too narrow. In the first chromatogram results we can see that with error 20 ppm it displays two peaks, the first smaller at retention time 0.56 min. This peak we can also see in both sample's chromatograms based on 20 ppm error, chromatogram number 2 and 4, but it is absolutely another compound. With 5 ppm error automatic system found peaks of Cystine in male (chromatogram 3) and also female urine sample (chromatogram 5). Signal intensity of the peak of the male and the female urine samples is ten times

larger than the intensity of the standard so it can be the reason why automatic system was able to identify Cystine in urine samples but not in the standard.

Figure 11: MS spectrum results, Cystine, male urine sample

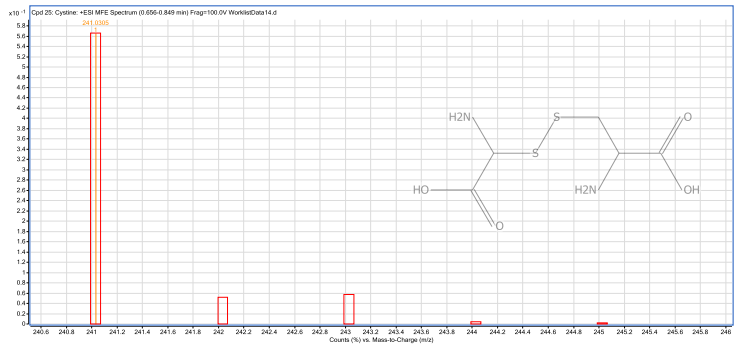
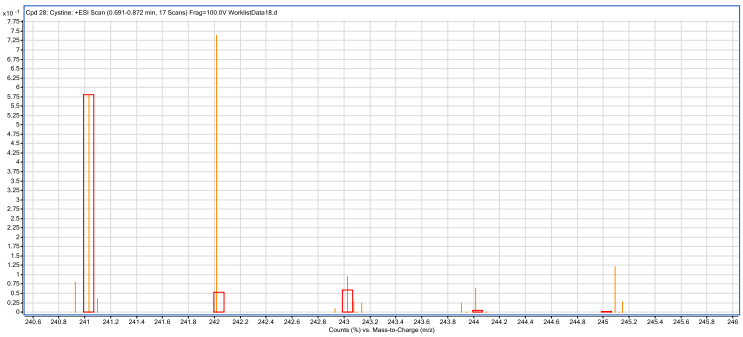


Figure 12: MS spectrum results, Cystine, female urine sample

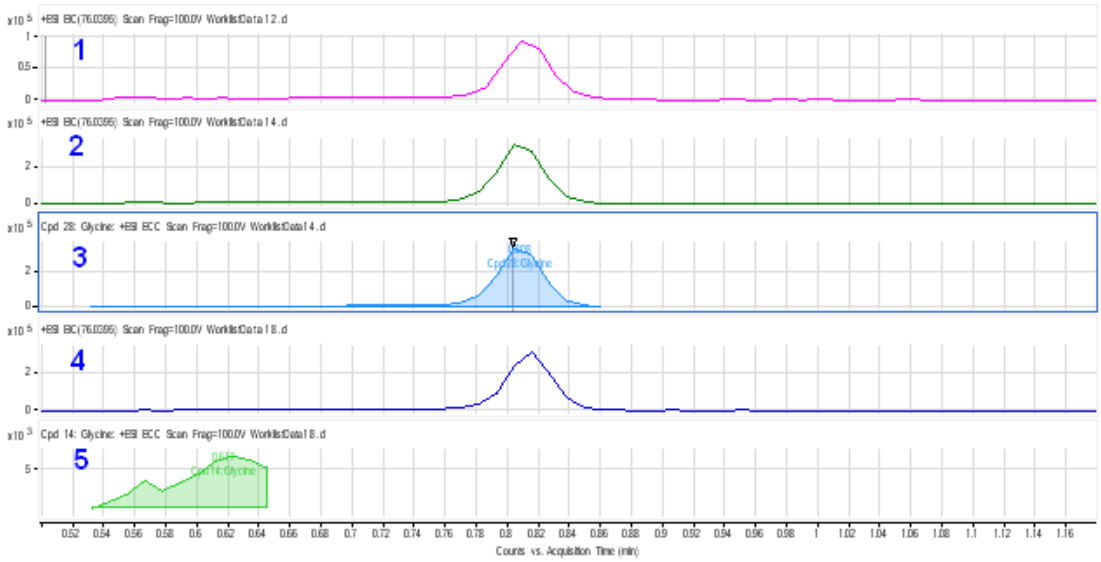


Spectrum of Cystine of male urine sample (figure 11) describes that the frames of all 4 expected isotopes are empty. Isotope abundances, and spacing between isotope peaks information can't be calculated as a positive point of identification and that is also why score of identification is only 46 %.

Different case shows spectrum of Cystine of female urine sample (figure 12). Intensity of all 4 expected isotopes is overloaded and that is why score of identification is also low 45.83 %.

5.2.1.3 Glycine

Figure 13: Chromatogram results; Glycine; amino acids standard; male and female urine samples

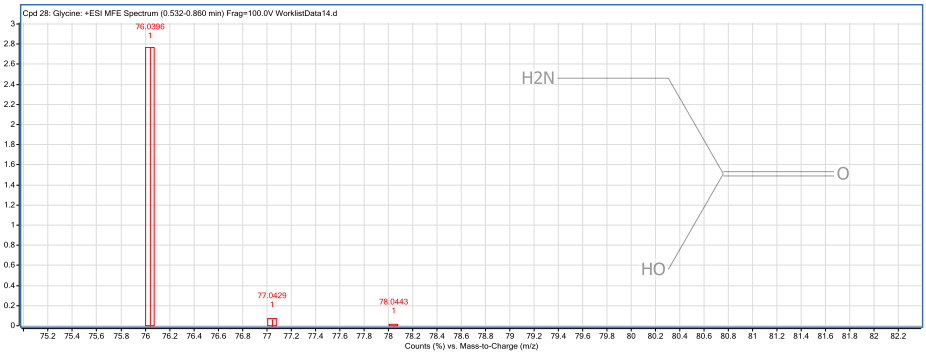


Legend:

1. Glycine found manually in the amino acids standard; Define chromatogram with allowed error 20 ppm
2. Glycine found manually in the male urine sample; Define chromatogram with allowed error 20 ppm
3. Glycine found automatically in the male urine sample; Find by molecular feature with allowed error 5 ppm
4. Glycine found manually in the female urine sample; Define chromatogram with allowed error 20 ppm
5. Glycine found automatically in the female urine sample; Find by molecular feature with allowed error 5 ppm

Glycine wasn't automatically found in the amino acids standard with 5 ppm error therefore manual extraction was used with error 20 ppm (the first chromatogram). With error window 20 ppm peak was found at RT 0.81 min. Position of the female urine sample peak found by automatic system (chromatogram 5) is different than the position of the peaks in chromatogram of male urine sample found by automatic system and by manual method (chromatogram 2, 3). With the change of error from 5 ppm to 20 ppm the peak of female urine sample (chromatogram 4) has the same position and the first peak which was automatically found is probably different compound.

Figure 14: MS spectrum results; Glycine; male urine sample

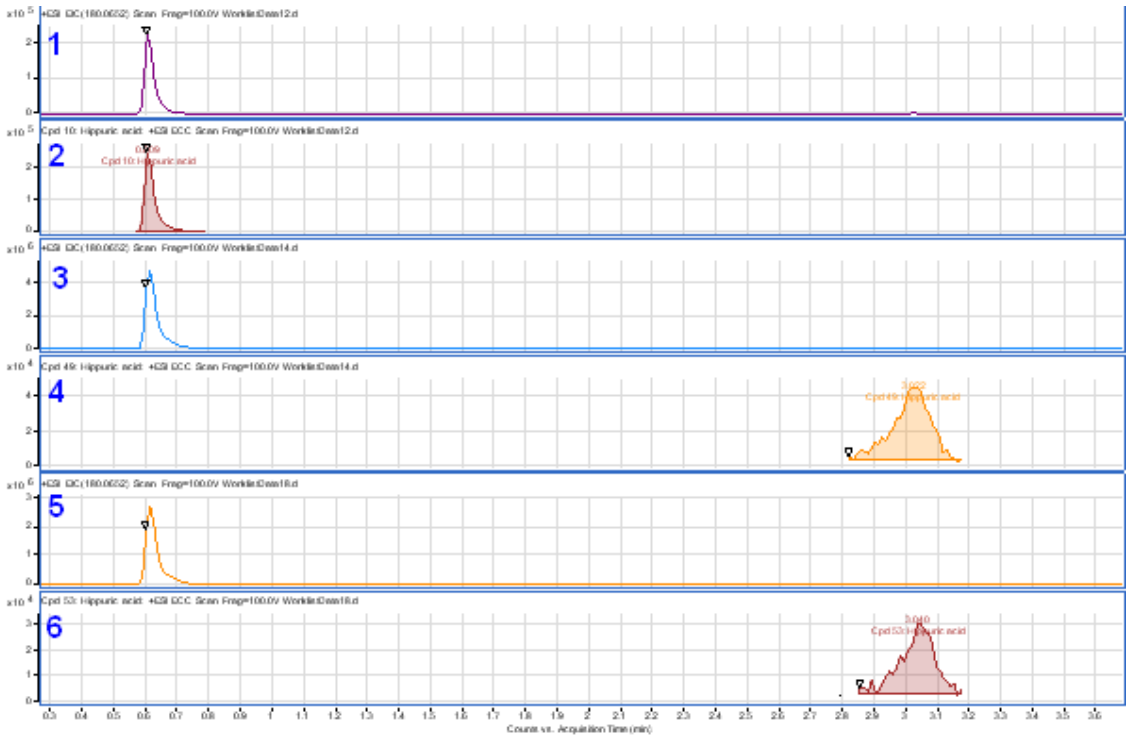


MS spectrum results of Glycine of male urine sample are giving good results for identification. All of the isotopes are well situated in the frames.

Automatic identification takes advantages of both the mass accuracy and mass spectral information. It includes monoisotopic mass, isotope abundances, and spacing between isotope peaks. Also thanks to these result identification is more accurate and with high probability this compound really is Glycine. In this case score of identification is 98.86 %.

5.2.1.4 Hippuric acid

Figure 15: Chromatogram results; Hippuric acid; amino acids standard; male and female urine samples



Legend:

1. Hippuric acid found manually in the amino acids standard; Define chromatogram with allowed error 20 ppm
2. Hippuric acid found automatically in the amino acids standard; Find by molecular feature with allowed error 5 ppm
3. Hippuric acid found manually in the male urine sample; Define chromatogram with allowed error 20 ppm
4. Hippuric acid found automatically in the male urine sample; Find by molecular feature with allowed error 5 ppm
5. Hippuric acid found manually in the female urine sample; Define chromatogram with allowed error 20 ppm
6. Hippuric acid found automatically in the female urine sample; Find by molecular feature with allowed error 5 ppm

In both urine samples automatic system was wrong. Hippuric acid was found automatically at retention time around 3.04 min. These two compounds (chromatogram 4 and 6) were eliminated according to the values acquired from manual identification. The right peak of Hippuric acid is at retention time around 0.609 min as automatic system found in standard (chromatogram 2) and the same results shows manual identification of standard

(chromatogram 1), and manual identification of male (chromatogram 3) and female urine sample (chromatogram 5).

Automatic identification was wrong due to the narrow error window because m/z values are different as figures 16 - 18 describe. With using 20 ppm error m/z values are suitable in this window and system can find right peak of Hippuric acid. Reason of this breakdown can be also high concentration of Hippuric acid in urine samples so the system is overloaded.

Figure 16 - 18: Spectrum preview, Hippuric acid, male urine sample (m/z=180.0650)

Figure 16: RT= 0,588 min; m/z= 180.0665

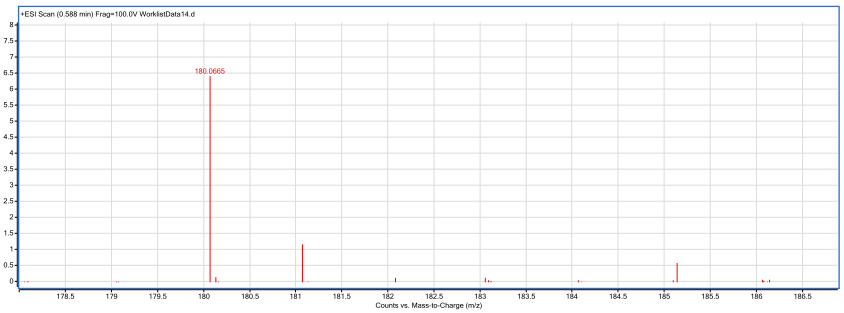


Figure 17: RT= 0.611 min; m/z= 180.0671

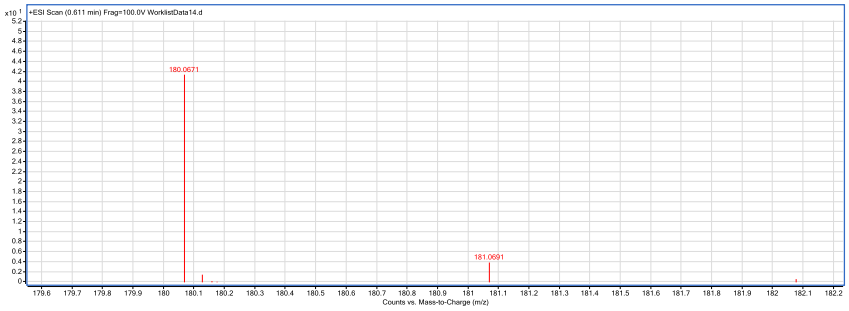
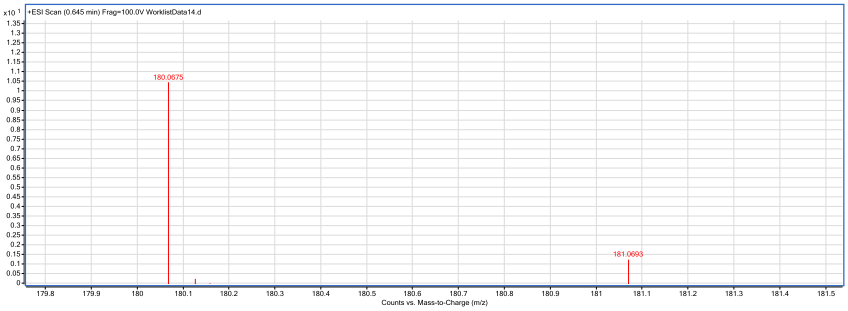
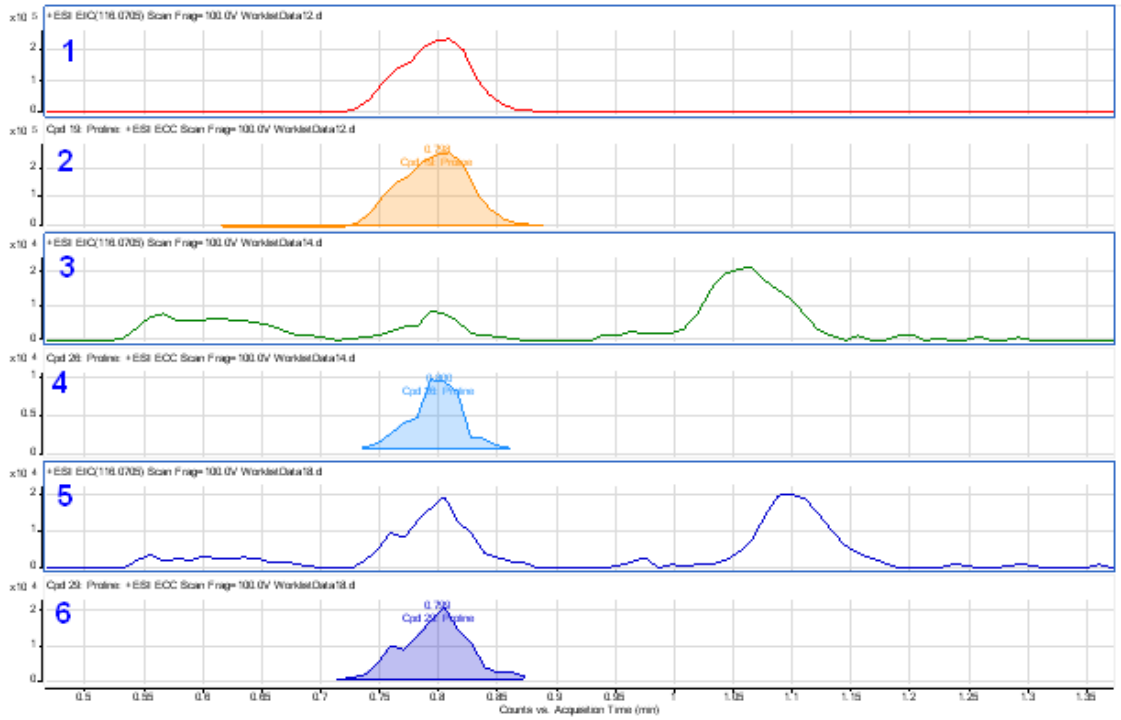


Figure 18: RT= 0.645 min; m/z= 180.0675



5.2.1.5 Proline

Figure 19: Chromatogram results; Proline; amino acid standard; female and male urine samples



Legend:

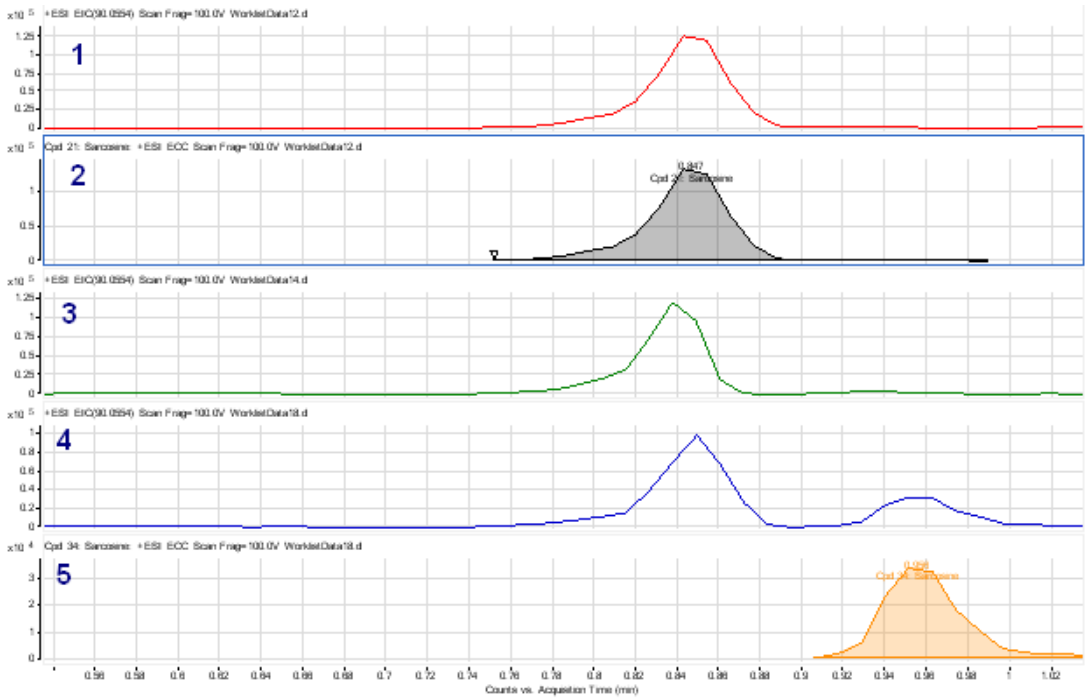
1. Proline found manually in the amino acids standard; Define chromatogram with allowed mistake 20 ppm
2. Proline found automatically in the amino acids standard; Find by molecular feature with allowed mistake 5 ppm
3. Proline found manually in the male urine sample; Define chromatogram with allowed mistake 20 ppm
4. Proline found automatically in the male urine sample; Find by molecular feature with allowed mistake 5 ppm
5. Proline found automatically in the female urine sample; Define chromatogram with allowed mistake 20 ppm
6. Proline found automatically in the female urine sample; Find by molecular feature with allowed mistake 5 ppm

This example shows that with wider range of error we cannot be sure which one of the peaks is the right one and if at least one of these peaks is the right one. Identification of the standard is without any problem (chromatograms 1 and 2) Also automatic system identified Proline in both male (chromatogram 4) and female (chromatogram 6) samples. But manual identification of Proline with using 20 ppm error (chromatograms 3 and 5) is

misleading. In the samples the concentration of Proline is unfortunately low and also it is the reason of troubles with identification.

5.2.1.6 Alanine

Figure 20: Chromatogram results, Alanine, amino acid standard, female and male urine samples



Legend:

1. Alanine found manually in the amino acids standard; Define chromatogram with allowed error 20 ppm
2. Alanine found automatically in the amino acids standard; Find by molecular feature with allowed error 5 ppm
3. Alanine found manually in the male urine sample; Define chromatogram with allowed error 20 ppm
4. Alanine found manually in the female urine sample; Define chromatogram with allowed error 20 ppm
5. Alanine found automatically in the female urine sample; Find by molecular feature with allowed error 5 ppm

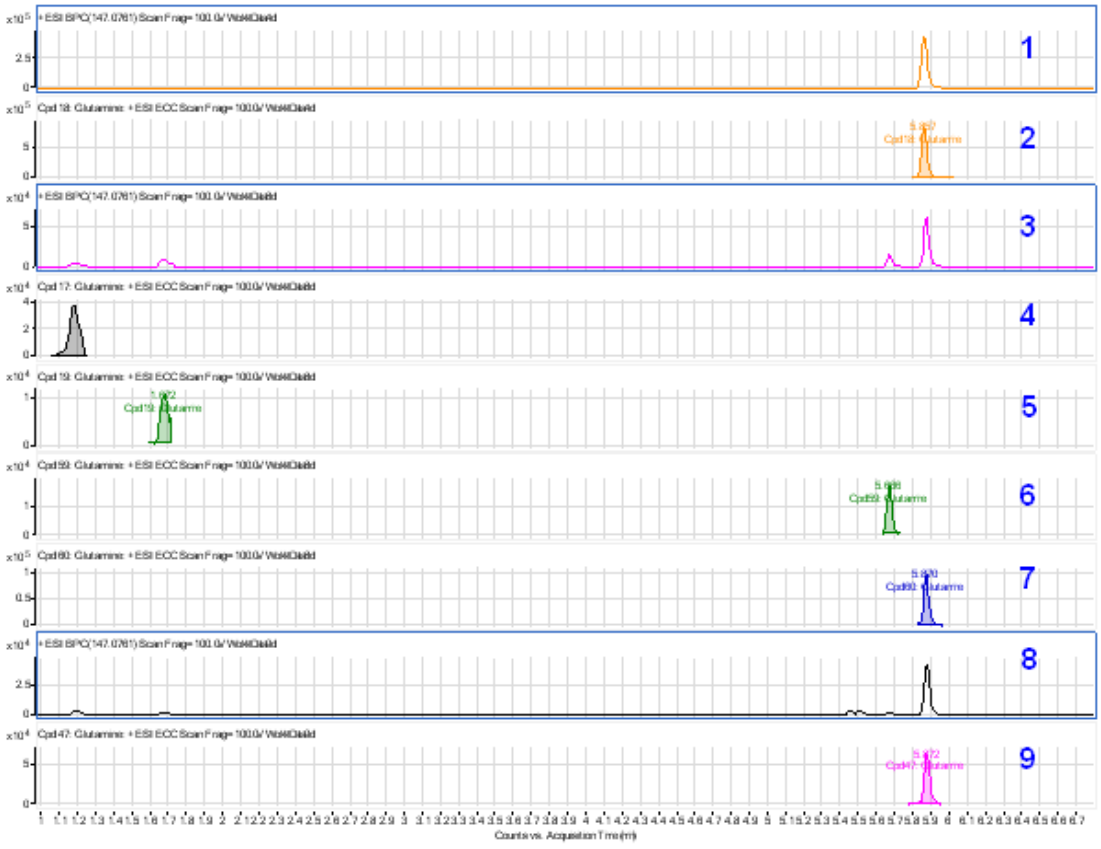
Also in this case automatic identification failed. In the male urine sample automatic system didn't find Alanine, but it was identified by manual identification with 20 ppm error (chromatogram 3). In female urine sample was identified Alanine automatically but this peak was late in comparison with the peaks of amino acids standard chromatogram and male urine sample.

By manual identification with allowed error 20 ppm the same peak was found (chromatogram 4). The compound found automatically in female urine sample (chromatogram 5) is not right Alanine.

5.2.2 HILIC COLUMN

5.2.2.1 Glutamine

Figure 21: Chromatogram results; Glutamine; amino acids standard; female and male urine samples



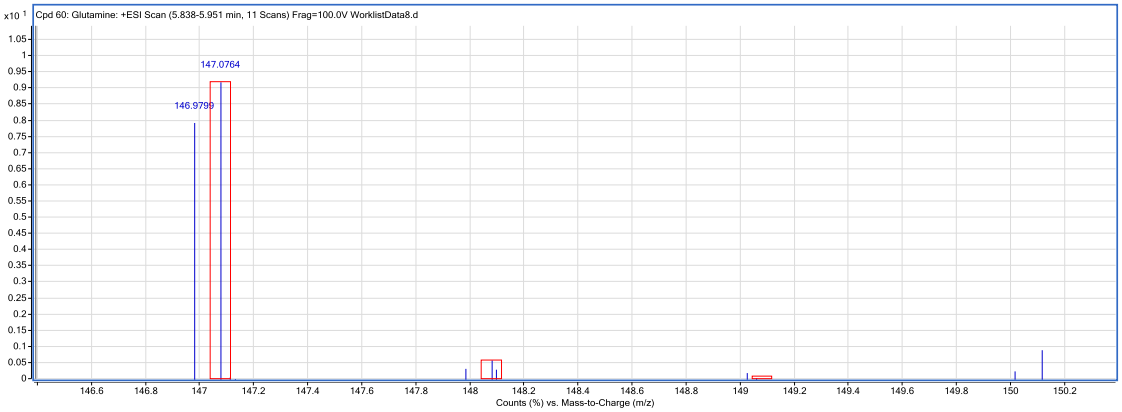
Legend:

1. Glutamine found manually in the amino acids standard; Define chromatograms with allowed error 20 ppm.
2. Glutamine found automatically in the amino acids standard; Find by molecular feature with allowed error 5 ppm.
3. Glutamine found manually in the female urine sample; Define chromatograms with allowed error 20 ppm.
4. Glutamine found automatically in the female urine sample; Find by molecular feature with allowed error 5 ppm.
5. Glutamine found automatically in the female urine sample; Find by molecular feature with allowed error 5 ppm.
6. Glutamine found automatically in the female urine sample; Find by molecular feature with allowed error 5 ppm.

7. Glutamine found automatically in the female urine sample; Find by molecular feature with allowed error 5 ppm.
8. Glutamine found manually in the male urine sample; Define chromatograms with allowed error 10 ppm.
9. Glutamine found automatically in the male urine sample; Find by molecular feature with allowed error 5 ppm.

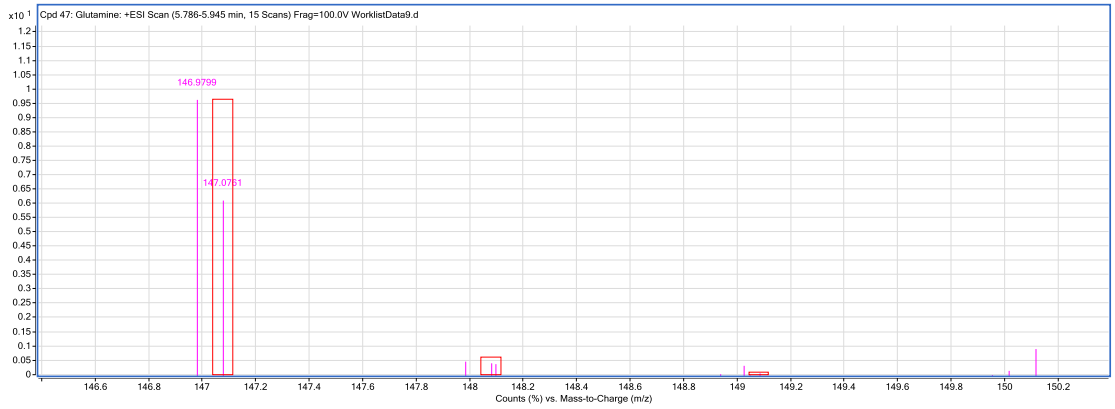
Glutamine is the example when the system found the same compound several times. In female urine sample 4 peaks for Glutamine were found automatically (chromatograms 4 - 7). In male urine sample one peak was found by automatic system. In comparison with amino acids standard (chromatogram 1) and manual identification methods (chromatograms 1, 3, 8) we can find out the right one at retention time 5.87 min.

Figure 22: MS spectrum results; Glutamine; male urine sample



Score of identification is 79.68 %

Figure 23: MS spectrum results; Glutamine, female urine sample



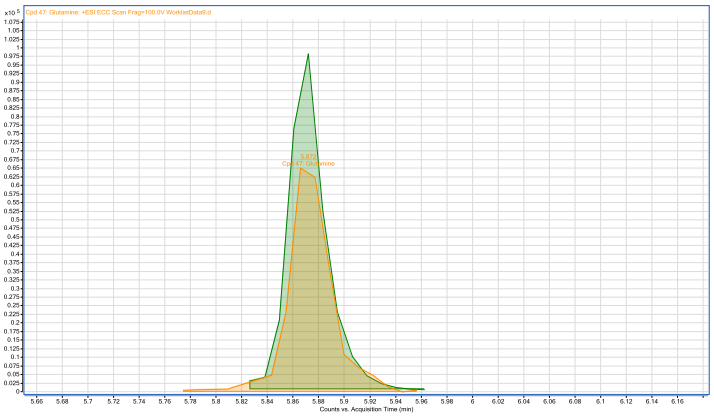
Score of identification is 86.44 %.

Positive role for identification in this case is that volume of peak in female urine sample is higher than volume of peak in male urine sample and the shape

of peak is symmetric. Next positive point is that every of isotopes are present and situated in the frames.

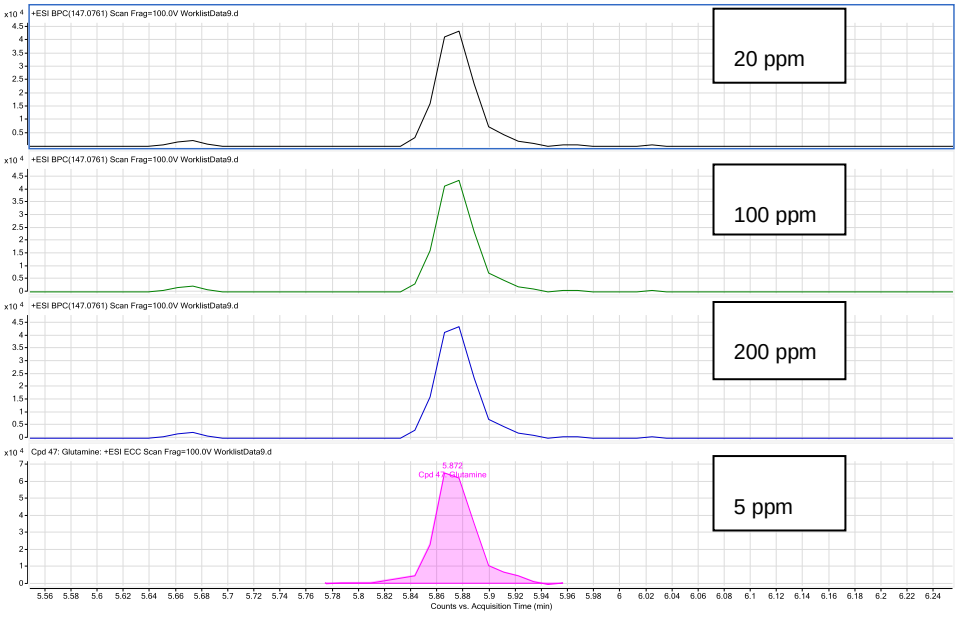
Peak of Glutamine in male urine sample is cut on the top of the peak (figure 24).

Figure 24: Glutamine; female (green) and male (orange) urine sample



The reason can be the m/z values are very different so they are not situated in the error range 5ppm. Figure 25 describes changes between applications of different tolerance.

Figure 25: Chromatograms with different expansion of m/z values

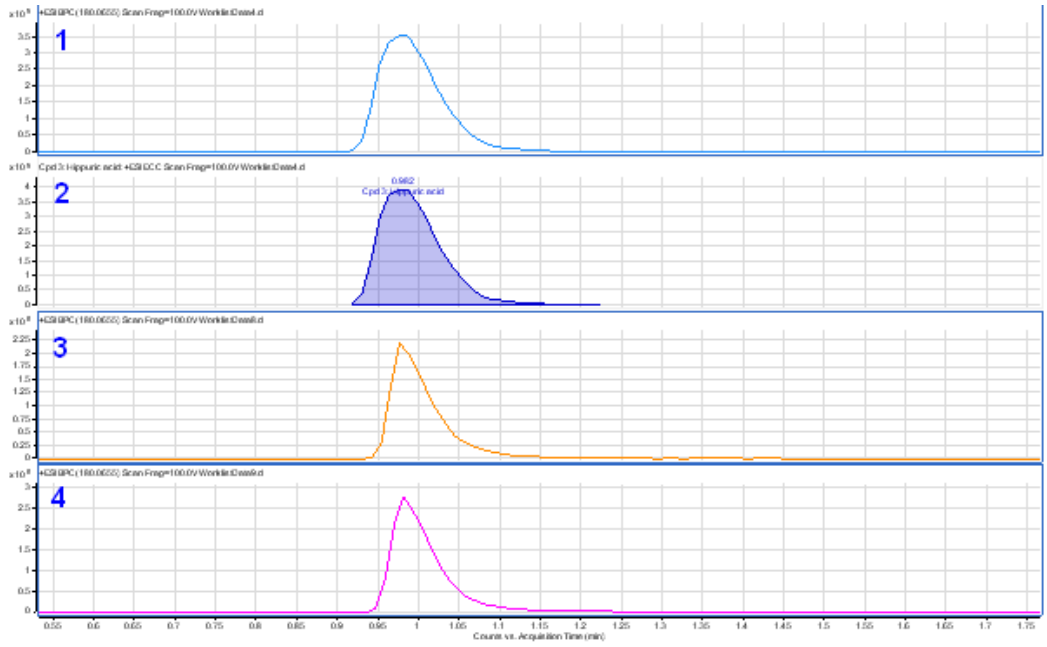


The shape of the Glutamine peak is almost the same with using different tolerance it means differences between m/z values are not main reason of breaking of the peak. With toleration 5 ppm the intensity of the peak is nearby

6.5×10^4 and with using expansion of m/z the intensity is around 4.4×10^4. The reason is the background is getting higher with higher error tolerance and the peak is smaller.

5.2.2.2 Hippuric acid

Figure 26: Chromatogram results; Hippuric acid; amino acids standard; female and male urine samples

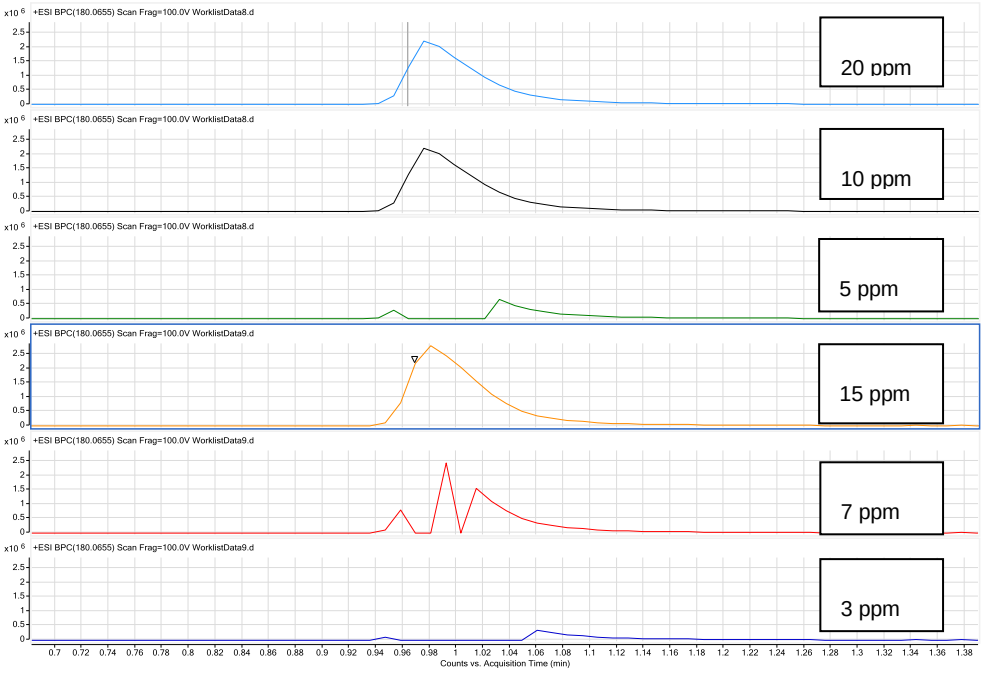


Legend:

1. Hippuric acid found manually in the amino acids standard; Define chromatograms with allowed error 20 ppm
2. Hippuric acid found automatically in the amino acids standard; Define chromatograms with allowed error 5 ppm
3. Hippuric acid found manually in the female urine sample; Define chromatograms with allowed error 20 ppm
4. Hippuric acid found manually in the female urine sample; Define chromatograms with allowed error 20 ppm

Hippuric acid wasn't found automatically in any urine sample even if the concentration of this compound is relatively high. The illustration (figure 27) of female urine chromatograms shows us the problem with accuracy.

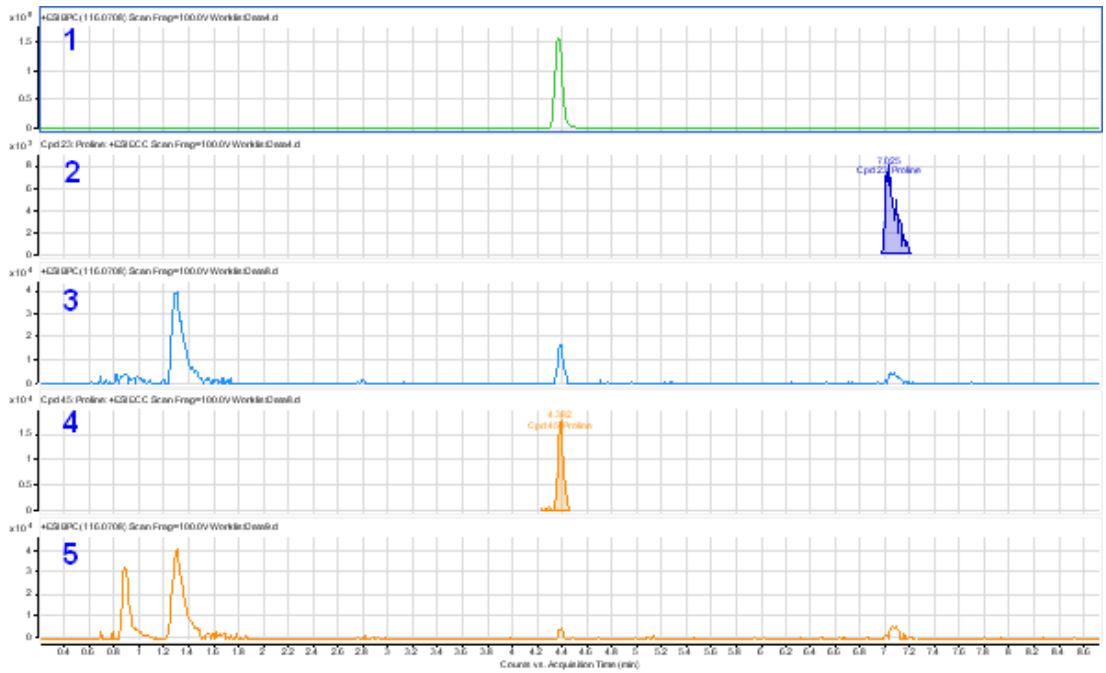
Figure 27: Chromatogram results, Hippuric acid, female urine



In the case of Hippuric acid reason of the incorrect identification can be signal is too high and the peak is overloaded. Detector can't measure precisely m/z values differ and error is getting higher. That is why with the lower error peak is missing or it is cut.

5.2.2.3 Proline

Figure 28: Chromatogram results; Proline; amino acids standard; female and male urine samples



Legend:

1. Proline found manually in the amino acids standard; Define chromatogram with allowed error 20 ppm
2. Proline found automatically in the amino acids standard; Find by molecular feature with allowed error 5 ppm
3. Proline found manually in the female urine sample; Define chromatogram with allowed error 20 ppm
4. Proline automatically in the female urine sample; Find by molecular feature with allowed error 5 ppm
5. Proline found manually in the male urine sample; Define chromatogram with allowed error 20 ppm

Automatic system found the wrong Proline in the amino acids standard (chromatogram 2). The reason of this mistake is also accuracy. The values of m/z are very different as following spectra previews (figures 29 - 31) show so it is not possible to satisfy 5 ppm error condition. In the female urine sample chromatogram automatic searching results are correct (chromatogram 4) but in the chromatogram from manual searching (chromatogram 5) there are many peaks and the volume of the peak at same retention time (4.4 min) as the peak in the amino acids standard is much smaller than other peaks in the same chromatogram.

Figure 29 - 31: Spectrum preview; Proline (m/z=116.0708); amino acids standard

Figure 29: RT=4.318 min; m/z=116.0709

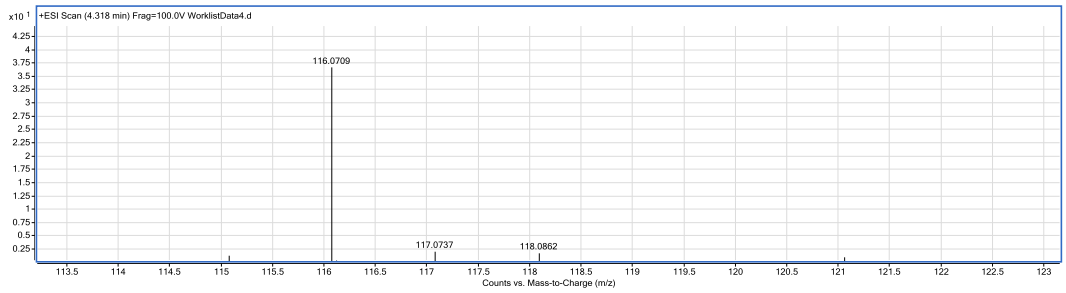


Figure 30: RT=4.374 min; m/z=116.0727

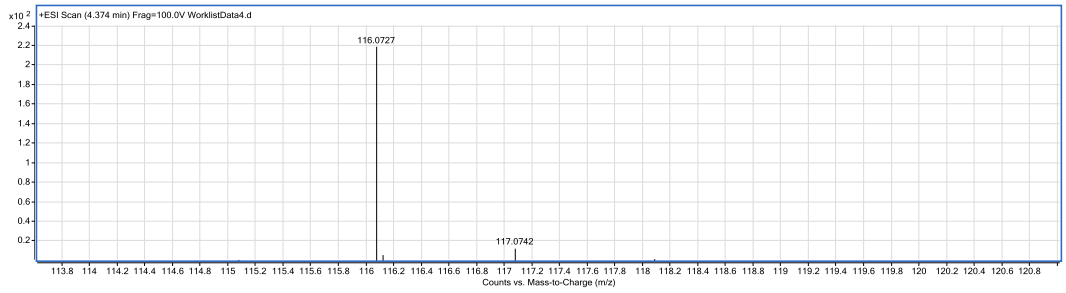
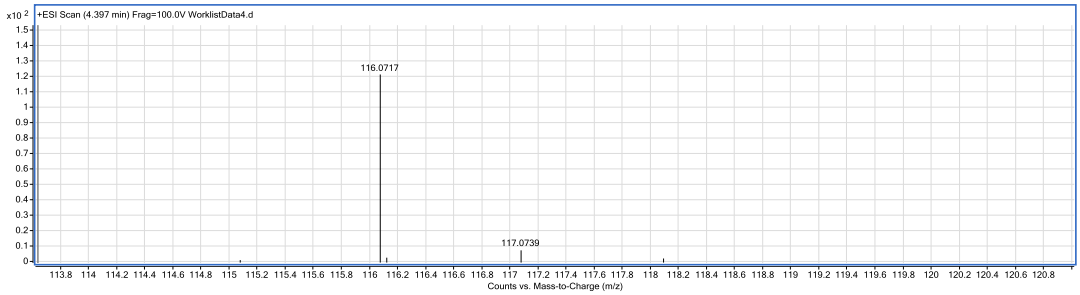
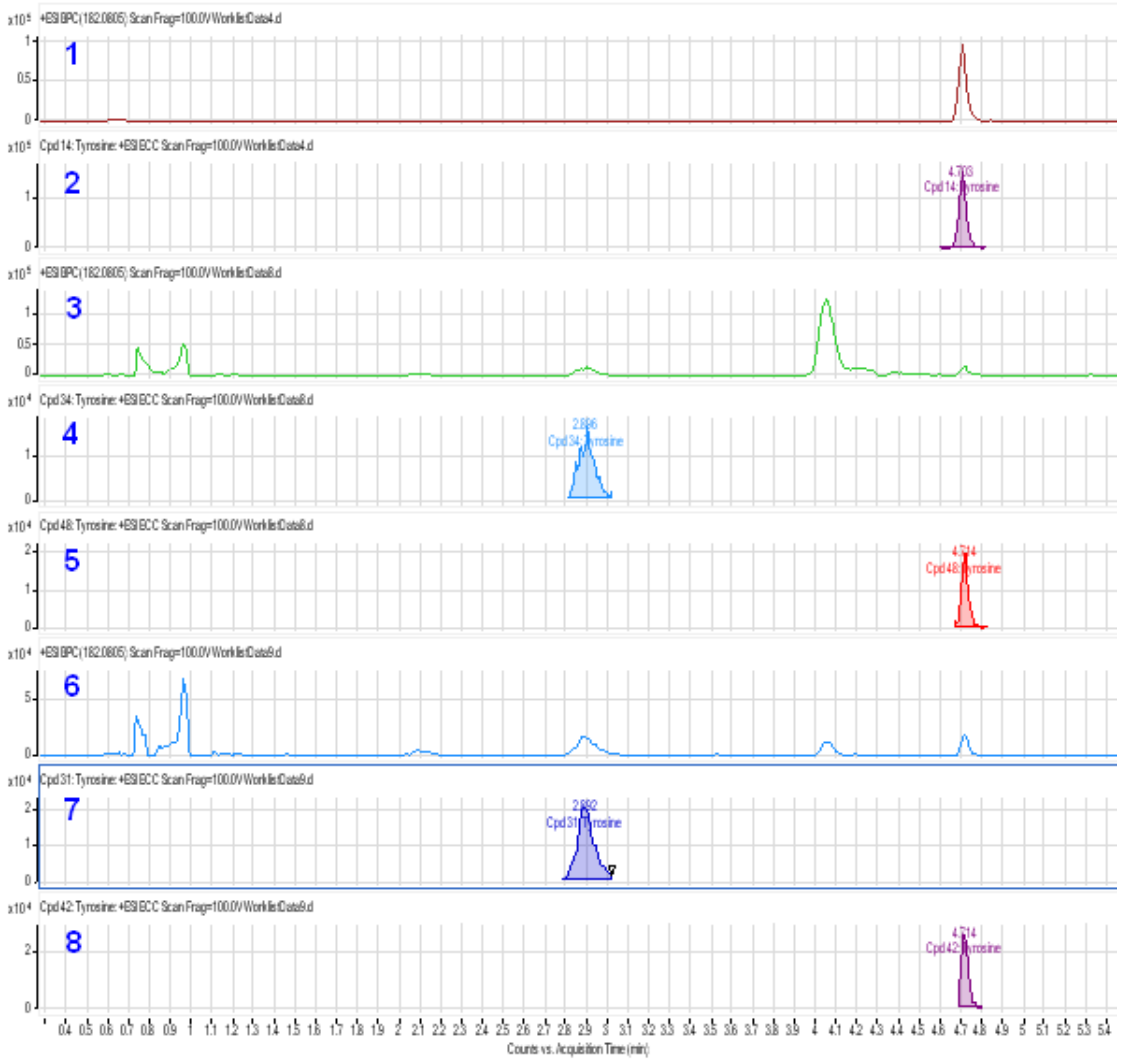


Figure 31: RT=4,397 min; m/z=116.0717



5.2.2.4 Tyrosine

Figure 32: Chromatogram results; Tyrosine; amino acids standard; female and male urine samples



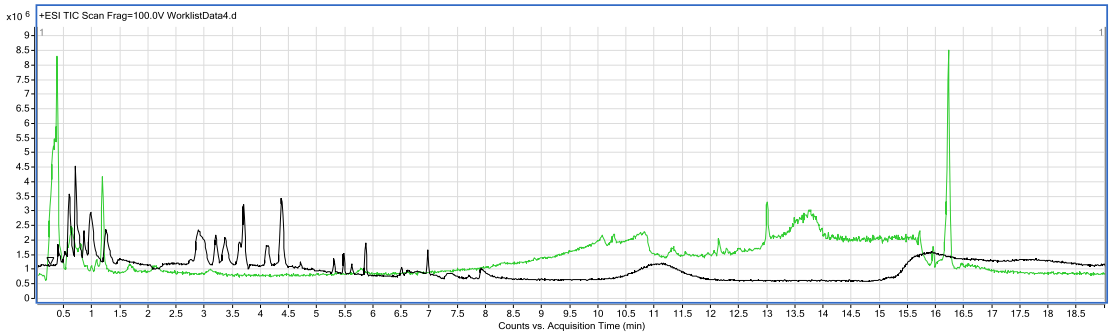
Legend:

1. Tyrosine found manually in the amino acids standard; Define chromatogram, allowed error 20 ppm
2. Tyrosine found automatically in the amino acids standard; Find by molecular feature, allowed error 5 ppm
3. Tyrosine found manually in the female urine sample, Define chromatogram, allowed error 20 ppm
4. Tyrosine found automatically in the female urine sample; Find by molecular feature, allowed error 5 ppm
5. Tyrosine found automatically in the female urine sample; Find by molecular feature, allowed error 5 ppm
6. Tyrosine found manually in the male urine sample; Define chromatogram, allowed error 20 ppm
7. Tyrosine found automatically in the male urine sample; Find by molecular feature with allowed error 5 ppm
8. Tyrosine found automatically in the male urine sample; Find by molecular feature with allowed error 5 ppm

Results of automatic identification are two Tyrosines in both male (chromatograms 4 and 5) and female (chromatograms 7 and 8) urine samples. Also chromatograms of urine samples from manual searching were not clear (chromatograms 3 and 6). According to the amino acids standard the peak at retention time 4.714 min was identified as the right one.

5.3 COMPARING OF THE COLUMNS

Figure 33: Amino acids standard, HILIC column (black line), F5 column (green line)



Results acquired from F5 column (green line) have the majority of the peaks of amino acids at the beginning of chromatogram. The high concentration of peaks at the same moment makes identification more difficult and sometimes confusing. HILIC column (black line) provides better separation and the distribution of the peaks is wider. On the figure 33 we can also see that separation by the HILIC column is much more suitable for amino acids.
Also the number of the identified compounds acquired by HILIC separation is larger as table 10 shows.

Table 10: Total number of compounds acquired from created database by F5 and HILIC column

	Female urine sample	Male urine sampe
F5 column	56	53
HILIC column	69	56

Table 11: F5 column, summary of selected compounds

Name	Formula	Mass	m/z	RT	Volume	Score
Cytidine	C9H13N3O5	243.0852	244.0924	1.151	353402	98.07
Hippuric acid	C9H9NO3	179.0579	180.0652	0.609	586258	99.03
Sarcosine	C3H7NO2	89.0481	90.0554	0.847	356904	86.47
Threonine	C4H9NO3	119.0584	120.0656	0.813	491770	78.21
Tryptophan	C11H12N2O2	204.0895	205.0968	5.778	1237933	99.28
Tyrosine	C9H11NO3	181.0735	182.0808	1.71	503015	84.27
Uracil	C4H4N2O2	112.0276	113.0349	0.56	173054	94.21

Table 12: HILIC column, summary of selected compounds

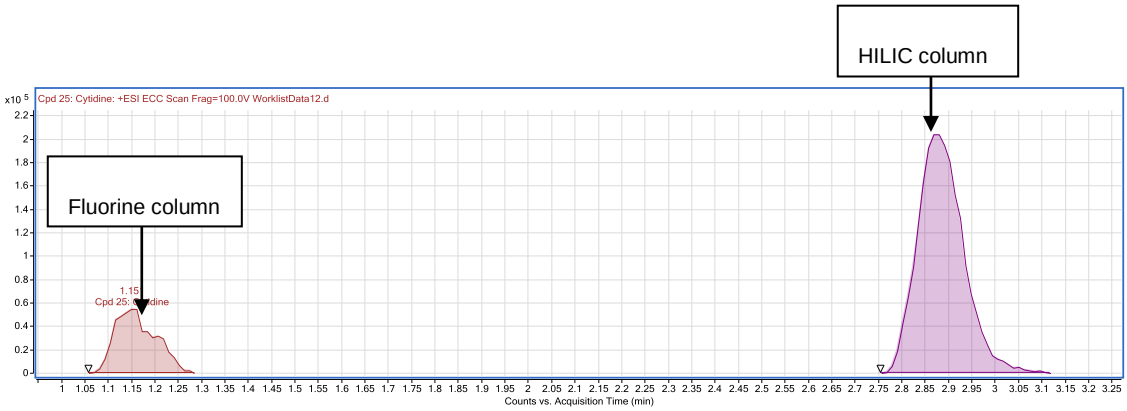
Name	Formula	Mass	m/z	RT	Volume	Score
Cytidine	C9H13N3O5	243.0853	244.0925	2.878	1449388	98.76
Hippuric acid	C9H9NO3	179.0582	180.0655	0.982	2128417	99.75
Sarcosine	C3H7NO2	89.0482	90.0555	5.294	500759	86.91
Threonine	C4H9NO3	119.0582	120.0655	5.488	109524	47.62
Tryptophan	C11H12N2O2	204.0898	205.0971	3.618	2145815	99.9
Tyrosine	C9H11NO3	181.0736	182.0809	4.703	393467	98.53
Uracil	C4H4N2O2	112.0278	113.0351	0.86	412229	86.02

Next figures show differences between volumes and retention times of the same compounds. It is obvious from chromatogram of HILIC column we found higher peaks there and separation is also better hence identification is easier.

5.3.1 CYTIDINE

Column	RT	Volume	Score
Florine	1.151	353402	98.07
HILIC	2.878	1449388	98.76

Figure 34: Chromatogram results, Cytidine, F5 and HILIC column

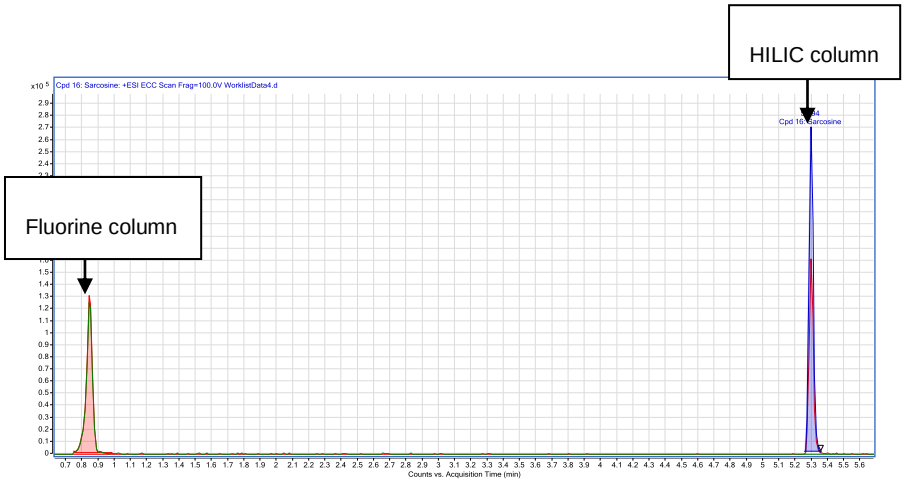


Peak of Cytidine found by HILIC column is four times larger than peak of Cytidine found by F5 column. Also the shape of the peak and symmetry is better with HILIC column. Even if the score of the identification is almost the same in both cases we can say that identification with HILIC column is much better in this case. Difference between retention times is 1.727 min.

5.3.2 SARCOSINE

Column	RT	Volume	Score
Florine	0.847	356904	86.47
HILIC	5.294	500759	86.91

Figure 35: Chromatogram results; Sarcosine; F5 and HILIC column

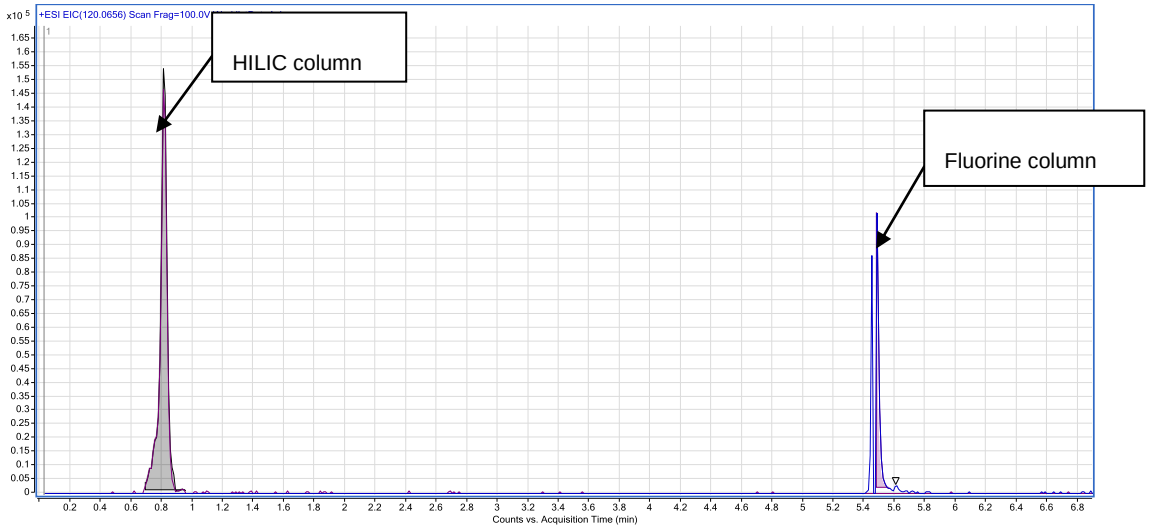


Also Sarcosine has a larger peak with HILIC column separation, around 1.4 times larger than peak with F5 column separation. The shape of the peak is satisfactory in both cases but symmetry is better with HILIC column. Score is nearly the same and the difference between RTs is around 4.4 min.

5.3.3 THREONINE

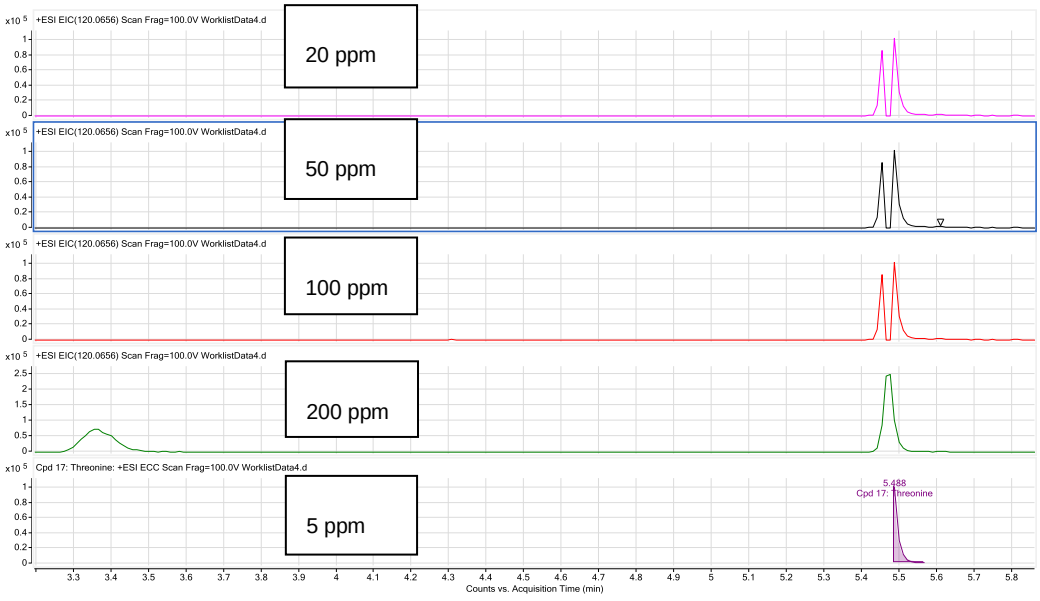
Column	RT	Volume	Score
Florine	0.813	491770	78.21
HILIC	5.488	109524	47.62

Figure 36: Chromatogram results, Threonine, F5 and HILIC column



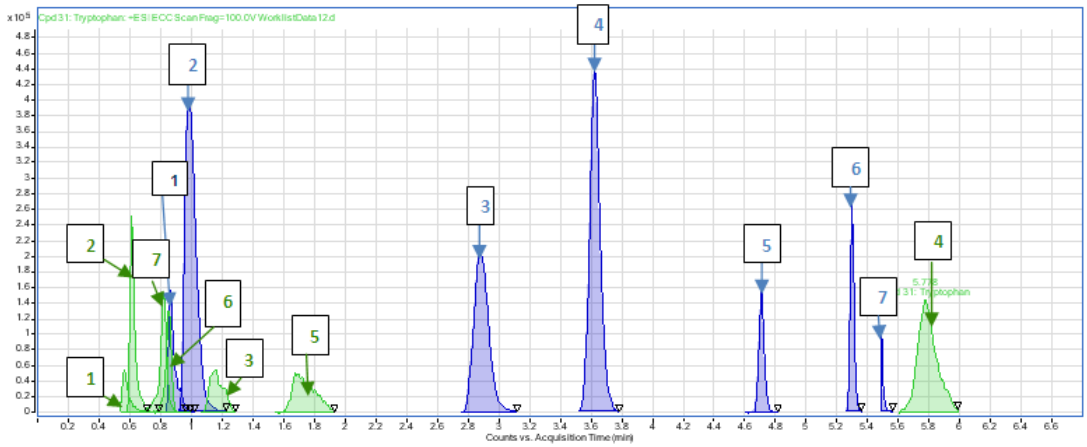
Results of Threonine are also better with HILIC separation method. Peak is larger, in good shape and symmetric. Peak of Threonine on F5 column is cut into smaller parts. Figure 37 describes if with different values of error range peak will be not divided. Strange is that score of the identification is better with F5 column. Difference between RTS is around 4.7 min.

Figure 37: Chromatogram results, Threonine, F5 column



Even if the error range is wide enough the peak of Threonine is still divided into two smaller peaks. Only when it was used error 200 ppm there is full peak but this peak is cut on the top and also second smaller peak at earlier retention time 3.35 min is present.

Figure 38: Chromatogram of seven common compounds on F5 (green) and HILIC (blue) columns



Separation of compounds on HILIC column is much better, with F5 column majority of amino acids are present at the beginning of chromatogram and the definition of the individual peak is more difficult. Also volumes of compounds are larger, score of identification is higher, and more compounds were identified on HILIC column. Results with HILIC column were clearer.

6 CONCLUSION

Metabolomics is very expanding field. As others “omics” sciences also metabolomics promise many benefits not only for the medicine. Even if high technologies are available and there is rapid progress of the methods and software, identification of the compounds is still demanding task. The goal is to find the method which enables to assign unambiguous elemental composition and to find maximum of the compounds contained in the sample.

Examples of the failure of the automatic identification by the Mass Hunter Qualitative Analysis (Agilent Technologies) are only representative demonstration of many others. With the HILIC column better results were achieved especially thanks to better retention of the amino acids. But also with HILIC column there are problems with the identification. It was used UPLC-MS method because in comparison to conventional HPLC-MS, UPLC-MS method showed better peak resolution, sensitivity, and greater S/N ratio. It is necessary to complement database with other data such as isotope ratios and RT or MS/MS spectra for unambiguous identification because mass alone is not enough. Significant impediment of the ability to include RT as a positive step of identification is variance of the RT due to the variations of the temperature, mobile phase composition, and column variability.

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8 SUPPLEMENTS

8.1 ABBREVIATIONS

ECG	Electro Cardio Graph
NMR	Nuclear Magnetic Resonance
MS	Mass Spectrometry
GC	Gas Chromatography
LC	Liquid Chromatography
m/z	Mass-To-Charge Ratio
GC/MS	Gas Chromatography–Mass Spectrometry Combination
CI	Chemical Ionization
MALDI	Matrix-Assisted Laser Desorption Ionization
HPLC	High-Performance Liquid Chromatography, sometimes as High-Pressure Liquid Chromatography
EI	Electron Ionization
ESI	Electrospray Ionization
APPI	Atmospheric Pressure Photoionization
DESI	Desorption Electrospray Ionization
DART	Direct Analysis in Real Time
NanoDESI	Nanospray Desorption Electrospray Ionization
ES	Electrospray
TOF	Time-of-Flight
ICR	Ion Cyclotron Resonance
E or ESA	Electric Sector
B	Magnetic Sector
Q	Quadrupole
IT	Ion Trap
FTICR	Fourier Transform Ion Cyclotron
FT-OT	Fourier Transform Orbitrap
C18	Octadecyl Carbon Chain
C8	Octyl Carbon Chain
F5	Pentafuorophenylpropyl
HILIC	Hydrophilic Interaction Liquid Chromatography

LC-MS	Liquid Chromatography–Mass Spectrometry
BEH	Ethylene Bridged Hybrid
Q-TOF	Hybrid Quadrupole Time-of-Flight
ICP-MS	Inductively Coupled Plasma Mass Spectrometry
METLIN	Metabolite Link database
PCDL	Personal Compound Database and Library
ECC	Extracted Compound Chromatograms
SOP	Standard Operating Procedure
UPLC/MS	Ultra Performance Liquid Chromatography – Mass Spectrometry Connection
RT	Retention Time
KEGG	Kyoto Encyclopedia of Genes and Genomes
FTMS	Fourier Transform Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
CAS	Chemical Abstract Service
ppm	Pars per Million
ACN	Acetonitrile
AA	Amino Acid
M	Male
F	Female
S/N	Signal-to-Noise Ratio

8.2 TABLE OF FIGURES

8.3 LIST OF TABLES