PALACKÝ UNIVERSITY OLOMOUC

Faculty of Science

Department of Biochemistry

Development of a HPLC-TMS screening method for diagnosing of inherited metabolic disorders

MASTER OF SCIENCE THESIS

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I hereby declare that this Master of Science thesis was written solely by myself on the basis of the cited references.

Olomouc, 22nd April 2011

Anna Barešová

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Jméno a příjmení autora	Bc. Anna Barešová
Název práce	Vývoj HPLC-TMS screeningové metody pro diagnostiku dědičných metabolických poruch
Typ práce	Magisterská
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Vedoucí práce	RNDr. David Friedecký, Ph.D.
Rok obhajoby práce	2011
Abstrakt	V experimentální části této práce je představena screeningová metoda pro dědičné metabolické poruchy (DMP) pomocí vysokoúčinné kapalinové chromatografie (HPLC) s detekcí tandemovou hmotnostní spektrometrií (TMS). Jsou zde srovnány různé chromatografické podmínky použité v průběhu vývoje metody a uvedeny výsledky optimalizace TMS a validace konečné metody. Potenciální přínos pro diagnostiku DMP je ilustrován analýzou vzorků močí od pacientů se známými DMP, kontrolních močí a vzorků se standardním přídavkem látek charakteristických pro vybraná onemocnění. V teoretické části jsou ve stručnosti popsány techniky HPLC a TMS a krátce uvedeno téma dědičných metabolických poruch.
Klíčová slova	vyskoúčinná kapalinová chromatografie, hmotnostní spektrometrie, dědičné metabolické poruchy, novorozenecký screening
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Abstract	A screening method for multiple inborn errors of metabolism by high performance liquid chromatography tandem mass spectrometry (HPLC-TMS) is presented in the experimental part of this thesis. Several chromatographic conditions used during method development are compared and results of TMS optimisation and final method validation are shown. The potential for application in screening for inborn errors of metabolism (IEMs) is demonstrated by measurement of urines of patients with known IEMs, control urines and samples spiked with metabolites characteristic for selected IEMs. In the theoretical part the used techniques of HPLC and mass spectrometry are described and the topic of IEMs is briefly introduced.
Keywords	high-performance liquid chromatography, mass spectrometry, inborn errors of metabolism, newborn screening
Number of pages	65
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Language	English

Contents:

Aims of the thesis:

1. Providing a brief introduction into mass spectrometry and inborn errors of metabolism and a review of chromatographic analyses of small molecules.

2. Optimisation of multiple reaction monitoring of selected compounds related to inherited metabolic disorders.

3. Development and validation of an ultra-high performance liquid chromatography – tandem mass spectrometric method for analysis of the chosen compounds in urine.

4. Analysing of urine samples of patients with inherited metabolic disorders and controls.

THEORETICAL PART

1 Mass spectrometry

Mass spectrometry (MS) is a widely applicable analytical technique. It may provide information about qualitative and quantitative composition of samples, structures of molecules, elemental composition, isotopic ratios of atoms or structure and composition of solid surfaces. In principle, ions of the analyte are formed and subsequently separated according to their mass-to-charge ratio (m/z).

A mass spectrometer consists of an ion source, one ore more successively linked mass analysers and a detector. Components are controlled and data processed by a computer (Fig. 1). Vacuum is maintained in the analyser and detector. Pressure in the ion source depends on the type of ionisation.

The following paragraphs give a brief overview of MS principles and are focused mainly on electrospray-ionisation quadrupole mass analysis because these were utilised in the practical part of this diploma thesis.

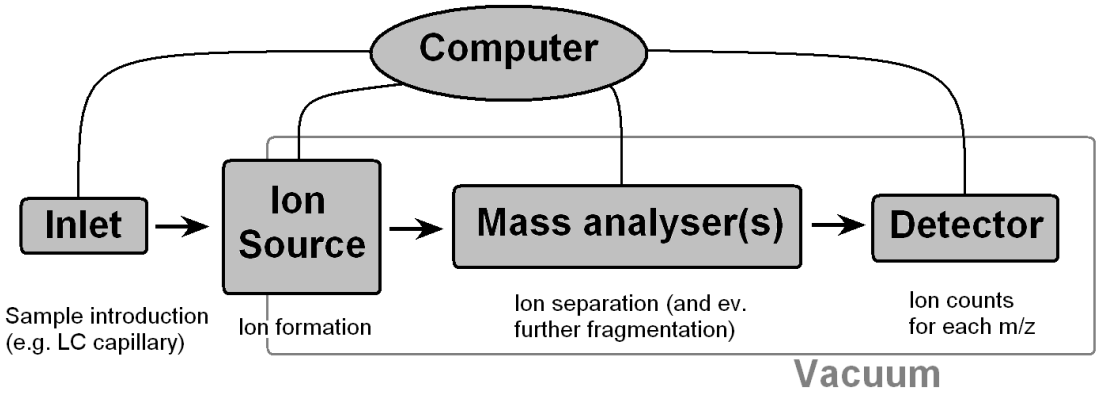

Fig. 1: Diagram of a mass spectrometer with atmospheric pressure ionisation.

1.1 Ionisation

Ions are generated from molecules in order to enable separation of compounds by application of electromagnetic field in the analyser.

In molecular MS two general groups of ion sources are recognised. Hard sources transfer a large amount of energy to ionised molecules, which are left in highly excited energy states. Relaxation is usually accompanied by excessive fragmentation of the molecule. Consequently, complex information about molecular structure is provided but the molecular ion may not be present in the spectrum. This, on the other hand, is typical for soft sources, which cause only limited fragmentation. In gas-phase sources the sample is first vaporised and subsequnetly ionised, while in desorption

sources ions are generated directly from the liquid or solid sample. Desorption sources are more convenient for thermally labile and large molecules because these may decompose prior to ionisation and it may be difficult to vaporise molecules with more than 1000 Da [Skoog, *et al.*, 1998 - p. 500].

Electron ionisation (EI) is a classical hard ion source. Ions are generated by the passage of high energy electrons close to vaporised sample molecules. Also chemical and field ionisation belong to gas-phase ion sources, but cause less fragmentation. Desorption sources include for example electrospray ionisation (ESI), thermospray ionisation, matrix-assisted laser desorption ionisation (MALDI), fast atom bombardment or secondary ion mass spectrometry. Today ESI is the most widely used method for ionisation of polar biomolecules up to high molecular weights, chemical ionisation is more suited for analysis of smaller and less polar compounds, MALDI applications are typical for protein analyses.

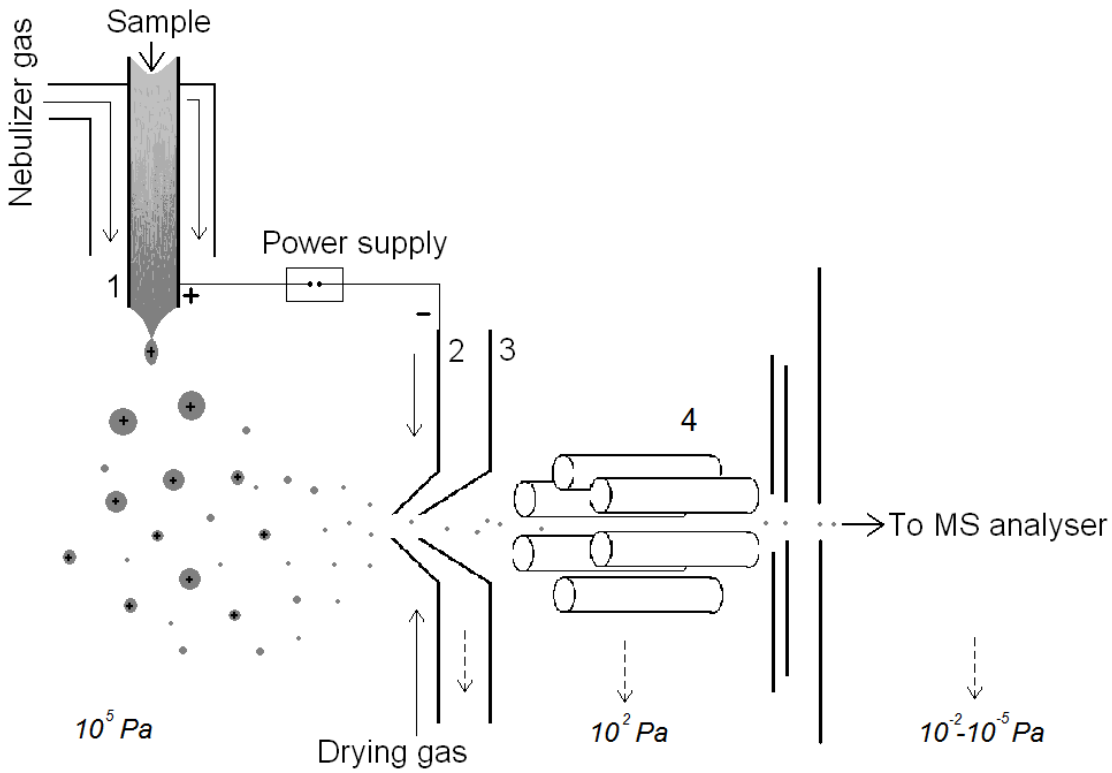

Fig. 2: Diagram of an orthogonal electrospray ion source. 1 – capillary, 2 – sample cone with orifice, 3 – skimmer, 4 – ion optics

As a typical soft ion source, ESI generally allows identification of the molecular ion and only very limited fragmentation. Ionisation takes place under moderate temperatures and atmospheric pressure and is convenient even for thermally unstable molecules. Large molecules with multiple ionisation sites, such as proteins, become

multiply charged, which enables their analysis even if their molecular weight lies outside the detection range of common analysers.

The sample solution is introduced into the ion source through a capillary surrounded by a needle with a high potential difference to the counter electrode forming the orifice. At the capillary tip liquid, usually pumped in µl/min flow rates, forms a Taylor cone from which charged droplets are emitted. These are guided by a potential and decreasing pressure gradient toward the orifice. Drying gas encourages solvent evaporation from the droplets (Fig. 2). When a droplet has shrinked to the point when its surface tension yields to the accumulated charge (Rayleigh limit), a coulombic explosion occurs, producing smaller droplets (Fig. 3). There are two models describing ion formation from microdroplets. The charged residue model assumes ion formation through complete evaporation of the solvent. According to the ion-evaporation model ions are formed by desorption from the droplet due to the electric field. Different mechanisms may play a role under different conditions [Gross, 2011 – pp. 582-583]. Ions are accelerated by a decreasing potential and pass as a beam focused by a set of electromagnetic lenses into the mass analyser.

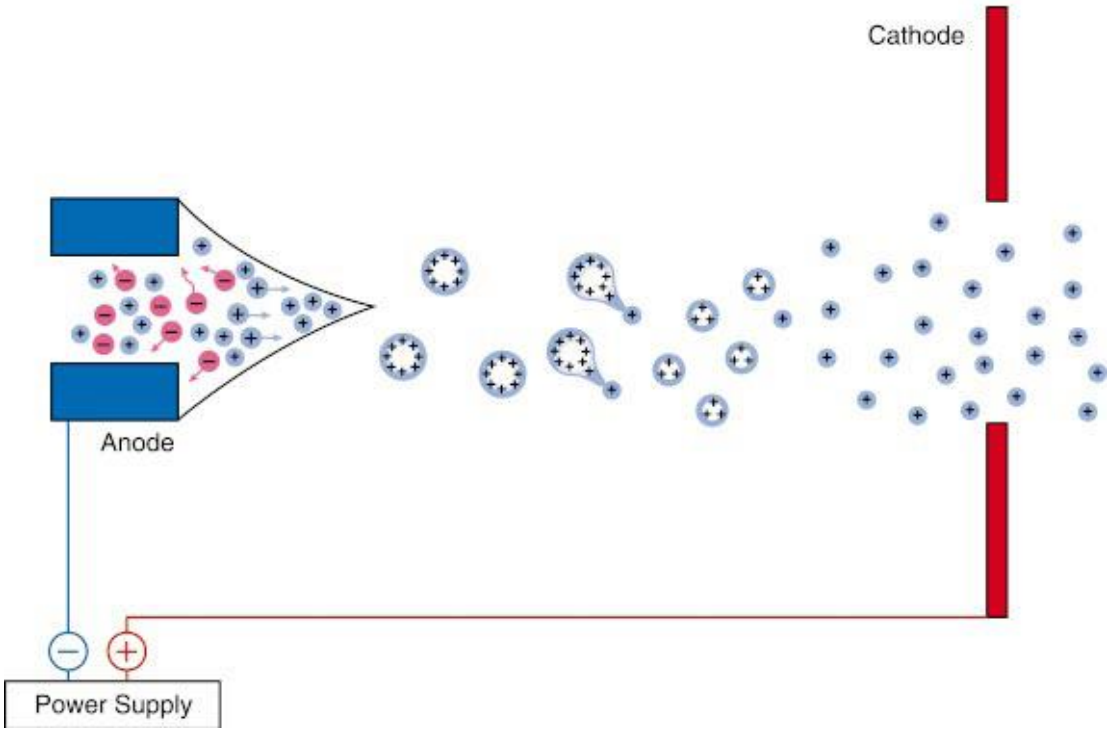

Fig. 3: Ion formation in electrospray ionisation [Griffith *et al.*, 2001].

1.2 Mass analysis

Trajectories of ions generated in the ion source are influenced by an electric or magnetic field in a way, that only ions of a certain m/z value reach the detector

at a time. Vacuum is strictly required for ion mass analysis. In higher pressures analyte ions would collide with present molecules and fragment or lose their charge.

There are several basic types of mass analysers. Sector instruments, which are the oldest type in use, offer high resolution and are suitable for exact mass analysis. Time-of flight (TOF) analysers are moreover theoretically unlimited in their mass range and therefore widely used in proteomics. So far, best resolution is obtained by ion cyclotron resonance, which represents the most expensive analysers. Quadrupole instruments have lower (usually unit) resolution but they provide high scan rates and are relatively cheap. In ion traps, ions of a selected m/z are accumulated in a quadrupole or between two electrodes (Orbitrap) and may be released at one point.

In a quadrupole instrument ions pass between four cylindrical or hyperbolical rods that serve as electrodes. Direct current (DC) of the same polarity is applied to opposite rods and radiofrequency alternating current (AC) is superimposed to each pair. Under a certain AC/DC potential ratio and a defined AC amplitude only ions of a distinct m/z pass through the analyser to the detector (Fig. 4).

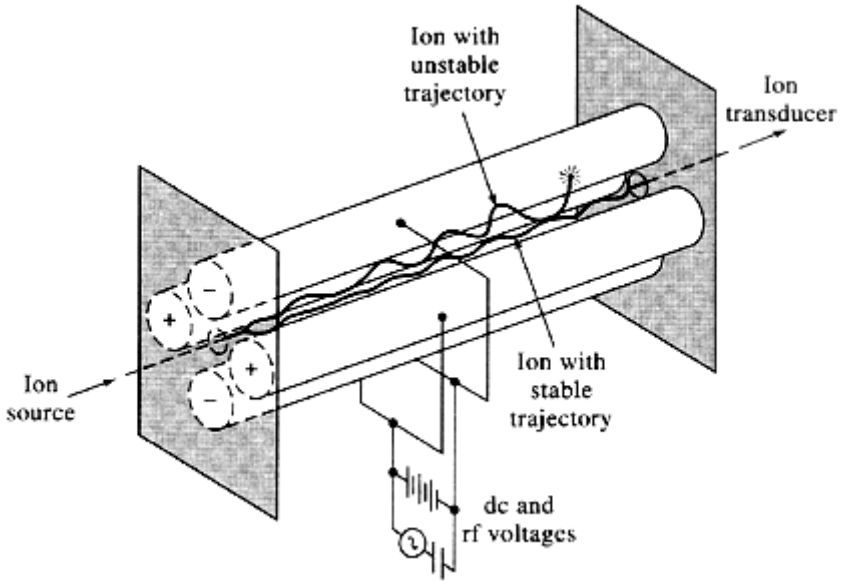

Fig. 4: Quadrupole mass analyser.
[http://www.clu-in.org/char/technologies/quadmass.htm]

In the case of cations oscillations caused by the AC field are increased by the rods with negative DC potential. Lighter (lower m/z) ions' oscillations are influenced to a greater degree. Therefore they hit the surface of the rods and lose their charge, while heavier (higher m/z) ions pass through. On the other hand, trajectories

of cations are destabilised by the positively charged pair of rods. Thus heavier ions are defocused while lighter ions, stabilised by the AC field, pass through to the other end. Therefore the pair of rods with the charge identical to passing ions acts as a low-pass filter, the pair with the opposite potential as a high-pass mass filter.

Quadrupoles (and preferably hexa- and octopoles) with AC only are employed as ion guides and collision cells. They transmit ions with little m/z discrimination and allow neutral molecules to be removed [Gross, 2011 - p. 153].

1.3 Performance of a mass spectrometer

Sensitivity of a mass analyser is defined as the detector response (expressed as the cumulative charge of ions of a certain m/z) related to their amount flowing through the ion source. It is expressed in C/µg. Sensitivity is one parameter affecting limits of detection (LD) of compounds, which are determined in individual methods. LD expresses the smallest measurable amount of the coumpound in a sample and is given at a certain signal-to-noise ratio (usually S/N = 3).

Apart from chemical noise resulting from impurities in the sample and tubing, there is electronic noise which is generated by fluctuations in the instrument's circuits and can be reduced by longer data acquisition.

Resolution may be calculated as $R = m / \Delta m$ (single charged molecules are assumed), where Δm is the difference between masses of compounds in two adjacent peaks or full width of the peak at half maximum height. Therefore, theoretically a quadrupole instrument with unit resolution will be able to reach R = 100 for ions with m/z = 100, but R = 10,000 for ions with m/z = 10,000. This is why resolution in MS is considered mass dependant. Resolution is determined by instrument settings (scan speed, type of mass selection), speed of the electronics and concentration of the sample (detector saturation).

Accuracy is affected by resolution, instrument calibration and its potential drift over time. It can be expressed as the difference between measured mass and theoretical mass divided by theoretical mass, and usually is expressed in ppm.

1.4 Ion detector

At the detector the beam of ions is transduced to an electrical signal, most frequently by electron multipliers. In high energy ion beams, such as in magnetic sector MS, ions emitted from the analyser have enough energy to eject electrons from the

dynode. Ion beams with less kinetic energy, as in quadrupoles, need to be accelerated before striking the surface of the electron multiplier [Skoog *et al.*, 1998 - p. 257].

1.5 Tandem mass spectrometry

Single MS identification of compounds is limited by the occurence of isomers which yield similar spectra by in source fragmentation. In tandem mass spectrometry (TMS) mass analysis is followed by fragmentation of the analyte and detection of product ions. This arrangement enables to monitor the molecular ion and its fragmentation in one step. While the molecular weight of two different compounds may be identical, fragmentation is likely to occur at different sites of the molecule. Thus TMS allows identification of compounds even in more complex mixtures.

The most common type of TMS is a triple quadrupole. From the ion source ions are accelerated into the first quadrupole (Q1), where molecular (precursor) ions are separated. The separated ions enter into a second quadrupole (q2), which has no DC applied to its rods and operates as a collision cell. Here precursor ions collide with atoms of a neutral gas and are broken into product ions in a process called collision induced dissociation. Product ions are then separated in the usual way in the third quadrupole (Q3).

In TMS diverse mass analysers may be linked in series. Combinations of different analyser types (e.g. Q-TOF) form a so called hybrid instrument. Besides TMS divided in space, as discussed so far, consequtive experiments may be carried out in time on analysers such as ion trap or cyclotron resonance.

TMS enables a variety of operation modes. For example, a precursor ion scan analyses all precursor (molecular) ions producing a fragment of a certain m/z. Similarly, a product ion scan detects fragments originating in one precursor ion. A constant neutral loss scan facilitates identification of compounds that easily lose an identical part of the molecule. Selected reaction monitoring (SRM) records only a certain precursor ion and its selected product ion (Fig. 5).

In contrast to TMS modes described above, a Q1 full scan monitors all m/z entering the mass analyser from the ion source and excludes the other quadrupoles. This mode of operation is less suited for quadrupole instruments because of their lower resolution than for example TOF analysers.

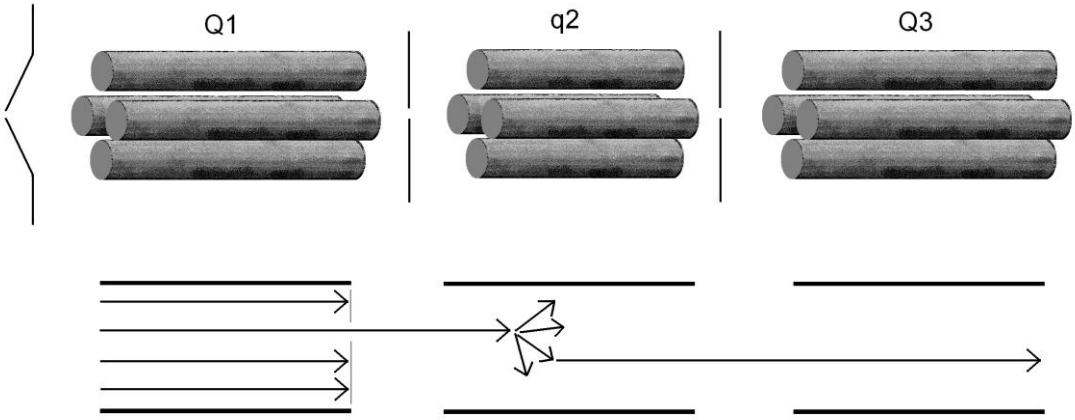

Fig. 5: Diagram of selected reaction monitoring on a triple quadrupole.

1.5.1 Multiple reaction monitoring

As mentionned above, in selected reaction monitoring Q1 and Q3 act as mass filters: Q3 detects one product of fragmentation of a precursor ion selected in Q1. Fragmentation takes place in q2 that acts as a collision cell. By switching among selected potentials multiple ion transitions may be sequentially acquired but time is not wasted by monitoring all m/z as in a full scan. Therefore multiple reaction monitoring (MRM) is widely applied to analysis of complex biological samples. Triple quadrupoles are favourable for MRM because of their ability of fast switching of m/z.

To a certain degree longer data acquisition for one ion transition (i.e. dwell time) increase sensitivity by increasing the signal-to-noise ratio. On the other side, longer dwell times lengthen the period of data acquisition for the whole set of ion transitions and in consequence reduce number of points in the chromatogram.

Enough time needs to be granted for complete removal of ions from one precursor from the collision cell before switching to the next m/z acquisition. If collision products leftovers from the previous precursor ion remain in the collision cell and the following ion transition has an identical product mass, a false signal may be observed (cross talk).

2 High Performance Liquid Chromatography

Principles of chromatography were discovered at the beginning of the 20th century by Michail S. Cvet, who employed adsorption column chromatography to separate plant pigments. Since then a number of chromatographic methods has been developed. General classes of gas, liquid and supercritical-fluid chromatography can be further distinguished by their separation mechanism (e.g. partition or adsorption, ion-exchange, size-exclusion chromatography).

In chromatography, the sample is carried in the mobile phase and separation of analytes occurs as the result of their various strength of interactions with the stationary and mobile phase. Components weakly retained by the stationary phase move faster and elute earlier than those held by a stronger interaction. Therefore, a sample applied to the chromatographic column separates into bands moving one after another. Detector response to the separated bands of compounds of interest creates peaks of the chromatogram (Fig. 6).

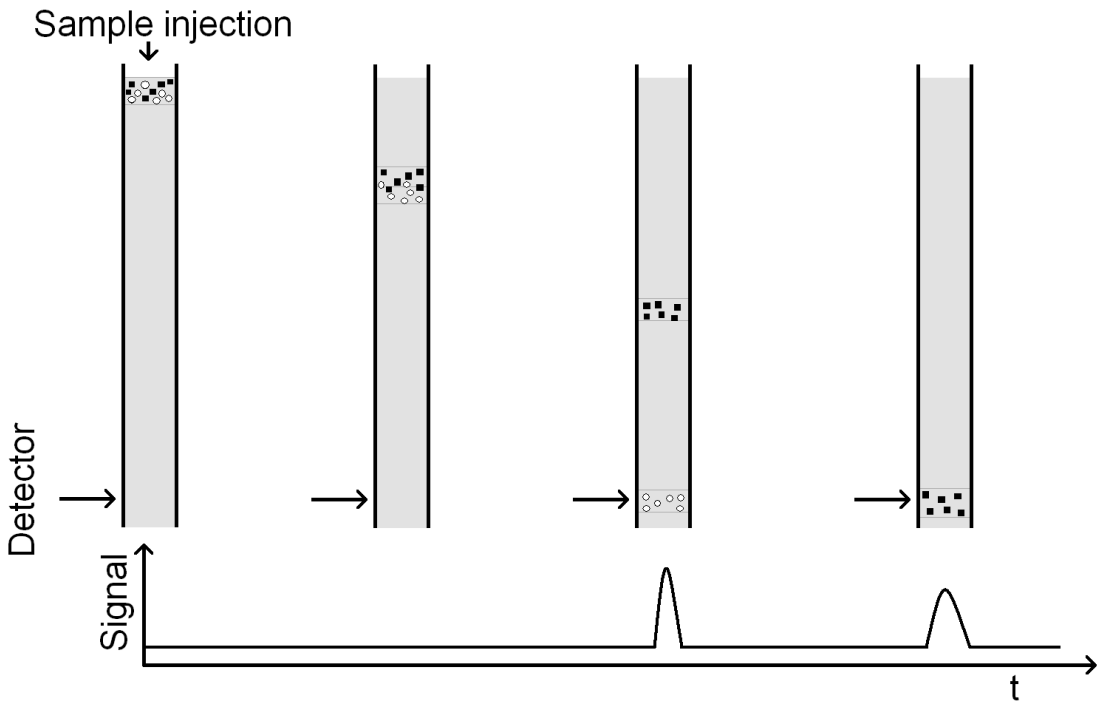

Fig. 6: Chromatographic separation of a two component mixture.

High performance liquid chromatography (HPLC) is a type of column liquid chromatography, which utilises very small column particles (3-10 μm) and operates at high pressures (up to 400 bar) in order to decrease the time of analysis and increase

separation eficiency and resolution. Further improvement has been achieved with ultra-high performance liquid chromatography (UHPLC), which utilises particles below 2 μm and pressures over 1,000 bar.

2.1 Principles of HPLC separation

2.1.1 Retention

Retention of a compound characterises its afinity to the stationary phase under given conditions and is described by comparison of its retention time to the column void time - the retention factor k.

$$k = \frac{t_r - t_0}{t_0}$$ (t_r…retention time; t_0…void time)

The void time (i.e. time required for an unretained molecule to pass the column) can be determined from a chromatogram as the first disturbance of the baseline, by injection of an unretained compound or estimated from column dimensions.

$$V_0 \approx \frac{1}{2} L d^2$$ (V_0 - void volume; L - column length; d - column diameter)

$$t_0 = \frac{V_0}{F}$$ (F - flow rate)

2.1.2 Efficiency

Column performance is characterised by its efficiency of separation (theoretical plate number N). A highly efficient column (large N) produces narrow peaks.

$$N = 5.54 \left(\frac{t_r}{W_{1/2}} \right)^2$$ ($W_{1/2}$ - peak width at half maximum height)

Column efficiency also may be expressed in terms of theoretical plate hight H.

$$H = \frac{L}{N}$$ (L - column length)

N increases with longer columns, smaller or core shell particles (shorter diffusion path), less extracolumn effects, lower mobile phase viscosity, higher temperature and, to a certain extent, lower flow rates (Fig. 7). Contributions to theoretical plate hight are described by the Van Deemter equation.

$$H = A + \frac{B}{u} + Cu$$ (A, B, C - terms of partial contributions; u - linear velocity)

Term A represents eddy diffusion (dispersion of molecules due to varied flow paths through the column packing), B longitudinal diffusion and C resistance

against mass transfer (time required to obtain equilibrium distribution of a compound between the stationary and mobile phase).

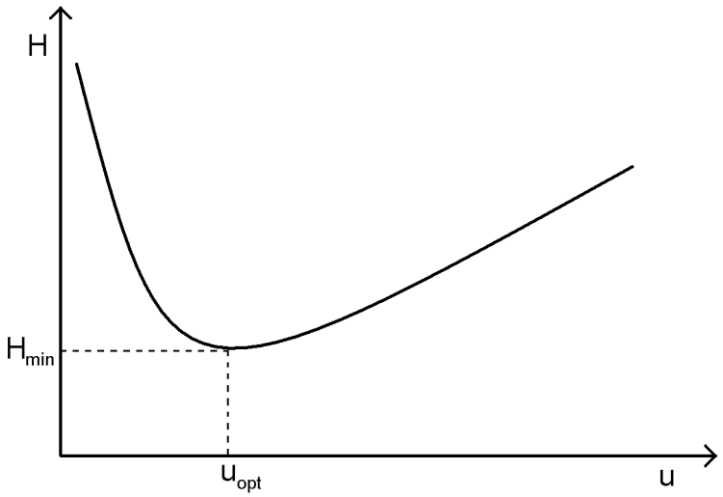

Fig. 7: Overall dependence of plate height on flow rate. (H_{min} – minimal plate height; u_{opt} – optimal flow rate)

2.1.3 Resolution

The distance between two separate peaks determines resolution of their separation.

$$R = \frac{2(t_1 - t_2)}{W_1 + W_2}$$ (t_1,t_2 – ret. time of peaks 1 or 2; W_1,W_2 - baseline peak widths)

For correct quantitation baseline separation si required, which corresponds to R > 1.5 for peaks of similar height. In the case of overlapping peaks determination of baseline widths may be unreliable and, similar to efficiency, resolution may be calculated more accurately from peak width at half maximum height.

$$R = \frac{1.18(t_2 - t_1)}{W_{1/2;1} + W_{1/2;2}}$$

Resolution is dependant on the retention of compounds of interest, efficiency of the column and selectivity of the analyte-column surface interaction. The impact of these conditions can be expressed by the following equation (k - average retention factor of two adjacent peaks; N - column plate count; α - separation factor).

$$R = \frac{1}{4}(\alpha - 1) \cdot \sqrt{N} \cdot \frac{k}{1 + k}$$

The separation factor α is a measure of the system‘s selectivity.

$$\alpha = \frac{k_2}{k_1}$$ (k_1,k_2 - retention factors of two adjacent peaks)

For complete separation of two peaks R = 1.5 is optimal. However, often separations with lower R are satisfactory. In this case relative heights of adjacent peaks need to be taken into account (Fig. 8).

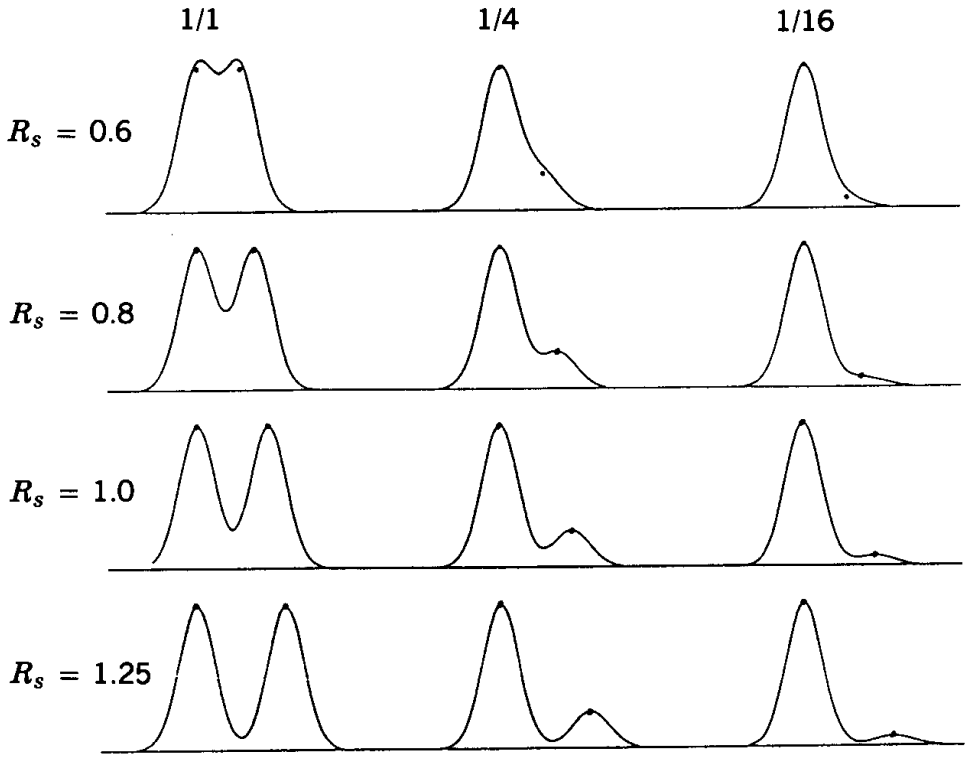

Fig. 8: Resolution curves of peaks with peak area ratios 1:1, 1:4 and 1:16, respectively. [Snyder *et al.*, 1997 - p. 25]

2.1.4 Optimisation of separation conditions

Retention and selectivity are influenced by conditions that affect distribution of the sample between the mobile and stationary phase, such as mobile or stationary phase composition or temperature. Usually resolution improves as the compounds elute later (i.e. k increases). However, excessive separation time is undesirable for practical applications and causes peak broadening due to analyte zone dilution. On the other hand, peaks of too weakly retained compounds may interfere with the initial baseline disturbance at t_0, which disables precise quantitation. A gradient of different mobile phase constituents may be essential in separation of compounds with distant chemical properties.

If two peaks overlap in spite of sufficient retention, resolution may be achieved by modifying the system's selectivity (α). Mobile phase selectivity depends on the type of interactions between molecules of the solvent and the analyte. Solvents can be characterised by their predominant interactions with the solute as forming hydrogen

bonds (acidic or basic) or dipolar. If for example a methanol (hydrogen-bond forming) containing mobile phase does not provide sufficient separation, acetonitril (dipolar) may be used to alter selectivity [Johnson & Vitha, 2010]. In the case of ionic compounds selectivity also depends on their ionisation state and changes in pK_a and can be improved by change of pH, buffer composition or concentration, organic phase content or addition of ion-pairing reagents.

Temperature control is a fundamental condition for reproducible analyses. Temperature influences retention, effectivity and selectivity by change of mobile phase viscosity, diffusion coefficient and equilibria of the analyte and may be used to improve performance [Hao *et al.*, 2008]. Increase in column temperature by 1°C usually decreases retention by 1 to 2% [Tran *et al.*, 2001, Snyder *et al.*, 1997 - p. 40]. Selectivity is affected especially in separations of ionic species [Dolan, 2002].

A radical change in selectivity can be achieved by replacing the analytical column.

2.2 Mechanisms of separation

General principles of chromatographic separation are applicable to any chemical compound but in a more detailed view separation of macromolecules may have specific characteristics because of their size and structure. Regarding the target analytes of the practical part of this thesis special consideration will be given to small molecules.

2.2.1 Partition and adsorption chromatography

Compounds may be separated according to their polarity. In normal-phase (NP) chromatography the stationary phase is polar and mobile phase consists of less polar organic solvents, whereas in reversed-phase (RP) chromatography stationary phase is non-polar and mobile phase contains water and/or more polar organic solvents. Therefore the most polar component of a mixture elutes last in NP and first in RP chromatography. The term normal-phase has its reason in history – first separations were carried out in this mode.

Unmodified silica may be used in NP separations but more often polar bonded residues are used, such as nitriles, diols or amines. Generally, NP-LC is suitable for water insoluble samples *et al.*, McLaren 2011] or very polar samples that are poorly reatained in RP-LC. Further, it may be advantageous for isomer separations or removal of non-polar contaminants. Presence of water and protic solvents affects negatively reproducibility of retention times because of deactivation of silica stationary phase

[Snyder *et al.*, 2010 - p. 393, Jandera, 2002, Lu & Rustum, 2009]. Polar modifiers, e.g. methanol or isopropanol, may be used deliberately to improve reproducibility [Younes *et al.*, 2011].

On the contrary to NP, RP-LC is applicable to a wide range of compounds including lipids, aromatic acids, saccharide derivates and aminoacids [Masoodi *et al.*, 2010, Lionetto *et al.*, 2008, Zuo *et al.*, 2008, van der Ham *et al.*, 2007, Waterval *et al.*, 2009]. Bonded alkyl groups (C8, C18) are a typical stationary phase. In relation to non-polar compounds longer alkyl chains result in higher retetion and separation efficiency. This is the reason why C18 stationary phases are probably the most frequently applied in HPLC. As for the mobile phase, a combination of acetonitrile and buffer or water is a common choice [Hou & Ding, 2010, Waterval *et al.*, 2009, van der Ham *et al.*, 2007]. In the case of water as polar component pH often is adjusted by weak acids, e.g. 0.1% formic or acetic acid [Clariana *et al.*, 2010, Perera *et al.*, 2010]. Gradients from 100% acetonitrile to 100% aqueous buffer may be applied [van der Ham *et al.*, 2007]. In some cases methanol was used as the organic component, for example in separations of purine and pyrimidine nucleosides, phenolic acids, flavonoids and coumarins [la Marca *et al.*, 2006, Novakova *et al.*, 2010]. A C8 modified hybrid particle (silica + polymer) column was used for example in a sensitive analysis of extracellular adenosine in cell culture medium [Van Dycke *et al.*, 2010]. Conversion of polar compounds prior to analysis may facilitate RP separation, as in the case of mevalonic acid transformed to mevalonic acid lactone to be analysed on a pentafluorophenyl column [Waldron & Webster, 2011].

Ionic species, which are too weakly retained on RP columns, may be separated by ion-pair chromatography (IPC). In this case stationary and mobile phase composition is in principle the same as in RP-LC, except of the presence of an ion-pairing reagent with opposite charge to the analyte. By reaction of the two ions, charge of the analyte is eliminated and its retention increased. A simultaneous analysis of several purine and pyrimidine bases and nucleotides, N-acetylated amino acids, dicarboxylic acids and other metabolites has been achieved on a C18 column by addition of tetrabutylammonium hydroxide as an ion-pairing reagent [Tavazzi *et al.*, 2005]. Nucleosides and nucleotides from food and beverages were separated by gradient elution of ammonium oxalate and acetonitrile in the presence of 1.25mM dihexylammonium acetate [Yamaoka *et al.*, 2010].

Aqueous normal phase (ANP) chromatography has overcome complications arising from polar solvent presence in NP by adding a defined amount of water to the mobile phase and represents an increasingly popular approach. A polar stationary phase (e.g. silica or its amine, amide or diol modifications) is used

in combination with a mobile phase composed of water and organic solvent. Partition of molecules takes place between a stationary layer of polar liquid held near the column surface and the less polar bulk of mobile phase. Other mechanisms, e.g. ion-exchange, also contribute to separation [Hao *et al.*, 2008]. Depending on sample, stationary phase and mobile phase composition retention in ANP varies between RP and NP-LC [McCalley & Neue, 2008]. Because of different interactions between solutes and stationary phase depending on mobile phase composition, minimal retention may be observed at intermediate organic/aqueous mobile phase ratios. This was found for pyridine separated on a bare silica column [Gritti *et al.*, 2009]. Type of stationary phase also strongly affects selectivity [McCalley, 2010].

Sometimes two overlapping areas are distinguished in ANP. While hydrophilic interaction chromatography (HILIC) contains a minority fraction of water in the mobile phase and separations are similar to NP-LC, methods using a major water portion are sometimes called per aqueous liquid chromatography (PALC) or reversed HILIC because retention of solutes becomes more RP like.

Both HILIC and PALC separations may be used for a wide range of compounds. Several column types were tested in HILIC mode in a metabolomic analysis of *Escherichia coli* extracts. An aminopropyl column with gradient elution by 20mM ammonium acetate (pH 9.45) and acetonitrile allowed analysis of 141 metabolites [Bajad *et al.*, 2006]. A bare silica column was used in HILIC mode for oxypurinol analysis [Stocker *et al.*, 2010]. Aqueous normal phase may be advantageous for polar compounds with low retention in RP-LC. For example, homocysteine, cysteine and methionine often elute with the void volume and suffer by matrix effects in mass spectrometry detection. By using a silica hydride-based stationary phase with graduent elution of water and acetonitrile with 0.1% formic acid improved retention was achieved [Hellmuth *et al.*, 2011]. On the other hand, HILIC may be suitable for analysis of non-polar compounds, such as neutral sphingolipids and cholesterol [Farwanah *et al.*, 2009].

2.2.2 Ion-exchange chromatography

In ion-exchange (IE) chromatography separation is based on exchange equilibria between ions in solution and those held by ionic interactions to the stationary phase. For a theoretical cation B^+ the equilibrium may be $RA^- H^+ + B^+ \Leftrightarrow RA^- B^+ + H^+$, elution therefore may be achieved by decreasing pH. The opposite is true for anion-exchange stationary phases.

Generally, multiply charged ions have higher affinity to the IE surface, retention also depends on the size of the hydrated ion. Typical cation-exchange resins contain

sulfonic or carboxylic acid groups, anionic exchangers tertiary or primary amines. Basically, IE mechanism may be important in normal phase and aqueous normal phase chromatography [Alonzi *et al.*, 2011, Hétu *et al.*, 2010].

2.2.3 Other chromatographic modes

Chiral stationary phases may be used for optical isomer separations. Such specifity may be useful in separation of biologically active compounds, either internal or pharmaceutical metabolites [Kang *et al.*, 2010, Zanitti *et al.*, 2010]. L-pipecolic acid was analysed on a chiral macrocyclic teicoplanin column using isocratic elution by methanol and water (60:40, v/v) [Rashed *et al.*, 2001]. In another assay separation of epimeric reduced monosaccharides was not achieved in RP on a amino modified column, but was possible by IE-LC [Wamelink *et al.*, 2005].

Chemical interactions are being minimised in size-exclusion (also gel-permeation) chromatography. Column packing is formed by a porous more or less inert material and mobile phase is used mainly as a carrier of the sample. Separation occurs by molecular size: small molecules penetrate deeper into the pores of the particles and are delayed in comparison to larger molecules, which omit the sinuous pores and take a straighter path to the detector. Size exclusion chromatography is suitable predominantly for separation of macromolecules, e.g. nucleic acids and proteins.

Specific interactions of the stationary phase and analytes are used in affinity chromatography, especially for isolation of a compound or as part of a purification process.

2.3 The column

Because of the high applied pressures, a HPLC tubing is usually made of stainless-steel, which is rugges and chemically resistant. A column is filled with particles or continuous porous material with chromatographically active surface.

2.3.1 Particle columns

Porous particles are used most commonly because of their convenient combination of efficiency, capacity and availability. With decreasing particle size efficiency improves, which allows the use of shorter columns and reduction of separation time. On the other hand, higher pressures need to be applied. Fused-core particles offer better efficiency and separation time thanks to reduction

of longitudinal and eddy diffusion [Guiochon & Gritti, 2011]. To achieve performance comparable to totally porous particles larger fused-core particles may be used, which limits increase in backpressure [Cunliffe & Maloney, 2007]. Perfusion particles contain large straight-through pores connected by narrower ones. Transition of mobile phase through the pores allows application of higher flow rates and improvement of efficiency without excessive increase in backpressure.

In most cases a column is packed with silica particles. Unmodified silica contains acidic free silanol groups that interact strongly with basic compounds. At high pH silica degrades and may cause collapse of the column packing [Kirkland *et al.*, 1995]. Depending on the type of silica, operation temperature and mobile phase, silica columns can be used up to pH 8 or 11. While high pH causes dissolution of the silica support, silane bonded phase is removed by hydrolysis in acidic conditions (pH below 3). Polymeration of silane stationary phase and sterical protection prevent damage of the column surface at low pH. Dense stationary phase bonding and endcapping is suitable for basic conditions [Kirkland *et al.*, 1997]. No volume changes of silica particles occur in any solvents and their surface can be easily modified.

Column particles are also made of polymers, such as cross-linked polystyrene or polymethacrylate. Their surface can act directly as stationary phase or may be further modified. Polymeric supports are stable in the whole pH range. If ionic ligands are added, they may be used in IE chromatography but RP separations are common as well [Wamelink *et al.*, 2005, Waterval *et al.*, 2009, Clariana *et al.*, 2010]. However, polymeric columns tend to have lower efficiency than silica and swell in some organic solvents [Snyder *et al.*, 2010 - p. 212].

Less often, other column supports are used. Zirconia polymer or carbon coated particles are useful in almost any pH and temperatures over 100°C [Nawrocki, 2003]. Use of porous graphitic carbon packing may be advantageous for stereoisomer or diastereoisomer separations [Snyder *et al.*, 2010 - p. 217].

In the last years, the predominant use of 5 µm particles subsides to smaller particle sizes, providing better efficiency and separation times. A weakness of particles below 2.5 µm is that they require use of smaller pore inlet frits and incline to clogging, which reduces their lifetime. Nevertheless, particles smaller than 2 µm have found use in many applications [Camera *et al.*, 2010]. Columns with inner diameter between 2.5–5 mm are suitable for most analytical uses. Solvent consumption decreases exponentially with smaller diameter and thus 2 mm columns are often used in HPLC-MS interfaces. In very small diameters (below 2 mm) separation efficiency may be limited by extracolumn peak broadening [Snyder *et al.*, 2010 - p. 240].

2.3.2 Monolithic columns

Another approach to column manufacturing resulted in monolithic columns. In this case the column is filled with continuous porous silica, a polymer or metal oxide. If maintaining the same active surface area as in a particle column, the ratio of mobile phase to column packing volume is severalfold increased and backpressures decrease significantly [Guiochon, 2007]. Alternatively, increased active surface area may be utilised to reach higher loading capacity [Abi Jaoudé & Randon, 2011]. So far monolithic columns have not surpassed the performance of particle packings [Manchón *et al.*, 2011]. The observed lack of efficiency may originate in low homogeneity of the chromatographic bed [Causon *et al.*, 2010]. Higher retention caused by larger active area may be compensated by increasing flow rate, and all in all, chromatographic conditions have to be adjusted when replacing a particle column with a monolithic one [Manchón *et al.*, 2011, Chirita *et al.*, 2011].

Efficiency up to 1,000,000 theoretical plates may be achieved by connecting capillary monolithic columns in a row or increasing column length [Miyamoto *et al.*, 2008, Ikegami *et al.*, 2004, Eghbali *et al.*, 2010, Miyazaki *et al.*, 2010]. Although this approach radically increases separation time, it is investigated in proteomics for its high resolution, efficiency and reduction of ionisation suppression by removal of contaminants and may even be advantageous with respect to total analysis time [Iwasaki *et al.*, 2010].

3 HPLC-TMS

Connecting HPLC with TMS allows detailed analyses of complex mixtures. However, to achive satisfactory results several aspects need to be taken into account.

1) Flow rates:

Several LC-MS interfaces were developed for introducing the relatively large volumes of mobile phase into the vacuum of the mass spectrometer. Nowadays, the most common solution are atmospheric pressure ion sources, e.g. electrospray, atmospheric pressure chemical ionisation (APCI), atmospheric pressure photoionisation (APPI). Thanks to a system of nebuliser and drying gases flow rates up to 1 ml/min may be introduced into the ion source [Stocker *et al.*, 2010, Van Dycke *et al.*, 2010, Waterval *et al.*, 2009]. Eventually, the flow may be split in order to exclude a part of the liquid from entering the ion source [Wamelink *et al.*, 2005]. On the other hand, too low flow rates may cause disturbances in the Taylor cone and discontinual ionisation.

2) Mobile phase compatibility:

Mobile phase composition and pH should be optimised for maximal ionisation of the analyte and minimal ionisation suppression and mass analyser contamination. Non-volatile mobile phase components precipitate in the ion source and decrease sensitivity of the instrument. Therefore ammonium acetate or ammonium formate are usual buffers in HPLC-MS [Bajad *et al.*, 2006, van der Ham *et al.*, 2007]. To facilitate evaporation and solute transition into the gas phase in the ion source solvents should have low surface tension and vaporization heat.

If non-volatile solvents or additives can not be avoided, special arrangements may reduce contamination of the analyser: A valve may by employed to introduce the flow into the ion source only around a certain retention time, or undesirable ions may be neutralised by an ion-pairing reagent and subsequently removed in the ion source.

3) Sample preparation:

Increased attention should be paid to sample purification to prevent contamination of the mass spectrometer, interfering aduct formation or ion suppression [Matuszewski *et al.*, 2003].

3.1 API 4000^{TM} LC-MS interface and mass analyser

Atmospheric pressure is maintained in the ion source of API 4000^{TM} (AB Sciex), which offers the possibility of interchangeable ESI and APCI. Pressure is decreased in the Q0 region by a rouging pump and high vacuum is maintained in the analyser part of the instrument by turbo pumps. While pressure is maintained automatically, several parameters may be adjusted for optimal TMS analysis with ESI.

Ion spray voltage applied to the inlet capillary affects ion spray stability and is optimised with regard to HPLC flow rate. Nebulizer gas (gas 1) also stabilises the Taylor cone and supports formation of smaller droplets. Solvent evaporation is encouraged by a heated gas stream (gas 2) aimed into the ionisation area (Fig. 9). Gas 2 is mainly important in higher HPLC flow rates (above 1 ml/min). Neutral molecules are prevented from entering the ion source by curtain gas flowing against the incoming ions.

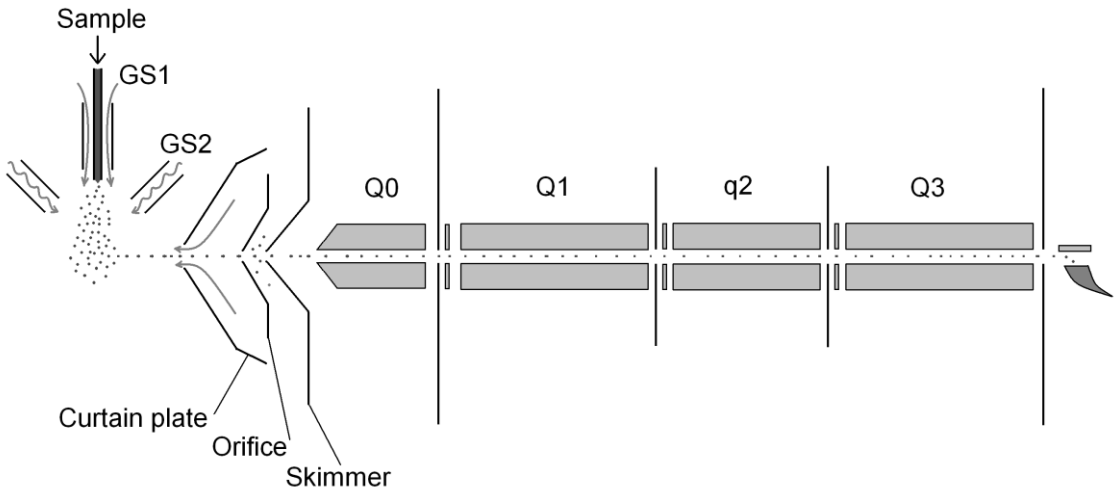

Fig. 9: Simplified diagram of an API 4000^{TM} instrument. GS1 = gas 1 (nebulizer gas), GS2 = gas 2 (drying gas), Q0 = ion guide in low vacuum cell, Q1 and Q3 = quadrupoles, q2 = collision cell.

Ions leave the ion source by the orifice to which declustering potential (DP) is applied. DP supports dissociation of ion clusters before entering the area of decreased pressure. Too high DP results in untimely fragmentation of the analyte. Ions passing through the orifice into vacuum tend to scatter. Too heavily deflected ions are prevented from entering the ion source by the skimmer. Entrance potential is applied to Q0 ion guide, which focuses ions before entering the analyser.

Precursor ions are analysed in the first quadrupole (Q1). A small amount of collision gas is introduced into the second quadrupole (q2) which operates

as a collision cell with AC potential only. Precursor ions are accelerated by the potential difference between Q0 and q2 (collision energy) and fragment by collision induced dissociation in q2. Collision cell exit potential (CXP, betweeen q2 and stubby lens before Q3) focuses and accelerates ions before they enter the third quadrupole (Q3). Similarly to short quadrupoles (stubby lenses) focusing ions before entering analyser quadrupoles, ions are leaded into the detector by a deflector lens.

To sum up, ion source voltage, gas 1, gas 2 and curtain gas flow, gas 2 temperature and declustering potential affect the amount of ions entering the mass analyser. Declustering potential, entrance potential, collision energy and CXP depend on the type of compound, but not on the HPLC flow rate. Because CXP is not mass dependent, DP and CE remain the most important parameters for compound optimisation.

4 Inborn errors of metabolism

Inborn errors of metabolism (IEMs) are caused by biochemical abnormalities with a genetic origin. In general, activity of an enzyme is altered, usually decreased, and changes in substrate or product concentrations may have a direct detrimental effect or accumulationg compounds may be transformed to toxic metabolites via alternative metabolic paths. Superfluous metabolites are usually excreted in urine. IEMs also may be caused by impaired metabolite transport, as is the case of creatine transporter deficiency or so called storage disorders.

A typical feature of IEMs is their low specifity of clinical symptoms. Very distant metabolic aberrations may present a similar clinical picture and one particular disorder may result in varied symptoms according to the degree of enzyme activity alteration and individual metabolic dispositions. Mutation in one allele only (heterozygous) usually causes less pronounced symptoms and sometimes may remained undiagnosed until adulthood. However, most IEMs manifest shortly after birth or during childhood, sometimes with serious impairment or fatal consequences.

With regard to the the metabolic defect, IEMs may be classified into several groups (Tab. 1). Disorders of intermediary metabolism are as a rule easily detectable by basic biochemical assays because they manifest by changes in lactate, ammonia, amino acid or organic acid concentration or the acylcarnitine profile. For example in galactosaemia (an IEM most often caused by galactose-1-phosphate uridyl transferase (E.C. 2.7.7.10) deficiency) galactose transformation to glucose is impaired (Fig. 10). This leads to elevation of galactose and its metabolites galactose-1-phosphate and galactitol concentrations. Galactose-1-phosphate is harmful to liver, kidney and brain, galactitol accumulates in the eye lens and inflicts development of cataract.

Tab. 1: Basic groups of inborn errors of metabolism.

Metabolic disorders of
Saccharides
Amino acids and peptides
Urea cycle
Organic acids
Fatty acids and ketogenesis
Citric acid cycle and oxidative phosphorylation
Purines and pyrimidines
Lysosomal storage disorders
Peroxisomal function
Porphyrins
Glycosylation and lipoproteins
Neurotransmitters

Failure to convert methylmalonyl-coenzyme A to succinyl coenzyme A by methylmalonyl-coenzyme A mutase (E.C. 5.4.99.2) may be caused by vitamin B_{12}

deficiency or genetic defects and results in elevated methymalonic acid in blood and urine. Several organic acidurias are linked to mitochondrial disorders. Ethylmalonic aciduria may be caused by short-chain-acylcoenzyme A dehydrogenase (E.C. 1.3.99.2) deficienty. The metabolism of pyruvate is closely related to the citric acid cycle and gluconeogenesis and its IEMs usually affect primarily the central nervous system, similarly as disorders of oxidative phosphorylation. While primary pyruvic and lactic acidurias result from IEMs of gluconeogenesis or oxidation of pyruvate, secondary ones may accompany a number of other organic acidurias.

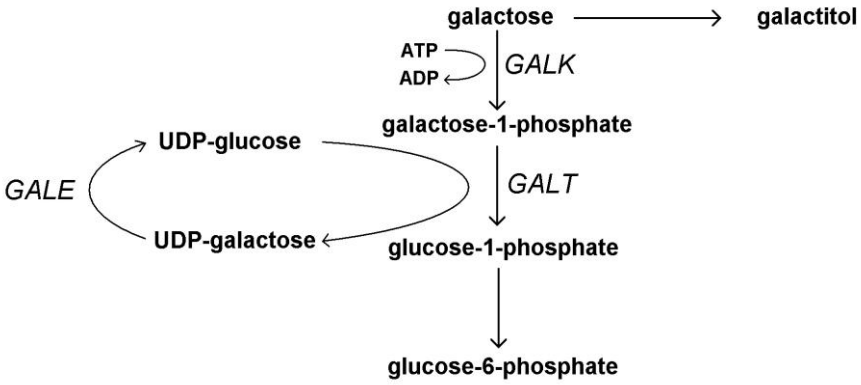

Fig. 10: Simplified diagram of galactose metabolism – enzymes whose deficiency causes galactosaemia are marked in italics. (GALE – UDP-galactose 4-epimerase, GALK – galactokinase, GALT - galactose-1-phosphate uridyl transferase, ATP – adenosine-5'-triphosphate, ADP – adenosine-5'-diphosphate)

Ornithine transcarbamoylase (OTC, EC 2.1.3.3) deficiency results in a metabolic block of the urea cycle and its most prominent symptom and major cause of health damage is hyperammonemia. Substrates of the enzyme accumulate and carbamoylphosphate is processed by aspartate transcarbamoylase in pyrimidine *de novo* synthesis, which leads to the characteristic increase in orotic acid concentration. Uracil concentration in urine may be elevated as well.

Increase in orotic acid not accompanied by hyperammmonemia may result from uridine-5'-monophosphate synthase (UMPS, E.C. 4.1.1.23) deficiency. The block of pyrimidine *de novo* synthesis leads to shortage of pyrimidine nucleotides and derangement in cell division. Uridine is administered to supply the body with lacking pyrimidines. One of the few IEMs with enhanced enzyme activity is 5-phosphoribosyl-1-diphosphate synthetase (PRPPS, E.C. 2.7.6.1) hyperactivity, in which the increased rate of purine *de novo* synthesis leads to excessive hypoxanthine and uric acid production. Uric acid has low solubility and tends to crystallise in joints and capillaries (gout) and cause kidney damage. However, most IEMs of purine and pyrimidine

metabolism are caused by an enzyme deficiency and lead to accumulation of the substrate, which has detrimental consequences for the organism. UMPS, involved in pyrimidine *de novo* synthesis, was mentionned above. Adenylosuccinate lyase (ADSL, E.C. 4.3.2.2), a bifunctional enzyme participating in purine *de novo* synthesis, removes a succinyl group from 5-aminoimidazole-4-(N-succinylocarboxamide) ribotide (SAICAR) or adenylosuccinate. In ADSL deficiency both respective ribosides are excreted in urine. Adenosine deaminase (ADA, E.C. 3.5.4.4) and purine nucleoside phosphorylase (PNP, 2.4.2.1) are enzymes of purine degradation. If the activity of one of them is diminished, its substrates may be found in increased amounts in urine. Xanthinuria may be caused by xanthine oxidase deficiency or molybdenum cofactor deficiency. In both cases the same step of purine degradation is blocked, xanthine accumulates and uric acid excretion decreases. Salvage plays an important role in purine metabolism by reutilising purine bases for nucleotide formation. Hypoxanthine-guanine phosphoribosyl transferase (HGPRT, EC 2.4.2.8) catalyzes the transfer of 5-phosphoribosyl-1-diphosphate (PRPP) to hypoxanthine or guanine, while adenine phosphoribosyl transferase (APRT, EC 2.4.2.7) to adenine. In HGPRT deficiency superfluous PRPP stimulates purine *de novo* synthesis and increased amounts of hypoxanthine and uric acid are excreted in urine, similar to PRPPS hyperactivity. Deficiency of APRT leads to accumulation of adenine, which is excreted in urine together with 8-hydroxyadenine and 2,8-dihydroxyadenine, products of oxidation of adenine by xanthine oxidase.

Differing metabolite profiles may point to distinct IEMs. So while concomittant elevation of 5,6-dihydrouracil, 5,6-dihydrothymine, uracil and thymine may point to dihydropyrimidine amidohydrolase (also dihydropyrimidinase – DHP, EC 3.5.2.2) deficiency, increased uracil together with orotic acid generally accompanies urea cycle disorders. Uracil also may originate from degradation of pseudouridine, which is normally present in urine, and amounts of the latter therefore should be monitored as well [Simmonds & van Gennip, 2003]. Thymidine phosphorylase deficiency leads to increase in thymidine and an unusual nucleoside 2-deoxyuridine, which is incorporated in mitochondrial DNA causing multiple deletions.

Defects of biosynthesis and degradation of complex molecules including purine, pyrimidine, steroid and porphyrin metabolism, storage and glycosylation disorders often cause disorders with slower onset but need to be diagnosed by specific methods. Infantile free sialic acid storage disease (ISSD) may be named as an example of lysosomal storage disorders. In ISSD increased amounts of sialic acid are excreted in urine.

So far not many IEMs of neurotransmitters are known. The common feature of cerebral creatine deficiency syndromes is decreased creatine concentration in the brain. They also may be identified by altered creatine and guanidinoacetate levels or creatine/creatinine ratio in urine.

Because of the uncertainty of clinical symptoms, analysis of metabolite concentrations and enzyme activity is essential for diagnosis. Newborn screening may contribute to uncovering an IEM and introducing efficient treatment before permanent health damage is inflicted. Nowadays, the czech national screening program includes monitoring of cystic fibrosis, congenital adrenal hyperplasia, congenital hypothyreosis, amino acid disorders including phenylketonuria and maple syrup urine disease, glutaric aciduria type I, isovaleric aciduria and several disorders of fatty acid metabolism. Supplementary newborn screening may be recommended for diagnosing of more rare IEMs. To set up a screening method, detecting of the particular IEM should be beneficial to the patient (i.e. effective treatment available). Furthermore the method should be cost-effective and minimise potential health risks and negative effects on the patient and their family.

EXPERIMENTAL PART

1 Introduction

Inborn errors of metabolism (IEMs) are caused by altered enzyme activity leading to accumulation or shortage of a metabolite. A direct way to asses possible health risks due to IEMs is measurement of metabolites in body fluids. Urine offers the advantage of less invasive sample collection in comparison to plasma or cerebrospinal fluid. Moreover, metabolites with high clearance may be more easily detected in urine than in plasma.

Screening for IEMs is an important precaution to uncover a latent metabolic disorder and prevent possible impairment of the patient. Diagnosing of IEMs not included in the national screening programme is performed in suspect cases according to presenting symptoms. These, however, may be rather ambiguous and several tests may be necessary. Simultaneous analysis of several metabolites therefore may simplify the diagnostic process. HPLC-TMS is well suited for such complementary screening methods by its ability to analyse complex mixtures.

Screening methods for various groups of metabolites in urine have been investigated. Thin-layer chromatography is the most common technique used for screening of mono- and disaccharides. In this way galactose, fructose, glucose, xylulose, arabinose, lactose and sucrose may be separated but quantity is only estimated [Shih *et al.*, 1991]. Two HPLC-TMS methods for analysis of monosaccharides and sugar alcohols were proposed for diagnosing of IEMs of sugar metabolism. A rapid screening method allowed quantification of sums of isomers, while more detailed information might be obtained by their separation under different chromatographic conditions [Wamelink *et al.*, 2005]. Reversed-phase HPLC with UV detection has been widely used for analysis of purines and pyrimidines [Vidotto *et al.*, 2003]. Several reversed-phase HPLC-TMS methods for analysis of purine and pyrimidine metabolites were developed. Various C18 columns were used, most often in combination with acidic amonium acetate - methanol gradients [la Marca *et al.*, 2006, van Lenthe *et al.*, 2000, Hartmann *et al.*, 2006]. A formic acid solution (pH 2.6) - methanol gradient was used for separation of pyrimidine *de novo* synthesis intermediates [van Kuilenburg 2004] and ammonium formate (pH 5) - methanol elution was used in a screening method for disorders of purine and pyrimidine metabolism [Ito *et al.*, 2000]. All these methods offered limits of detection in the µM range and separation times from 15 to 20 min. Capillary electrophoresis with UV or MS detection also was found suitable for analysis of purines and pyrimidines [Haunschmidt *et al.*, 2008] and their nucleotides, in both acidic and alkalic conditions [Friedecký *et al.*, 2007, Soga *et al.*, 2007]. A 30 min reversed-phase UHPLC-TMS method for measurement for underivatised aminoacids in plasma and

urine samples has succesfully replaced a commercial amino acid analyser for routine analysis [Waterval 2009]. Ionisation efficiency of related dicarboxylic acids was improved by benzofurazan derivatization in a fast UHPLC-TMS method [Al Dirbashi *et al.*, 2007]. Volatile organic acids may be favourably analysed by gas chromatography (GC) with MS or flame ionisation detection [Buchanan & Thoene, 1991, Lo *et al.*, 2010, Yamaguchi *et al.*, 2001]. Extraction (usually liquid-liquid or solid phase extraction or ion-exchange chromatography) and derivatisation (in order to increase volatility) usually are essential before GC-MS analysis [la Marca & Rizzo, 2011]. Distinct HPLC-TMS methods were presented for analysis of creatine and its metabolites, pipecolic acid and sialic acid [Carling *et al.*, 2008, Rashed *et al.*, 2001, van der Ham *et al.*, 2007]. Recently practical potential of an ion-pairing HPLC-diode array detection method for several purines, pyrimidines, organic acids and other metabolites was demonstrated on samples of patients with IEMs [Lazzarino *et al.*, 2011].

In this thesis a widely targeted HPLC-TMS screening method is presented for analysis of 24 purine and pyrimidine metabolites, creatine, guanidinoacetate, galactose, galactitol and several organic acids. Analysis of these compounds may contribute to diagnosing various IEMs.

2 Material and methods

Standards, Chromasolv® deionised water and solvents of HPLC quality were obtained from Sigma-Aldrich (St. Louis, USA) except of adenine and creatinine purchased from Lachema (Brno, Czech Republic) and uric acid, glucose, guanidinoacetate and pipecolic acid obtained from Merck (Darmstadt, Germany). N-carbamyl-β-aminoisobutyrate was prepared by alkalic hydrolysis of dihydrothymine as published previously [van Kuilenburg *et al.*, 1999].

Standard mixtures were prepared by dissolving standards in water at concentrations yielding a signal-to-noise ratio 20. Urine samples were diluted with initial mobile phase to obtain creatinine concentration 1 mM, centrifuged (5 min / 14,500 *g*) and the supernatant was analysed directly or stored at -20°C. ERNDIM quality control samples of urine spiked with metabolites characteristic for selected IEMs were processed in the same way.

Separation was performed using UHPLC (Ultimate 3000, Dionex, Sunnyvale, USA). Following reversed phase elution conditions were tested on a Kinetex 2.6 µm C18, 2.1 x 150 mm column (Phenomenex, Torrance, USA): 1) eluent A: 25mM ammonium acetate (pH 6.6), eluent B: A + methanol (1:1, v/v); 2) A: 25mM ammonium

formate (pH 6.9), B: A + methanol (1:1, v/v); 3) A: 0.1% formic acid; B: A + methanol (1:1, v/v). In all cases the elution gradient started at 100 % A for 1 min, followed by a linear decrease to 0 % A at 6 min, 0 % A was held for 3 min, succeeded by a 4 min final wash with 100 % A. The flow rate was 150 µl/min. During separation temperature was held at 30°C. Optimal separation was achieved by a Luna 3 µm NH2, 2 x 150 mm column (Phenomenex, Torrance, USA) in HILIC mode. Gradient elution by 20mM acetic acid titrated by ammonium hydroxide to pH 9.45 (eluent A) and acetonitrile (eluent B) was applied at 250 µl/min. Final gradients are shown in Tab. 1. All separations were carried out at 30°C. Injection volume was 1 µl and total analysis time 13 min.

Tab. 1: Elution gradients in HILIC (Luna NH2 column) for detection by positive (+) and negative (-) mode TMS.

(+)	Time	% B
	0 - 2 min	85
	4 min	75
	6 – 8 min	5
	9 – 13 min	85

(-)	Time	% B
	0 min	85
	2 – 9.5 min	5
	10 - 13 min	85

Detection was performed by an API 4000^{TM} quadrupole tandem mass spectrometer (Applied Biosystems, Foster City, USA) with electrospray ionisation. Ion transitions and TMS potentials were optimised automatically, DP and CE are shown in Tab. 2, EP was set to 10 V or -10V in positive or negative mode, respectively. Solutions of 0.1µM standards were diluted ten times by an acidic (20mM formic acid + methanol 1:1, v/v) or alkalic (20mM ammonium acetate, pH 9.4 + acetonitrile 1:1, v/v) buffer. Ion source temperature was 350°C, capillary voltage 4500 V, nitrogen was used as nebulizer gas (40 psi), auxiliary gas (40 psi), curtain gas (15 psi) and collision gas (6 psi). Dwell times were chosen according to TMS sensitivity for compounds. Retention time and characteristic ion transitions were used for compound detection by MRM.

Data were processed using Analyst 1.5 (Applied Biosystems, Foster City, USA).

Linearity, precision, recovery and limits of detection were determined by measuring spiked urine samples. Pooled urine (n = 10) and urine samples were diluted by initial mobile phase to obtain creatinine concentration after addition of standard mixtures of 1 mmol/l, 5 µM 8-bromoadenosine was used as internal standard.

Standard solutions of 20 mM were prepared for creatinine, 5 mM for uric acid, 1 mM for creatine, lactate, pseudouridine (psUR), 5,6-dihydrouracil (DHU) and guanidinoacetate (GAA) and 200 µM for the rest of analytes. Calibration solutions were prepared by diluting standard solutions with initial mobile phase to 10 mM, 4 mM and 1 mM for creatinine, 2.5 mM, 1 mM and 0.5 mM for uric acid, 500 µM, 200 µM and 50 µM for creatine, lactate, psUR, DHU and GAA and 100 µM, 40 µM and 10 µM for the other analytes. Calibration solutions and mixtures were added to equal volumes of pooled urine with 2mM creatinine. Urine blanks were prepared by adding initial mobile phase (1:1, v/v). Calibration curves were constructed from differences between spiked urine samples and urine blank. Limits of detection for individual ion transitions were determined as concentration at S/N = 3.

Precision was calculated from pooled urine aliquots to which equal volumes of standard solutions were added to yield final concentrations of 5 mM, 2.5 mM, 250 µM or 50 µM for creatinine, uric acid, the mixture of creatine, lactate, psUR, DHU and GAA or the rest of compounds, respectively. Samples were prepared in 6 replicates and measured in one day.

Recovery was determined from 5 different urine samples added by equal volume of standard solutions at two concentration levels. Spiked concentrations of standards were 10 mM and 2 mM for creatinine, 2.5 mM and 0.5 mM for uric acid, 500 µM and 50 µM for the mixture of creatine, lactate, psUR, DHU and GAA and 100 µM and 10 µM for the rest of compounds.

Ion suppression was investigated by post-column infusion of 1µM 2-deoxyguanosine and adenosine at a flow rate of 5 µl/min on different urine samples. For 2-deoxyguanosine both optimal ion transitions were chosen from the negative mode, for ion suppression analysis in the positive mode Q1 mass was 268.0 Da and Q3 mass 152.1 Da.

3 Results

3.1 Optimisation of TMS parameters

Five most intense ion transitions and their TMS parameters were obtained by direct infusion of compounds diluted in an acidic or alkalic buffer and verified by HPLC-TMS analysis of standard mixtures. Two ion transition were selected for each compound. One is used for quantitation, the ratio of both for peak identity confirmation. In the case of pyruvate, thymidine and ethylmalonate only one acceptible ion transition

was obtained (Tab. 2). In the case of orotic acid and homovanillate the second ion transition has very low limits of detection.

Tab 2: Selected ion transitions and optimised declustering potential (DP) and collision energy (CE). The first ion transition is considered primarily, the second one to peak confirmation. (Q_1 – mass of precursor ion, Q_3 – mass of product ion)

Compound	Mode	Q_1 (Da)	Q_3 (Da)	DP (V)	CE (V)
adenine	+	136.0	92.1	56	39
	-	133.9	106.8	-60	-24
xanthine	-	150.8	107.8	-60	-22
	-	150.8	80.1	-60	-30
hypoxantine	+	137.1	110.1	56	29
	-	134.8	91.8	-60	-22
2,8-dihydroxyadenine	-	165.7	122.9	-55	-16
	-	165.7	79.9	-55	-24
Uric acid	-	166.7	126.7	-55	-10
	-	166.7	84.9	-55	-12
adenosine	-	265.9	133.7	-40	-10
	+	268.1	119.0	46	60
guanosine	-	281.9	149.9	-75	-24
	-	281.9	132.7	-75	-38
inosine	-	266.8	134.8	-55	-24
	+	269.1	109.9	36	53
2-deoxyadenosine	+	252.1	135.9	51	19
	+	252.1	116.9	51	19
2-deoxyguanosine	-	265.9	149.9	-70	-24
	-	265.9	132.9	-70	-36
2-deoxyinosine	+	253.1	136.8	31	11
	+	253.1	109.9	31	51
succinyladenosine	-	382.1	206.0	-60	-20
	-	382.1	134.0	-30	-30
thymine	+	127.1	84.0	76	23
	+	127.1	81.9	76	27
uracil	-	110.8	67.1	-50	-24
	+	113.0	70.1	61	23
5,6-dihydrothymine	+	129.0	111.9	61	15
	+	129.0	84.3	61	19
5,6-dihydrouracil	+	115.1	55.2	61	29
	+	115.1	98.0	61	17

Compound	Mode	Q_1 (Da)	Q_3 (Da)	DP (V)	CE (V)
5-hydroxymethyluracil	-	141.0	123.0	-60	-20
	-	141.0	80.0	-60	-20
orotic acid	-	154.8	110.9	-35	-14
	+	157.1	68.0	61	31
thymidine	+	243.2	127.0	30	10
uridine	-	243.2	110.0	-60	-20
	-	243.2	200.0	-60	-20
2-deoxyuridine	-	227.0	183.8	-60	-20
	+	229.2	113.0	30	10
pseudouridine	-	243.2	153.0	-60	-20
	+	245.2	209.0	60	10
N-carbamoyl-b-alanine	+	133.0	115.0	41	13
	-	131.0	88.0	-60	-10
N-carbamoyl-b-aminoisobutyrate	+	147.1	129.0	30	10
	-	145.0	102.0	-30	-10
guanidinoacetate	+	118.0	76.0	41	17
	+	118.0	72.0	41	21
creatine	+	132.1	90.0	36	17
	+	132.1	86.0	36	15
creatinine	+	114.1	86.1	51	17
	+	114.1	72.0	51	23
lactate	-	88.9	43.0	-40	-16
	-	88.9	45.2	-40	-14
pyruvate	+	89.0	48.0	31	11
methylmalonate	-	116.8	73.0	-35	-12
	-	116.8	54.9	-35	-28
ethylmalonate	-	130.7	87.0	-25	-10
galactitol	-	180.9	88.9	-50	-20
	-	180.9	101.0	-50	-18
galactose	-	178.9	88.8	-20	-10
	-	178.9	71.1	-20	-22
sialic acid	-	307.9	86.8	-55	-24
	-	307.9	169.8	-55	-20
pipecolic acid	+	130.0	84.2	60	10
	+	130.0	55.4	51	43
homovanillate	+	183.1	136.9	46	15
	-	180.8	136.9	-40	-10
vanillylmandelate	-	196.9	137.8	-50	-16
	-	196.9	153.0	-50	-12

3.2 HPLC method development

Initially, a Kinetex C18 column was used with elution by 25 mM ammonium acetate (pH 6.6) and methanol. This condition offered good separation for most analytes within a 13 min run. However, several analytes eluted near the void time, which was 2.2 min (Fig. 1). Sialic acid and N-carbamoyl-b-alanine were not retained by the column at all, other compounds possibly affected by the initial disturbance were guanidinoacetate, creatine and N-carbamoyl-b-aminoisobutyrate. Overall retention further slightly decreased in elution by 25mM ammonium formate (pH 6.9) and methanol, but improved in the case of a gradient of 0.1% formic acid. Nevertheless, guanidinoacetate was not retained under these conditions and several other compounds, including creatinine, eluted shortly after the void volume.

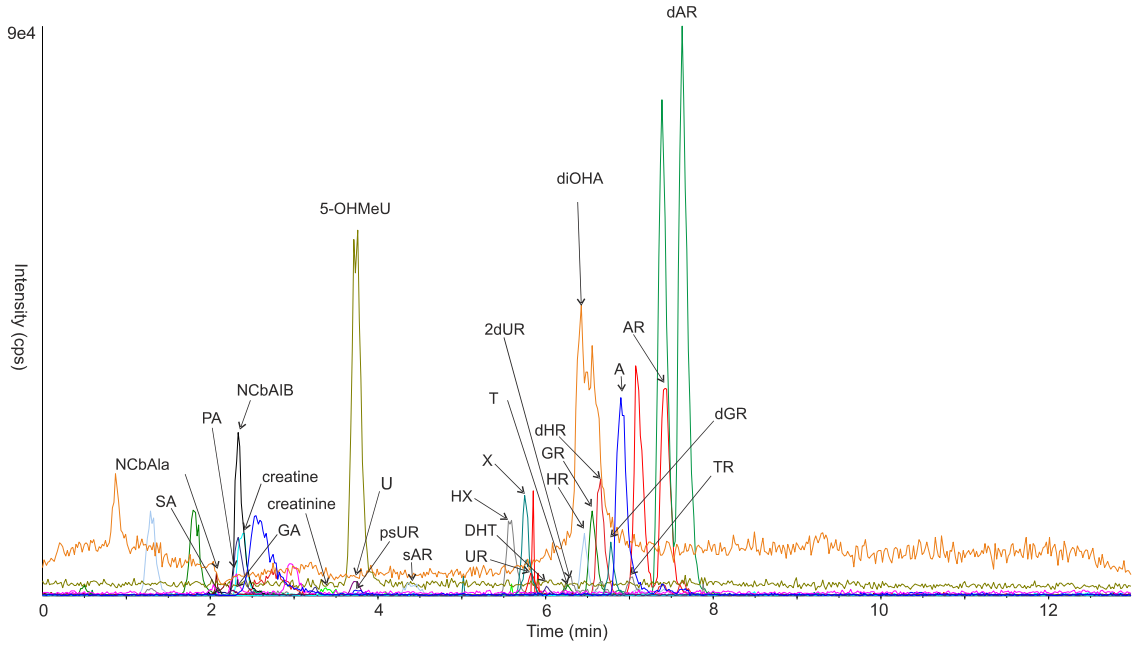

Fig. 1: Chromatogram of a standard mixture analysis on Kinetex C18 column wit ammonium acetate (pH 6.6) – methanol elution in positive mode. (SA – sialic acid, NCbAla – N-carbamoyl-b-alanine, PA – pipecolic acid, NCbAIB - N-carbamoyl-b-aminoisobutyrate, GAA – guanidinoacetate, 5-OHMeU - UR – 2-deoxyuridine, galOH – galactitol, U – uracil, UR – uridine, 5OHMeU – 5-hydroxymethyluracil, U – uracil, psUR – pseudouridine, sAR – succinyladenosine, UR – uridine, DHT – 5,6-dihydroxythymine, HX – hypoxanthine, X – xanthine, T – thymine, dUR – 2-deoxyuridine, HR – inosine, GR – guanosine, dHR – 2-deoxyinosine, diOHA – 2,8-dihydroxyadenine, A – adenine, AR – adenosine, dAR – 2-deoxyadenosine, dGR – 2-deoxyguanosine, TR - thymidine)

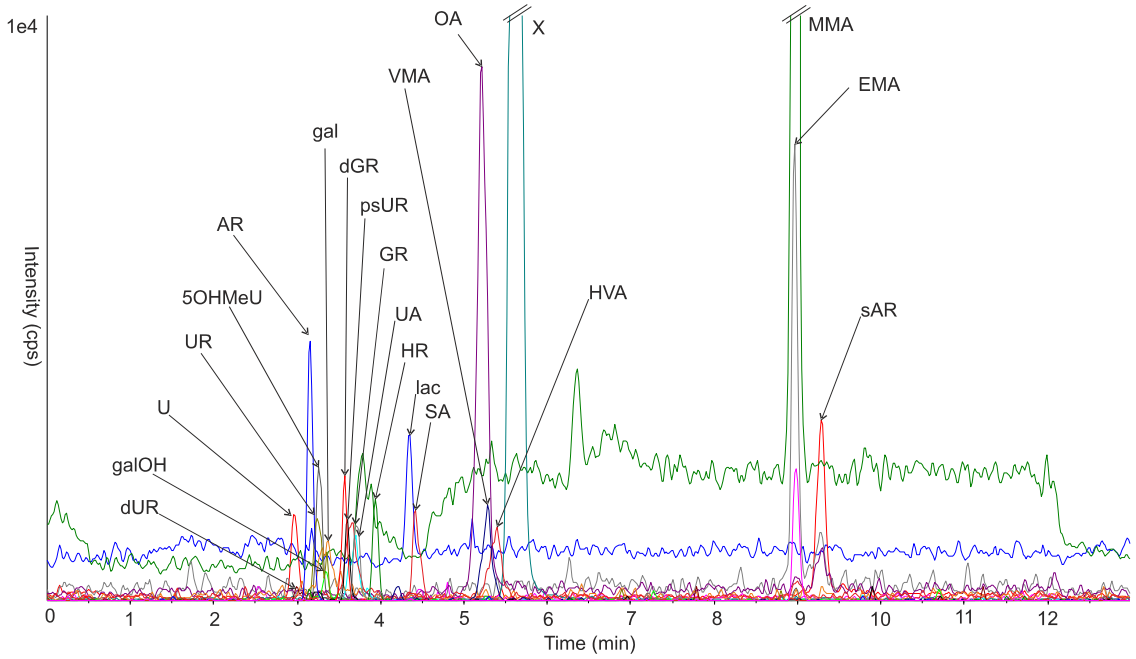

Fig. 2: Chromatogram of a standard mixture analysis under final conditions in negative mode. (dUR – 2-deoxyuridine, galOH – galactitol, U – uracil, UR – uridine, 5OHMeU – 5-hydroxymethyluracil, AR – adenosine, gal – galactose, dGR – 2-deoxyguanosine, psUR – psudouridine, GR – guanosine, UA – uric acid, HR – inosine, lac – lactate, SA – sialic acid, VMA – vanillylmandelic acid, OA – orotic acid, X – xanthine, HVA – homovanillic acid, MMA – methylmalonic acid, EMA - ethylmalonic acid, sAR – succinyladenosine)

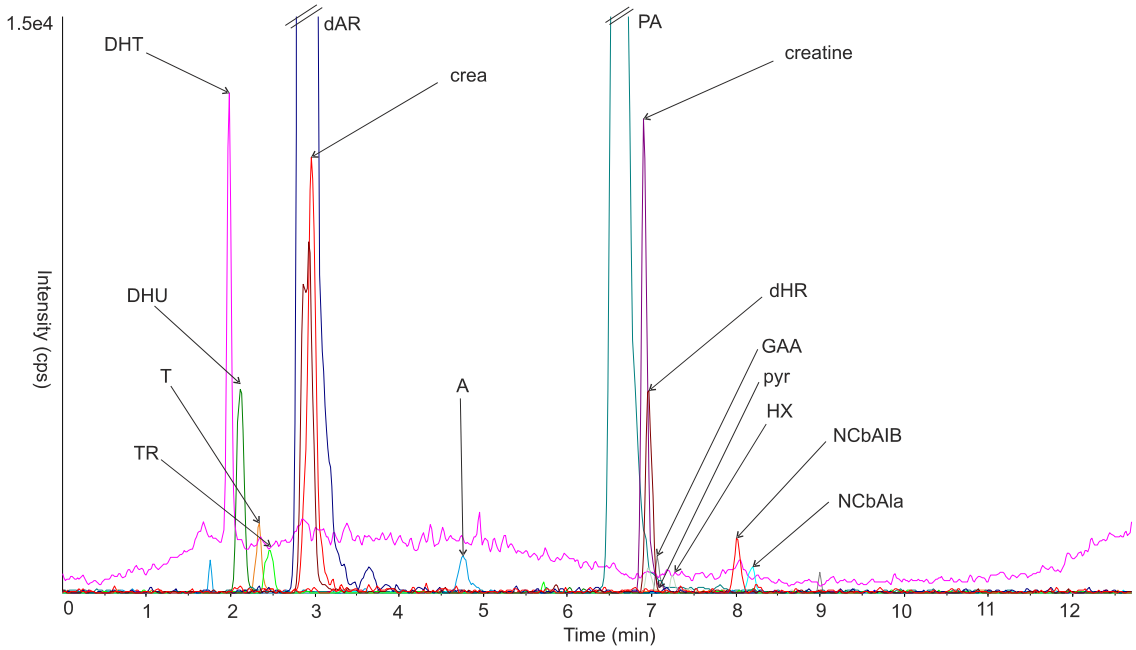

Fig. 3: Chromatogram of a standard mixture analysis under final conditions in positive mode. (TR – thymidine, T – thymine, DHU – 5,6-dihydrouracil, DHT – 5,6-dihydrothymine, dAR – 2-deoxyadenosine, crea – creatinine, A – adenine, PA – pipecolic acid, dHR – 2-deoxyinosine, GAA – guanidinoacetate, HX – hypoxanthine, NCbAla – N-carbamoyl-beta-alanine, NCbAIB – N-carbamoyl-b-aminoisobutyrate)

To increase retention of polar compounds a modified HILIC metod using an aminopropyl column [Bajad *et al.*, 2006] was employed with ammonium acetate (pH 9.45) - acetonitrile elution. Initial separations were carried out on a Luna 3 µm NH2, 1 x 150 mm column (Phenomenex) at a flow rate of 50 µl/min, with void time 1.7 min. Analytes showed sufficient retention except of 5,6-dihydrothymine, 5,6-dihydrouracil and thymine with retention times 2.0 min, 2.2 min and 2.4 min, respectively. Excellent separation of compounds with similar structure was achieved during these 25 min analyses. The elution gradient was modified to optimise separation and shorten time of analysis and a column of the same type with 2 mm diameter was chosen. Final gradients modified according to retention times distribution in positive and negative MRM detection are given in Tab 1. Although 2,8-dihydroxyadenine MRM transitions were optimised, it was not detected after HPLC separation on the aminopropyl column. Under final conditions one run including column equilibration lasted 13 min (Fig. 2 and 3).

3.3 Method validation

Intraday precision was determined from spiked pooled urine prepared in six replicates. Coefficients of variation (CV) of peak areas were between 3.38% (guanidinoacetate, 118.0→76.0) and 39,26% (homovanillate, 183.1→136.9), CV of adenine (133.9→106.8) peak area was 63.61%. CV of retention times were lower than 1.00%, except for 2-deoxyinosine (1.13%) and adenine in positive mode (1.59%). Retention times of compounds are given in Tab. 3.

Linearity was assessed on pooled urine samples spiked with standard solutions at 4 concentration levels. Most compounds showed linear response intensity ($R^2 \geq 0.9$) in the tested concentration range. Pseudouridine and lactate were linear only up to 250 µM and no linear relationship was found for pyruvate and uric acid. During sample preparation uric acid precipitated in concentrations higher than 125 µM. Limits of detection (LD) were below or within the normal concentration range (Tab. 3) of the majority of metabolites. Higher LDs were observed for thymine, 5-hydroxymethyluracil, 5,6-dihydrouracil and 5,6-dihydrothymine.

Recovery was calculated from five different urine samples spiked at two concentration levels and processed in duplicates. Extreme variations of recovery (up to hundreds of percent) were observed in both ionisation modes. Creatinine displayed rather constantly reduced recovery values (41.31-63.84% at the higher concentration level).

Tab. 3: Retention times (t_r) and limits of detection (LD) of chosen ion transition for all compounds optimised in the final HPLC-TMS method. (n.d. – not determined)

Compound	Mode	Q1 (Da)	Q3 (Da)	t_r (min)	LD (uM)
adenine	-	133.9	106.8	3.5	0.215
xanthine	-	150.8	107.8	5.0	0.991
hypoxantine	+	137.1	110.1	7.1	0.655
uric acid	-	166.7	126.7	3.9	n.d.
adenosine	-	265.9	133.7	3.2	0.042
guanosine	-	281.9	149.9	3.8	0.169
inosine	-	266.8	134.8	3.9	0.189
2-deoxyadenosine	+	252.1	135.9	3.3	0.022
2-deoxyguanosine	-	265.9	149.9	3.6	0.041
2-deoxyinosine	+	253.1	136.8	6.8	0.045
succinyladenosine	-	382.1	206.0	9.7	0.436
thymine	+	127.1	84.0	2.5	16.187
uracil	+	110.8	67.1	3.1	2.852
5,6-dihydrothymine	+	129.0	84.3	2.0	9.534
5,6-dihydrouracil	+	115.1	98.0	2.2	95.339
5-hydroxymethyluracil	-	141.0	123.0	3.3	1.415
orotic acid	-	154.8	110.9	5.3	0.221
thymidine	+	243.2	127.0	2.7	0.135
uridine	-	243.2	200.0	3.3	0.455
2-deoxyuridine	+	229.2	113.0	3.1	0.622
pseudouridine	-	243.2	153.0	3.6	0.101
N-carbamoyl-b-alanine	+	133.0	115.0	8.7	0.674
N-carbamoyl-b-aminoisobutyrate	+	147.1	129.0	8.5	3.947
guanidinoacetate	+	118.0	76.0	7.2	0.507
creatine	+	132.1	90.0	7.0	0.077
creatinine	+	114.1	86.1	3.4	0.967
lactate	-	88.9	43.0	4.4	18.382
pyruvate	+	89.0	48.0	7.1	5.703
methylmalonate	-	116.8	73.0	9.7	0.227
ethylmalonate	-	130.7	87.0	9.7	0.062
galactitol	-	180.9	88.9	3.4	0.033
galactose	-	178.9	88.8	3.2	0.091
sialic acid	-	307.9	86.8	4.5	0.250
pipecolic acid	+	130.0	84.2	6.8	2.027
homovanillate	-	180.8	136.9	5.9	4.732
vanillylmandelate	-	196.9	137.8	5.9	0.595

Correlation to 8-bromoadenosine as internal standard did not reduce coefficients of variation. Large variations in internal standard peak area were observed, presumably as a result of variable ionisation suppression.

3.4 Matrix effects

Evaluation of matrix effects was achieved by post-column infusion of an adenosine and 2-deoxyguanosine solution (Fig. 4). All analysed urine samples showed similar profiles of matrix effects.

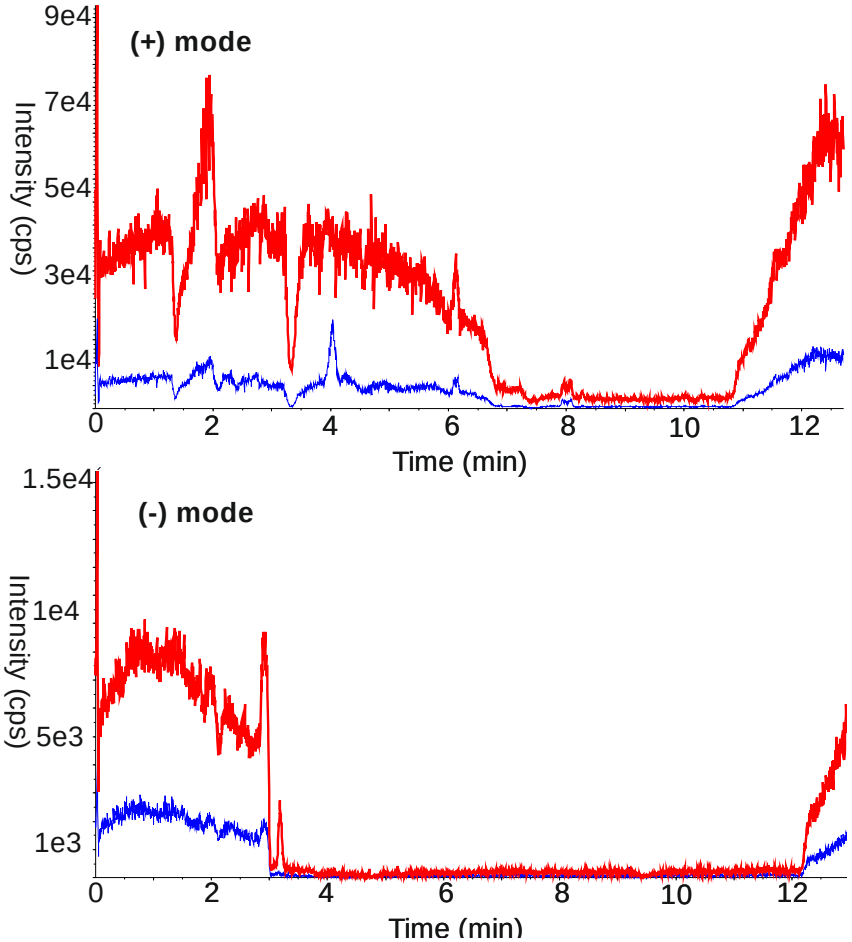

Fig. 4: Ion suppresions – example of an analysis of a urine sample with post-column infusion of 2-deoxyguanosine (red) and adenosine (blue).

In the positive mode several minor fluctuations in ionisation (intensity of response) were observed in the initial 7 min, with major ionisation suppression periods around 1.3 min (void time) and 3.3 min, which corresponds to retention time of 2-deoxyadenosine and creatinine. In the chromatogram of adenosine the peak of adenosine present in the urine sample can be seen at t = 4 min. At physiological concentrations adenosine is detected only in positive mode. Between 6.5 and 11 min

a period of strong ionisation suppression was observed, intensity of response decreased to 1/10. Considerable ionisation suppression (decrease to 1/20 of original intensity) was observed from 3 to 12 min in the negative mode. At t = 3.3 min a peak in the chromatogram of 2-deoxyguanosine is visible, in other parts of the chromatogram response intensity remained constant.

3.5 Analysis of quality control and urine samples

Samples of spiked urine used in the ERNDIM quality control scheme were analysed in positive and negative mode. Results of all compounds except of uric acid showed a linear correlation to median values obtained from laboratories participating in the quality control programme.

Chromatograms of 5,6-dihydrothymine and uracil can be seen on Fig. 5, increase in both compounds in comparison to control urine samples is apparent. Together with elevated levels of 5,6-dihydrouracil and thymine, these compounds are found in DHP deficiency or dihydropyrimidine dehydrogenase (EC 1.3.1.2) deficiency, in which also 5-hydroxymethyluracil concentration tends to rise. Correlation coefficients (R^2) of our data compared with ERNDIM results for 5,6-dihydrothymine, 5,6-dihydrouracil, thymine, uracil and 5-hydroxymethyluracil were 0.966, 0.756, 0.936, 0.969 and 0.976, respectively. Amounts of pseudouridine, which is normally present in urine, also were in accordance with official values. (Data not shown.) Pipecolic acid, which may be useful for identification of peroxisomal disorders, was not detected in control urines but clearly visible in spiked urine samples (Fig. 6). Increased amounts of adenosine and 2-deoxyadenosine, as excreted in ADA deficiency, are illustrated on Fig. 7 on spiked quality control samples. In a similar way, spiked guanosine, 2-deoxyguanosine, inosine and 2-deoxyinosine, which are characteristic for PNP deficiency, correlated well to official quality control results (R^2 = 0.976, 0.723, 0.886 and 0.974, respectively; Fig. 8).

Comparable correlations of official results and our data were found for homovanillic acid, typical for some neuroendocrine tumours presenting usually in children under age of 5, adenine, thymidine, 2-deoxyuridine, xanthine, hypoxanthine, orotic acid, lactate, sialic acid, guanidinoacetate, creatine and creatinine. (Data not shown, for brief information about IEMs related to these metabolites see chapter 4 of the theoretical part.)

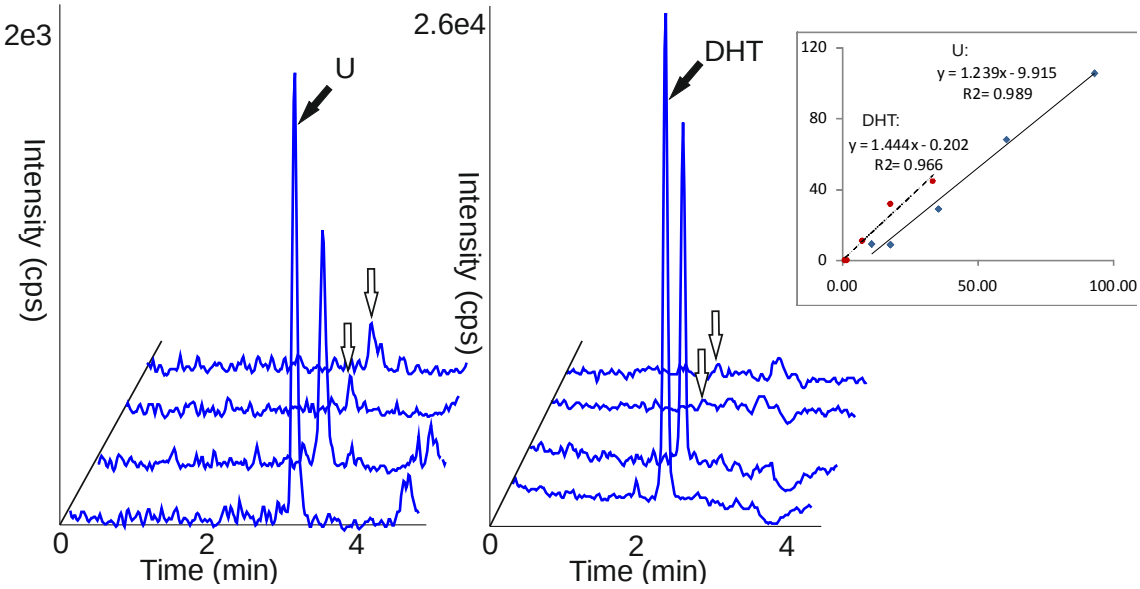

Fig. 5: Chromatograms of spiked urines (two lower lines) and control urines (two upper lines) of uracil (U, neg: 110.8→67.1), 5,6-dihydrothymine (DHT, pos: 129.0→111.9). Black arrows mark increased metabolite concentrations, white arrows peaks (or their absence) in control samples. Graphs show correlation of acquired data of all five measured QC samples with official results.

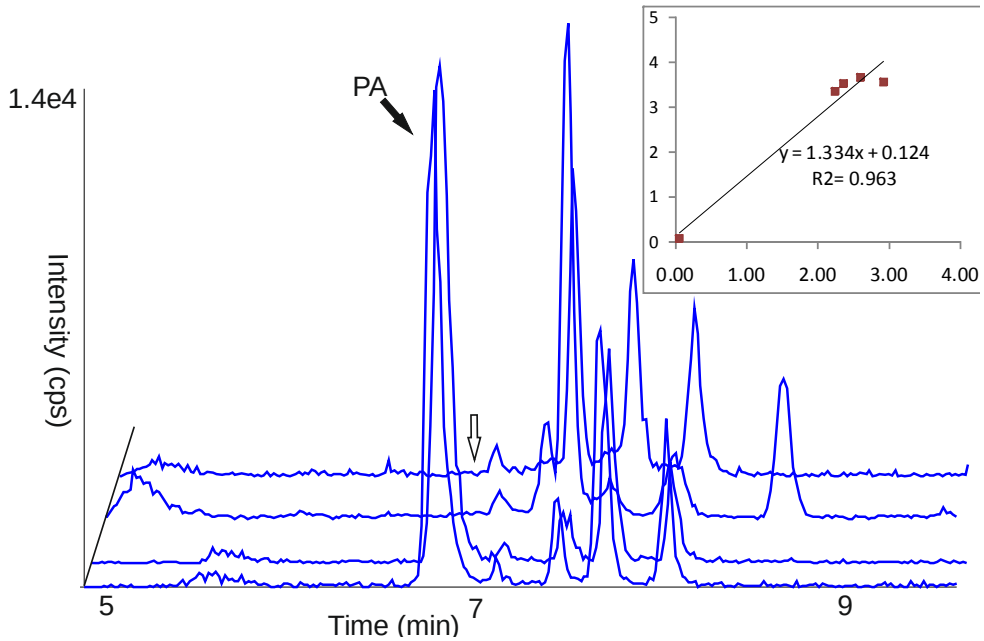

Fig. 6: Chromatograms of spiked urines (two lower lines) and control urines (two upper lines) of pipecolic acid (PA, pos: 130.0→84.2). Black arrows mark increased metabolite concentrations, white arrows peaks (or their absence) in control samples. The graph shows correlation of acquired data of all five measured QC samples with official results.

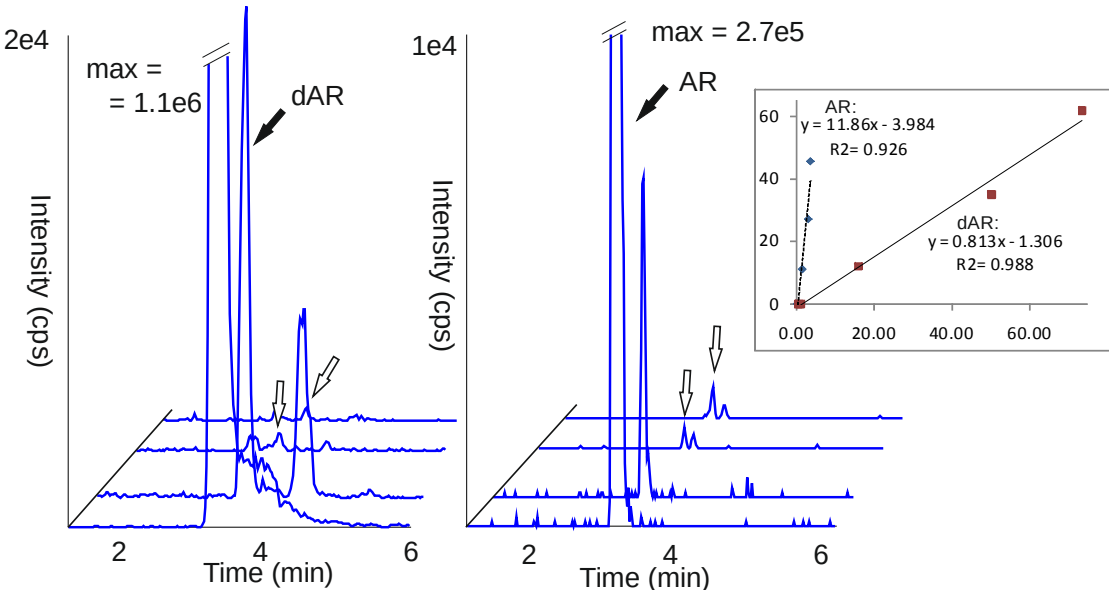

Fig. 7: Chromatograms of spiked urines (two lower lines) and control urines (two upper lines) of 2-deoxyadenosine (dAR, pos: 252.1→116.9) and adenosine (AR, neg: 265.9→133.7). Black arrows mark increased metabolite concentrations, white arrows peaks (or their absence) in control samples. Graphs show correlation of acquired data of all five measured QC samples with official results.

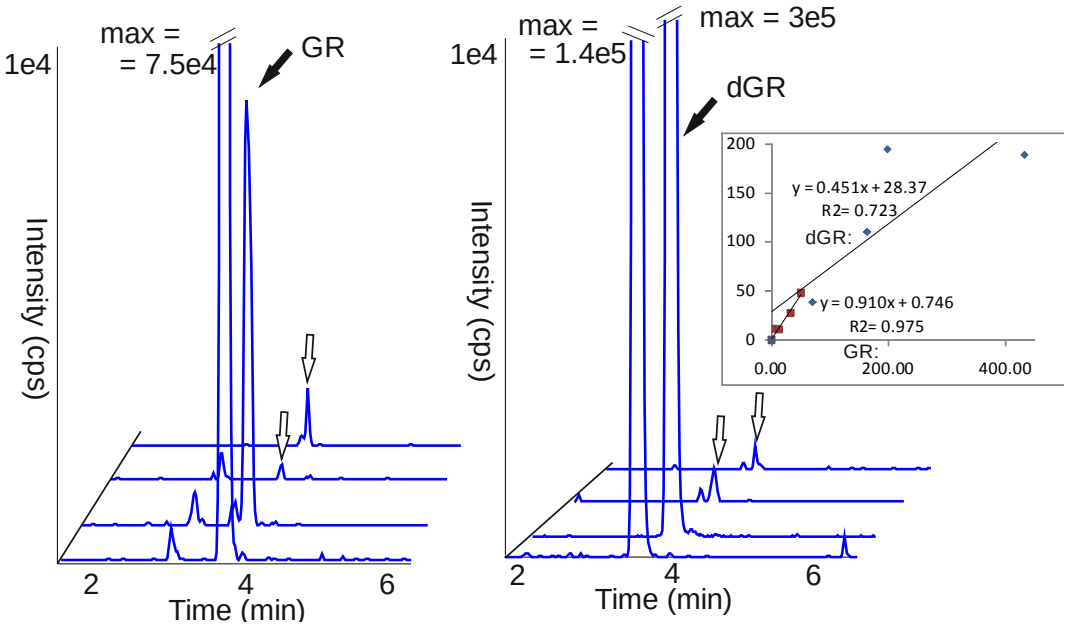

Fig. 8: Chromatograms of spiked urines (two lower lines) and control urines (two upper lines) of 2-deoxyguanosine (dGR, neg: 265.9→149.9) and guanosine (GR, neg: 281.9→149.9). Black arrows mark increased metabolite concentrations, white arrows peaks (or their absence) in control samples. Graphs show correlation of acquired data of all five measured QC samples with official results.

Urine samples of patients with known IEMs were analysed and their chromatograms compared to control samples (n = 40). An increase in galactitol and more prominently in galactose was observed in urine of a galactosaemic patient. No significant aberration was found in urine of a treated galactosaemic patient diagnosed earlier (Fig. 9). A strong elevation of uridine and increased amounts of orotic acid were observed in urine of a patient with ornithine transcarbamoylase (OTC, EC 2.1.3.3) deficiency. The ion transition for uridine is also sensitive to pseudouridine, which is normally present in urine and can be seen as the following peak in Fig. 10. Increased xanthine content was confirmed in urine of a patient with xanthinuria. In this sample also elevated 2-deoxyinosine was found (Fig. 11). In urine of a patient with lactic aciduria no increase in lactate was found, but considerably elevated galactitol was detected. No difference was found between urine of a patient with ethylmalonic aciduria and control samples. Elevated methylmalonic acid was confirmed in urine of a patient with methylmalonic aciduria (Fig. 12).

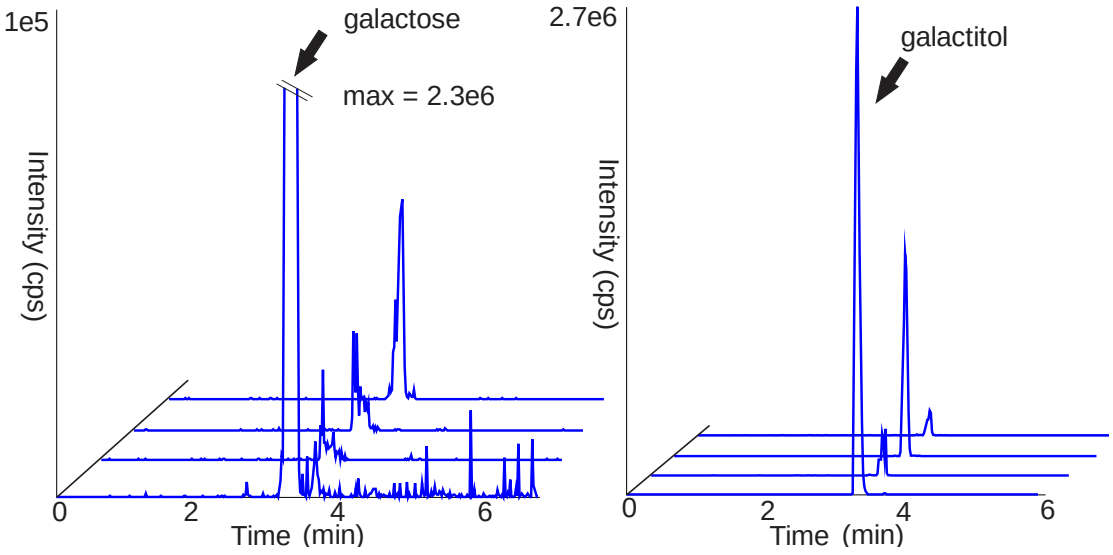

Fig. 9: Comparison of galactose (neg: 178.9→88.8) and galactitol (neg: 180.9→88.9) in urine of a galactosaemic patient (black arrow) and control samples.

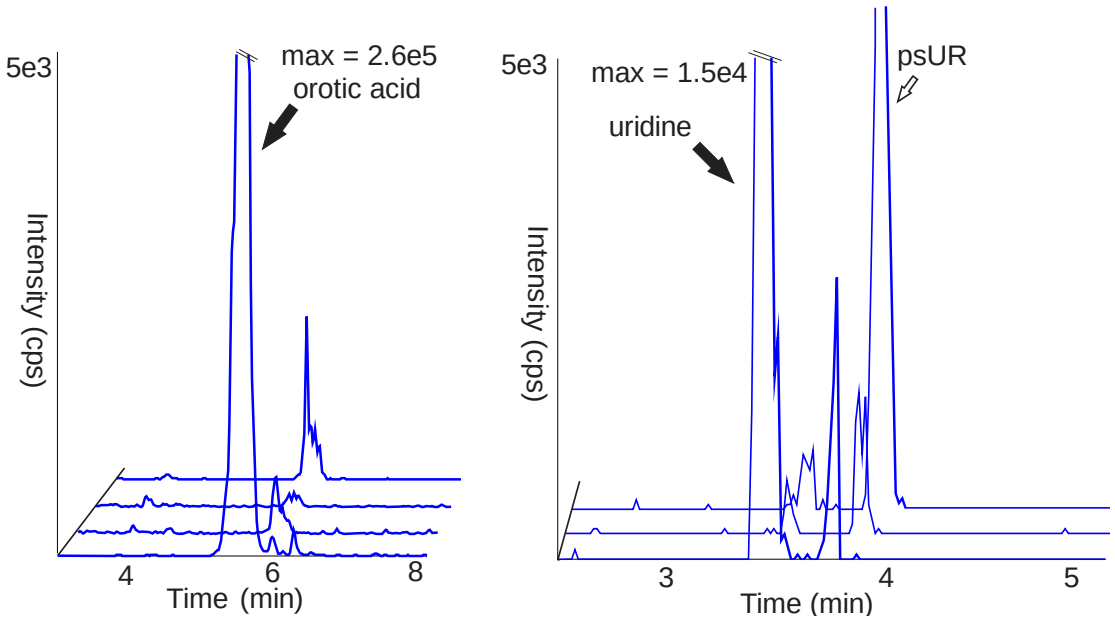

Fig. 10: Comparison of orotic acid (neg: 154.8→110.9) and uridine (neg: 243.2→110.0) in urine of an OTC deficient patient (black arrow) and control samples.

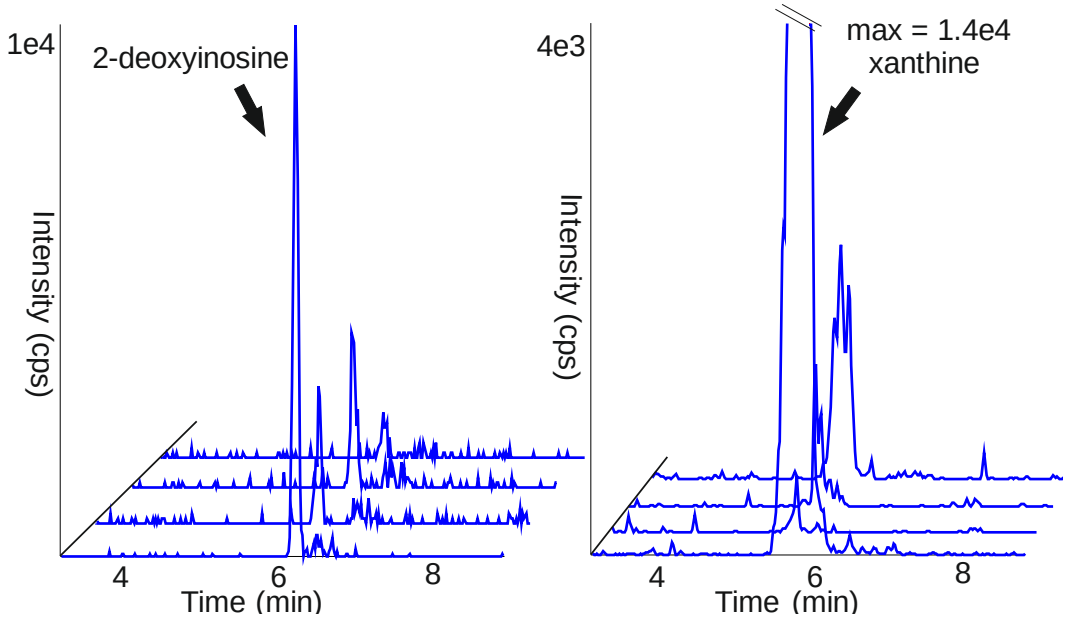

Fig. 11: Comparison of xanthine (neg: 150.8→107.8) and 2-deoxyinosine (pos: 253.1→136.8) in urine of a xanthinuric patient (black arrow) and control samples.

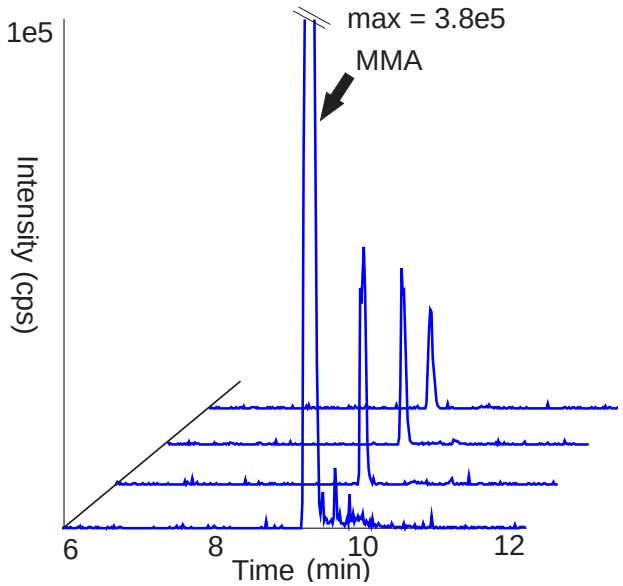

Fig. 12: Comparison of methylmalonic acid (MMA, neg: 116.8→73.0) in urine of a patient with methylmalonic aciduria (black arrow) and control samples.

4 Discussion

Since the initial choice of a C18 column did not provide sufficient retention for several compounds, an earlier published HILIC method [Bajad *et al.*, 2006] was adapted. This method was originally optimised for analysis of 141 metabolites in *Escherichia coli* extracts and one run lasted 40 min in positive and 50 min in negative mode. For our purpose the flow rate was increased, the elution gradient was adjusted and analysis duration was cut down to about one half. Final conditions were achieved by further reduction of analysis time and modifying the gradients in positive and negative mode in order to reflect the distribution of retention times of measured compounds.

In comparison to UV-vis or potentiometry TMS offers a superior differentiation potential by conveying information about molecular weight and structure (fragmentation). However, this quality should not be overestimated, especially in analyses of complex biological samples. Compounds with a similar structure may suffer from analogous fragmentation patterns. Moreover, monosaccharide epimers and other isomers such as uridine and pseudouridine have the same molecular wieghts. In the case of larger molecules fragmentation may result in product ions identical to another metabolite present in the sample. So, as a rule, ribosides yield their corresponing nitrogenous base as one of their product ions. Ion transitions of 2-deoxyribosides are likely to respond to nucleosides as well because the only distinction of deoxyribosides is a lacking oxygen atom. Another more complex example may be

found in the cleavage of succinate from succinyladenosine. Succinate, apart from appearing in urine in physiological conditions, is an isomer of methylmalonic acid. In the presented method most of these potential interferences were eliminated by HPLC separation.

Galactose was separated from glucose and mannose, also present in urine, in the 25 min long analysis but not in the final method. The measured galactose therefore should be rather considered a total of urine hexoses. In relation to IEMs, galactosaemia with its incidence of 1 : 60 000 is one of the most common metabolic defects of monosaccharides. Hypoxanthine and inosine are not resolved in the negative mode but can be distinguished in the positive mode. Other potential interferences were sufficiently resolved.

In the case of overlapping retention times of compounds with a common ion transition (i.e. isobaric effects) peak identity can be determined more accurately by calculating the ratio of two ion transitions.

The presented method is not suitable for analysis of uric acid because of its precipitation during sample preparation. In a similar way 2,8-dihydroxyadenine is presumed to precipitate from the standard solution because of its low solubility.

Considerable matrix effects were observed during analysis of urine samples in positive ionisation mode. This is in accordance with previous findings that positive mode electrospray ionisation is more susceptible to ionisation suppressions than other ionisation techniques, such as APCI, APPI or negative mode ESI [Taylor, 2005]. However, the most impressive ionisation suppression, which occurs over a longer period, may be attributed to the mobile phase composition. In both modes the onset of this period comes closely after the increase in ammonium acetate buffer content to 95% (v/v). Similar ion suppression in gradient elution with an ammonium acetate buffer, albeit less pronounced, was observed in analysis of fructose and sorbitol by APCI-TMS [Liang *et al.*, 2005].

The presented method offers good reproducibility of retention times and analytes, with the exception of pyruvate and uric acid, showed linear response in the range of expected concentrations. Uric acid precipitation is caused by dilution with initial mobile phase which contains 85% of acetonitrile (v/v). Dilution of urine samples is important for reduction of contamination of the mass spectrometer. The use of another solvent may be considered, but some precipitation is likely occur during separation anyway. Limits of detection were satisfactory in most compounds, higher values were observed for several uracil and thymine derivatives that are generally known for their limited ionisation. However, in IEMs concentrations are elevated to such a degree that is clearly distinguishable from normal samples. Because of ion

suppresion mentionned before, correction to 8-bromoadenosine as the only internal standard turned out unsufficient and determination and precision were unacceptable. Validation of the method should be performed with a larger number of internal standards. Isotope labelled analogs for every analyte would be an optimal solution. However, satisfactory results may be obtained with internal standards covering various stages of ion suppression.

Urine samples of patients with known IEMs were analysed and typical aberrations were easily identified in comparison to controls. All three known disorders of galactose metabolism may be detected by higher galactose and its metabolite galactitol excretion in urine. We found increased galactose and galactitol in urine of one galactosaemic patient, while no difference to controls was observed in another sample of a galactosaemic patient under treatment, which as a rule consists of a low-galactose diet. Significant elevations in orotic acid, typical for OTC deficiency, were observed in urine from a patient with this disorder. The concurrent increase in uridine may result from the enhanced pyrimidine *de novo* synthesis. In comparison to control samples significantly larger amounts of xanthine and a less pronounced increase in another purine 2-deoxyinosine were found in urine from a xanthinuric patient and elevated methylmalonic acid levels in urine from a patient with methylmalonic aciduria. No differences against controls were found in urines from a patient with ethylmalonic aciduria and another with lactic aciduria. The samples had been analysed by routine GC-MS screening and a modest increase in ethylmalonic acid and lactate were observed. This discrepancy may be caused by different sample preparation using derivatisation by silylation and measurement by GC-MS technique.

Urine samples mimicking IEMs were analysed and data obtained by the presented method exhibited linear correlation with results from accredited laboratories for all compounds included in the quality control programme except of uric acid.

Conclusions

In the theoretical part a brief introduction into mass spectrometry, HPLC of small molecules, with special attention paid to mechanisms of separation and chromatographic columns, and inborn errors of metabolism was presented. Examples of IEMs related to the method presented in the experimental part were given.

A method for analysis of 35 compounds in urine by HPLC-TMS was described in the experimental part of this thesis. It includes metabolites with diagnostic importance in IEMs of purine, pyrimidine, galactose and creatine metabolism and several organic acids. The method requires minimal sample preparation and offers fast analyses. Its suitability for qualitative screening was documented by measuring pathological urine samples and urine samples imitating IEMs. However, it is not applicable for uric acid analysis and improvement of validation parameters and use of several internal standards is necessary for routine application and correct quantification. The method offers great potential for expanding the spectrum of analytes by simple optimisation and addition of ion transitions.

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Abbreviations

α	separation factor
AC	alternating current
ADA	adenosine deaminase
ADSL	adenylosuccinate lyase
ANP	aqueous normal-phase
APCI	atmospheric pressure chemical ionisation
APPI	atmospheric pressure photoionisation
APRT	adenine phosphoribosyl transferase
CE	collision energy
CV	coefficient of variation
CXP	collision cell exit potential
dAR	deoxyadenosine
DC	direct current
DHU	5,6-dihydrouracil
DNA	deoxyribonucleic acid
DP	declustering potential
dGR	deoxyguanosine
DHP	dihydropyrimidine amidohydrolase = dihydropyrimidinase
dHR	deoxyinosine
EI	lectron ionisation
ERNDIM	European research network for evaluation and improvement of screening, diagnosisand treatment of inherited disorders of metabolism
ESI	electrospray ionisation
GAA	guanidinoacetate
GALT	galactose-1-phosphate uridyl transferase
H	theoretical plate height
HGPRT	hypoxanthine guanine phosphoribosyltransfrase
HILIC	hydrophilic interaction chromatography
HPLC	high-performance liquid chromatography
IE	ion-exchange
IEM	inborn error of metabolism
IPC	ion-pair chromatography
ISSD	infantile free sialic acid storage disease
LD	limit of detection
k	retention factor

MRM	multiple reaction monitoring
MS	mass spectrometry
m/z	mass to charge ratio
N	number of theiretical plates
NP	normal-phase
OTC	ornithine transcarbamoylase
PALC	per aqueous liquid chromatography
PNP	purine nucleoside phosphorylase
PRPP	5- phosphoribosyl-α-pyrophosphate
PRPPS	phosphoribosylpyrophosphate synthetase
psUR	pseudouridine
Q (q)	quadrupole (quadrupole used as a collision cell)
R	resolution
R^2	correlation coefficient
RP	reversed-phase
SAICAR	5-aminoimidazole-4-(N-succinylocarboxamide) ribotide
sAR	succinyladenosine
SRM	single reaction monitoring
S/N	signal-to-noise ratio
t_r	retention time
t_0	void time
TMS	tandem mass spectrometry
TOF	time-of-flight
UHPLC	ultra-high performance liquid chromatography
UMPS	uridine monophosphate synthase
XDH	xanthine dehydrogenase