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University of Helsinki
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Laboratory services, HUSLAB

Development of liquid chromatography mass spectrometric methods for quantification of metabolites from cellular level to clinical biomarkers

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ACADEMIC DISSERTATION

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“Scientific advancement should aim
to affirm and to improve human life”

Nathan Deal

Contents

List of original publications

This thesis is based on the following original publications referred to in the text by their Roman numerals.

I Tohmola, N.*, Ahtinen, J.*, Pitkänen, J-P., Parviainen, V., Joenväärä, S., Hautamäki, M., Lindroos, P., Mäkinen J. & Renkonen, R. On-line high performance liquid chromatography Measurements of extracellular metabolites in an aerobic batch yeast (*Saccharomyces cerevisiae*) culture. *Biotechnol Bioproc E* **2011**; 16: 264-72.

*) Equal contribution

- NT participated in cell cultivations and sample collecting, performed the MS runs and data analysis and participated in the writing of the manuscript.

II Tohmola N., Itkonen O., Sane T., Markkanen H., Joenväärä S., Renkonen, R. & Hämäläinen E. Analytical and preanalytical validation of a new mass spectrometric serum 5-hydroxyindoleacetic acid assay as neuroendocrine tumor marker. *Clin Chim Acta* **2014**; 428: 38-43.

- NT developed and validated the assay, collected the patient samples, performed the MS runs and data analysis and wrote the manuscript.

III Tohmola N., Johansson A., Sane T., Renkonen R., Hämäläinen E. & Itkonen O. Transient elevation of serum 5-HIAA by dietary serotonin and distribution of 5-HIAA to serum protein fractions. *Ann Clin Biochem* **2014**. *Published online.*

- NT participated in the planning and performing of the study, analysed the distribution study samples and data and wrote the manuscript.

IV Tohmola N., Itkonen O., Turpeinen U., Joenväärä S., Renkonen R. & Hämäläinen E. Preanalytical validation and reference values of mass spectrometric assay of serum vanillylmandelic acid for screening of catecholamine secreting neuroendocrine tumors. *Clin Chim Acta* **2014**. *Resubmitted after revision.*

- NT developed and validated the assay, collected the patient samples, performed the MS runs and data analysis and wrote the manuscript.

Abbreviations

5-HIAA	5-hydroxyindole acetic acid
5-HTP	5-hydroxytryptophan
3MT	3-methoxytyramine
AADC	Aromatic acid decarboxylase
ACTH	Adrenocorticotropic hormone
AKG	α-ketoglutarate
ALDH	Aldehyde dehydrogenase
ALDR	Aldehyde reductase
APCI	Atmospheric chemical ionization
APPI	Atmospheric photo ionization
AUC	Area under curve
BPG	Bisphosphoglycerate
CA 19-9	Carbohydrate antigen
CID	Collision induced dissociation
CIT/ICIT	Citrate/Isocitrate
CgA	Chromogranin A
COMT	Catechol-O-methyltransferase
CV	Coefficient of variation
DA	Dopamine
DHEA	Dehydroepiandrosterone
DOPA	3,4-dihydroxy-L-phenylalanine
DOPAC	3,4-dihydroxyphenylacetic acid
E	Epinephrine
ESI	Electrospray ionization
FT	Fourier transform
G1P	Glucose 1-phosphate
G6P	Glucose 6-phospate
F16P	Fructose 1,6-phosphate
HILIC	Hydrophilic interaction chromatography
HPLC	High performance liquid chromatography
HVA	Homovanillic acid
IS	Internal standard
IT	Ion trap
OD	Optical density
PEP	Phosphoenolpyruvate
PNMT	Phenylethanolamine N-methyltransferase

PP	Pancreatic polypeptide
m/z	Mass-to-charge ratio
MAL	Malate
MALDI	Matrix assisted laser desorption ionization
MAO	Monoamine oxidase
MEPS	Microextraction by packed sorbent
Met (MN)	Metanephrine
MHPG	3-methoxy-4-hydroxyphenylglycol
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MRM	Multiple reaction monitoring
NE	Norepinephrine
NET	Neuroendocrine tumor
NMR	Nuclear magnetic resonance
Nor (NMN)	Normetanephrine
NORIP	Nordic reference interval project
NP	Normal phase
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantitation
LOD	Limit of detection
LOQ	Limit of quantitation
Q	Quadrupole
QqQ	Triple quadrupole mass spectrometer
r^2	Coefficient of determination
RE	Relative error
RI	Refractive index
RIA	Radioimmunoassay
ROC	Receiver operator characteristics
RP	Reversed phase
SPE	Solid phase extraction
TOF	Time-of-flight
TPH	Tryptophan hydroxylase
TSH	Thyrotropin
TYR	Tyrosine
ULOQ	Upper limit of quantitation
VMA	Vanillylmandelic acid

Abstract

Metabolites are low molecular weight compounds participating in different functions of cellular systems. Metabolites can be used as diagnostic biomarkers for numerous diseases. Liquid chromatography tandem mass spectrometry (LC-MS/MS) is a powerful tool in quantification of metabolites from various sample matrices. Good sensitivity and specificity are the main benefits of the technique. Mass spectrometry is commonly used in industry, drug research and clinical diagnostics. Extensive validation of newly developed analytical methods will construct the basis to a reliable assay, and it is significant especially when analysing e.g. patient samples.

The aim of this study was to develop quantitative assays for metabolites from biological samples for biomedical research and clinical diagnostics. We designed and constructed an on-line high performance liquid chromatography (HPLC) equipment and validated an assay for direct quantification of extracellular metabolites from cell cultivation. Automated sampling for LC-MS/MS analysis of intracellular metabolites was connected to the on-line system. The on-line analysis improves the methodology and shortens the time of analysis. Furthermore, a frequent sampling data can provide valuable information about physiological indications in various cell cultivations. On-line HPLC is suitable for various biotechnological applications because of its ability to monitor and collect data during cell cultivation.

We developed and validated LC-MS/MS assays for neuroendocrine tumor (NET) biomarkers 5-hydroxyindole acetic acid (5-HIAA) and vanillylmandelic acid (VMA) from human serum. Generally, urinary HPLC assays are used for the determination of NET markers. HPLC assays have certain limitations and 24-h urine collection is laborious. Our LC-MS/MS assays are specific, fast and well suited for diagnostics of NETs. Furthermore, guidelines for urine collection advise to refrain from serotonin-containing foods for three days before sample collection. We showed that such a diet restriction before serum 5-HIAA assay is not necessary. Instead, one day serotonin-free diet before sampling is sufficient because the half-life of 5-HIAA in circulation was found to be 1.3 hours.

All assays developed during this study were sensitive and had a wide linear range. Our serum 5-HIAA LC-MS/MS assay is routinely used for the analysis of NET patient samples at the Helsinki University Central Hospital Laboratory, HUSLAB. Serum VMA LC-MS/MS assay will be in routine use in the HUSLAB in near future. Furthermore, On-line HPLC Ltd, (Helsinki, Finland) has commercialized the on-line HPLC equipment developed in this study.

1 Review of the literature

1.1 Introduction

Liquid chromatography (LC) combined to mass spectrometry (MS) is a powerful tool for the analysis of various compounds, e.g. small molecular weight metabolites from different sample matrices. The number of LC-MS/MS instruments has increased in clinical chemistry laboratories during the past decade. Metabolite data is used to understand biochemical functions of cellular systems, and biomarker invention. Recent development in mass spectrometry techniques has contributed to the quantification of metabolites. Furthermore, there is a need for improved assays in clinical diagnostics.

In this study, we used LC and LC-MS/MS methods to develop and validate assays for metabolites from biological samples. The main aim was that the newly developed assays would be useful both in research and clinical diagnostics.

1.2 The metabolites

Metabolites are a group of low molecular weight intermediates and products of metabolism. Generally, these include organic species like amino and fatty acids, carbohydrates, hormones, vitamins and lipids[1]. Metabolites can be divided into endogenous and exogenous metabolites and the term metabolome includes all metabolites of an organism. Endogenous metabolites are inherent compounds participating in general metabolic reactions like glycolysis, citric acid cycle and the pentose phosphate pathway. They have a role in the signalling, growth and normal function of a cell, in defence and in interactions with other organisms[2,3]. Exogenous metabolites are formed as part of the biochemical process of degrading and eliminating exogenous compounds such as drugs, dietary components or environmental pollutants[1]. The size of a metabolome is enormous. A relatively simple species of yeast, the *Saccharomyces cerevisiae,* contains almost 600 metabolites[4] while the human metabolome database[5] contains detailed information of over 40 000 small molecule metabolites found in the human body. Metabolite data can help in understanding biochemical functions of complex cellular systems. In metabolite analysis, research data is used for phenotypic[6] and genotypic analyses[7], biomarker determination[8-10] drug intervention[11], nutrigenomics[12], clinical diagnostics[13], metabolic engineering[14] and systems biology[15]. A substantial part of metabolite research is focused on finding new biomarkers for diseases and development of analysis methods for metabolite biomarkers. New

analysis methods can be exploited in drug research, diagnostics or other medical applications.

1.2.1 Metabolites as biomarkers

According to the National Institutes of Health's Biomarkers Definition Group, the term biomarker means "a characteristic that is objectively measured as an indicator of normal biological processes, pathogenic processes or a pharmacological response to a therapeutic intervention"[16]. Biomarkers can be categorized into four different groups according to their use, i.e. diagnostic, predictive, metabolic and outcome biomarkers[17]. They can be used in the prediction, detection and classification of a disease or to determine the dose of medication. Metabolite biomarkers are used e.g. in screening of inborn errors in metabolism[18,19] and testosterone measurement in clinical diagnostics[20]. Biomarker discovery is important in the field of medicine. Recent developments in metabolite profiling techniques have facilitated the discovery of new biomarkers[21]. However, a promising new biomarker is not necessarily a useful biomarker. The path of validation and implementation of a new biomarker is demanding (Fig. 1).

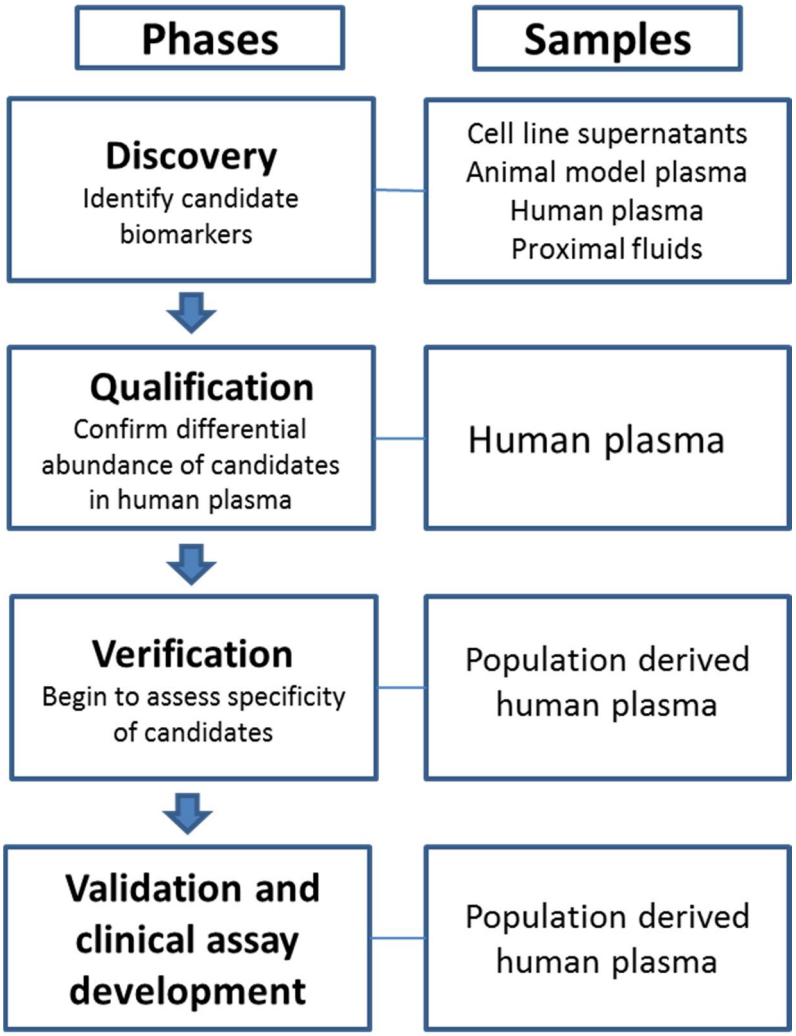


Figure 1. Biomarker validation process (modified from Rifai et al.[22]).

1.2.2 Analysis of metabolites

In clinical chemistry laboratories quantification of diagnostic biomarkers is based on several assay principles. The main principles include photometry, enzymatic assays, immunological assays, electrophoresis, chromatography and MS. For example, glucose and cholesterol are assessed by enzymatic assays coupled to photometric techniques by automatized clinical chemistry analyzers[23]. Immunological assays are proven to be efficient with good sensitivity and specificity e.g. for analysing thyroid hormones and cancer biomarkers. Serum thyrotropin (TSH) is a protein biomarker used as the primary screening test for thyroid dysfunctions. It is usually determined by automated immunoanalysers[24,25]. A radioimmunoassay (RIA) has shown good sensitivity in the analysis of hyperandrogenism and polycystic ovary syndrome biomarker dehydroepiandrosterone (DHEA) and its sulphate metabolite (DHEA-S) from serum[26]. Ease of use, high sample throughput and possibility of automation are advantages of these methods in clinical laboratory.

Recent advances in mass spectrometry technology have contributed to the development of new and better assays for disease biomarkers or therapeutic drug monitoring. For example, unspecific immunoassays are not recommended for the analysis of steroid hormones[27]. Also, LC-MS assays of immunosuppressants administered to prevent of transplant rejection have shown better specificity than immunological assays[28]. The high specificity and sensitivity of mass spectrometric detection and the possibility to combine multiple analyses into one MS equipment (multiplexing)[29] enable improvement of assays. However, the use of MS techniques requires highly skilled laboratory staff. Manufactures are developing improved MS software and analytical kits. MS kits for common analytes like immunosuppressants[30] or steroid hormones[31] have been introduced for diagnostics.

Novel biomarkers are constantly needed and metabolites are a possible source for discovery. Screening and identification of new metabolites is based on two main techniques; nuclear magnetic resonance (NMR) or MS[32] in stand-alone mode or coupled to modern separation techniques such as gas chromatography[33,34], liquid chromatography[35,36] or capillary electrophoresis[37,38]. NMR is an efficient technique for structural analysis and it is used for fingerprinting of large amounts of metabolites[39]. However, it is less sensitive than MS and thus requires a larger sample sizes[40].

1.3 High pressure liquid chromatography

Liquid chromatography (LC) is an important tool in metabolite analysis[41]. LC analysis is robust and rapid to perform, has good repeatability and is relatively easy to automate and connect to a mass spectrometer or other detection devices. The chemical properties of the compounds of interest are various. Therefore, different chromatographic separation techniques have been developed and are commercially available. Usually two types of stationary phases with several modification options are used; inorganic silica or organic polymer phase[42]. The stationary phase pore size in the LC columns is usually 80–300 Å and the size of the particles is 3–5 µm. Furthermore, the column length may vary from 30 to 250 mm[43,44]. In HPLC, analytes are separated by using operational pressures of 50–350 bar. The separation is based on interaction of analytes between the stationary and mobile phases[44]. Ultra High Performance Liquid Chromatography (UHPLC) is a relatively new technique and has gained popularity in metabolite discovery in particular[45,46]. The difference between HPLC and UHPLC is that in UHPLC smaller particle and column sizes are utilized (inner diameter of 1–2.1 mm) and separation of analytes occurs under very high pressure. The advantage of UHPLC is the narrow peaks, high peak capacity and short analysis times leading to increased sensitivity and sample throughput[47]. For

example, the UHPLC-MS protocol was used to produce global metabolic profiles from human urine[48].

1.3.1 Reversed phase chromatography

On the basis of publications cited in the PubMed[49] reversed phase chromatography (RP) has been by far the most employed technique in metabolite analysis (Fig. 2).

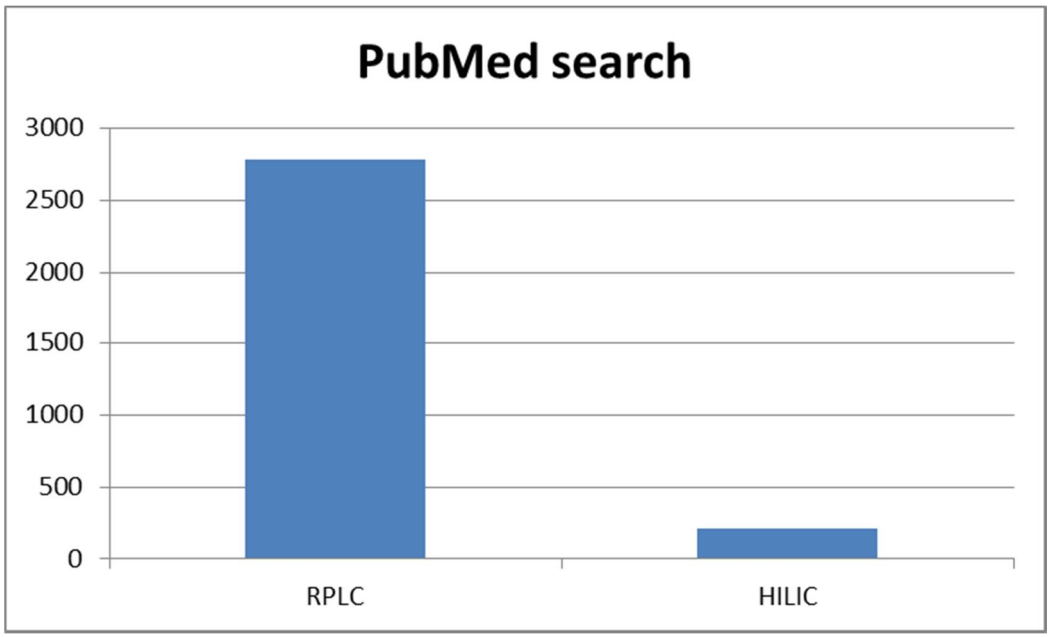


Figure 2. PubMed search results with words ”reversed phase chromatography” or “hydrophilic interaction chromatography” and “metabolites”. Abbreviations: RPLC: reversed phase liquid chromatography, HILIC: Hydrophilic interaction chromatography.

In RP, the stationary phase is a hydrophobic carbon chain covalently bound to solid silica or polymer and the separation is based on hydrophobicity of the sample molecules[44]. By increasing the content of the organic eluent, hydrophobic molecules can be eluted from the column. The eluents used in RP are often volatile and connecting to electrospray ionization (ESI) and MS is thus easy. The disadvantage of RP is its weak capability to bind polar molecules[50].

1.3.2 Hydrophilic interaction chromatography

Hydrophilic interaction chromatography (HILIC) was first introduced in the 1970s[51], but it became common in metabolite analysis in the 2000th century. HILIC is a variant of normal phase (NP) chromatography and its separation mechanism is based on hydrophilicity of the molecules. It is usually an alternative in cases where RP is not able to separate polar compounds. The separation is founded on partitioning of the compounds into hydrophilic

stationary phase, hydrogen bonding and weak electrostatic interactions[52]. Manufacturers are offering a wider selection of specifically designed HILIC stationary phases with diverse functionalities to improve selectivity and retention of polar compounds. Unmodified bare or hybrid silica materials are the most popular phases. The most common mobile phase eluent is acetonitrile and the elution of the analytes is achieved by a water gradient. To improve retention, buffering salts like ammonium acetate and formiate are used in HILIC as they are compatible with MS[53]. The major advantage of HILIC is the possibility to use organic solvents in sample preparation without a vaporization step before chromatography. HILIC is used e.g. in the determination of levosulpiride from human plasma[54] and neurotransmitters from primate cerebral cortex[55].

1.4 Mass spectrometry

The first mass analyzer was manufactured in 1912[56] and since then the number of mass analyzers has multiplied[56,57]. In mass spectrometry, the sample is first ionized and the ions are then separated based on their mass-to-charge ratio (*m/z*) values. The use of mass spectrometric techniques has become more and more popular in medical laboratories during the past decade[58]. Liquid chromatography tandem–mass spectrometry (LC-MS/MS) is nowadays a standard tool in clinical chemistry laboratories. This technique has good specificity and sensitivity, wide dynamic range and robustness[59]. Its major applications in clinical laboratories are vitamin assays (especially D-vitamin)[60,61], steroid hormone assays[62-64] and therapeutic drug monitoring[65,66]. The strengths, weaknesses, opportunities and threats (SWOT analysis) of LC-MS/MS analysis in clinical diagnostics are presented in Table 1.

Table 1. SWOT analysis of LC-MS/MS in clinical diagnostics. SWOT is a tool for auditing an organization, its environment and its processes. The strengths and weaknesses are internal factors; opportunities and threats are external factors. (Modified from van den Ouweland et al.[59]).

Strengths	**Weaknesses**
♦ High sensitivity ♦ High specificity ♦ High speed of development at low costs of new assays when compared to immunoassays by in vitro diagnostics (IVD) companies ♦ Possibility to measure multiple analytes in the same sample simultaneously ♦ Multiplexing opportunity ♦ Versatility ♦ Near reference methodology in routine setting ♦ Compatible with automated sample handling configurations	♦ Relatively high instrument cost ♦ Serial (batch-wise), non random-access operation ♦ Need for highly skilled personnel for method development, validation, operation and troubleshooting ♦ Lack of clearly defined quality regulations ♦ Limited sample throughput in conventional set-up ♦ Limited experience of IVD requirements from MS vendors
Opportunities	**Threats**
♦ Progress towards more user-friendly instruments ♦ Adoption of MS technology by major IVD companies ♦ Broader availability of IVD approved kits for LC-MS/MS analysis ♦ Quantitative measurement of peptides and proteins ♦ Profiling metabolically related metabolites	♦ Speed of development of new instruments ♦ Difficulty in finding skilled technicians and experience at an academic level ♦ Lack of commitment from major IVD companies ♦ Regulatory bodies applying restrictions on using home-brew assays for diagnostic purposes ♦ Competition from innovations in immunoassays or from the introduction of new technologies

1.4.1 Electrospray ionization

There are several different ionization techniques in MS i.e. atmospheric pressure photo ionization (APPI)[67] and atmospheric pressure chemical ionization (APCI)[68], but electrospray ionization (ESI) is the most commonly used in metabolite research[40,69]. In ESI, analytes are ionized directly from the solution, so it is easy to connect to the LC system. ESI is a robust technique and tolerates high buffer concentrations. The main advantage of ESI is its suitability for ionization of small and large polar biomolecules[70]. However, APCI and APPI are more compatible for non-polar compounds[71]. The sample is sprayed through a high voltage capillary producing positively or negatively charged ions. Due to the high pressure and voltage, the liquid is dispersed into small droplets. Nebulizer gas produces turbulence that assists in the formation of the droplets. Repulsion makes the charges attempt to the surface and the neutral dissolvent molecules evaporate from the drops at the same time. The charge density increases in the drops and when it reaches the maximum the drops decompose into smaller ones. Eventually, only ions which fly to the mass analyzer are left[72,73] (Fig. 3).

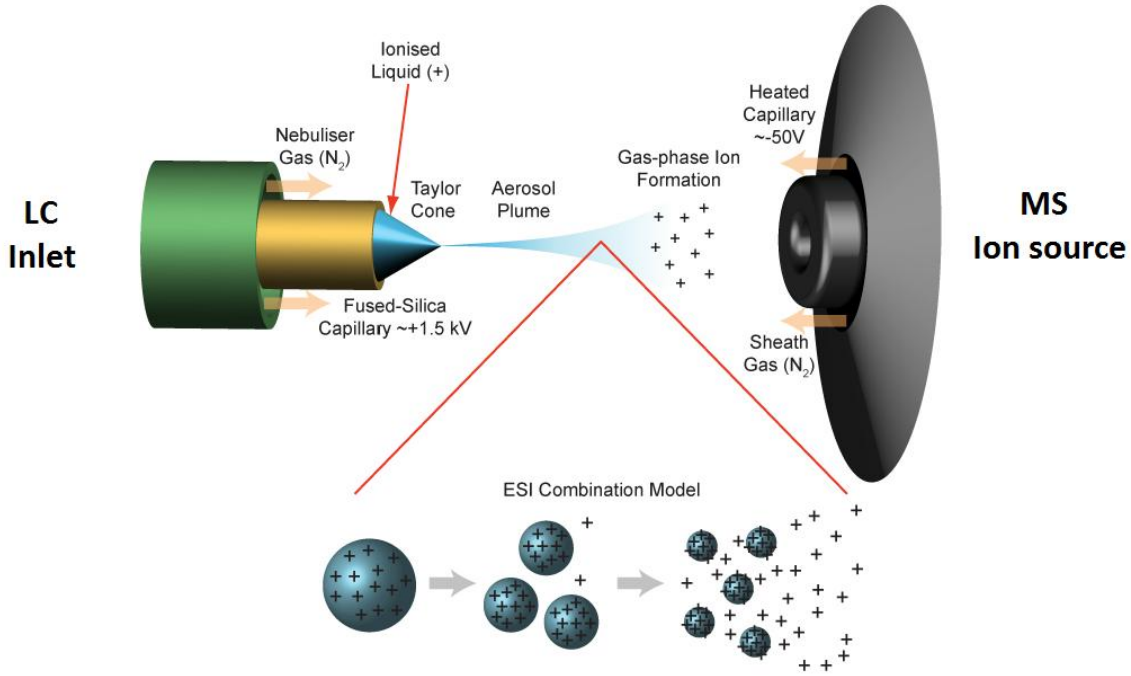


Figure 3. Principle of the ESI (modified from www.lamondlab.com[74]).

Analytes of interest compete with other sample molecules in the ionization process. Some additives, like formic acid, can be added to improve the positive ionization of the analyte[75]. Ionization in ESI can provide singly or multiply charged compounds. Generally, larger molecules e.g. peptides are multiply charged. The composition of eluent, buffer, pH, flow rate and concentration of the analyte of interest also affect to ionization[76-78].

1.4.2 Mass analyzers

Mass analyzer is the part of an MS instrument where ions are separated based on their *m/z* values. Mainly five different types of mass analyzers have been used in the analysis of metabolites, i.e. quadrupole (Q), ion trap (IT), time-of-flight (TOF), Fourier transformer (FT) and Orbitrap mass analyzers[44,79]. These analyzers have different strengths and weaknesses from the point of metabolite analysis. MS instruments vary in size, price, resolution, mass range and their ability to perform tandem mass spectrometry (MS/MS) experiments[40,57].

1.4.3 Triple quadrupole and MS/MS

The triple quadrupole mass analyzer (QqQ) is the working horse in absolute quantification. A QqQ consists of three quadrupoles; Q1, Q2 and Q3. The first Q1 and the last Q3 are operated as mass analyzers and Q2 as a collision cell where molecules can be fragmented (Fig. 4). The Q1 and Q3 can be used to scan or isolate ions of interest. When desired, ions leaving Q1 can be fragmented in the collision cell before entering Q3[80]. In Q1 and Q3 the ions can be separated by their *m/z* values[57]. A quadrupole consists of four quadrupole rods that have opposite voltages[81]. The electromagnetic field between the rods causes a wave motion of arriving ions. Stable ions start to vibrate with small amplitude and fly through the quadrupole. Ions with high vibration amplitude are not stable within the quadrupole and collide to the quadrupoles or walls of the instrument[71]. In the collision cell, ions undergo collision with inert gas (e.g., helium, nitrogen, argon, xenon) molecules. The transfer of kinetic energy from the stream of collision gas causes fragmentation of ions. This process is called collision-induced dissociation (CID)[82]. CID is the most often applied ion fragmentation method in metabolite analysis, but ion fragmentation can also be induced by techniques called electron capture induced dissosiation[83] or surface induced dissosiation[84].

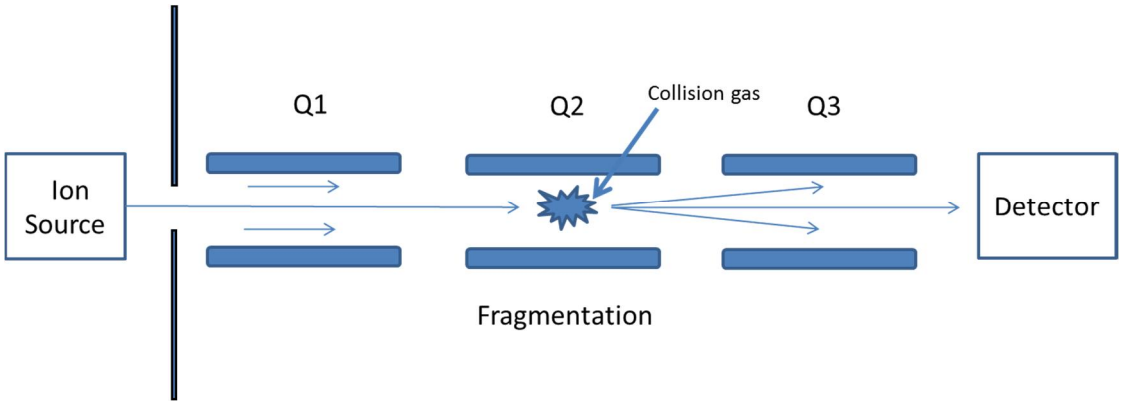


Figure 4. Schema of a triple quadrupole mass spectrometer.

Compared to high resolution analyzers, the major advantages of triple quadrupole analyzers are the relatively low cost and small size, robustness, wide dynamic area and ease of use and maintenance. Triple quadrupoles can work in different scanning modes (Fig. 5). For example the TOF and Q-TOF instruments are not able to operate in multiple reaction monitoring mode. On the other hand, triple quadrupole analyzers have a low resolution and limited mass range, usually within *m/z* 0-2000[85].

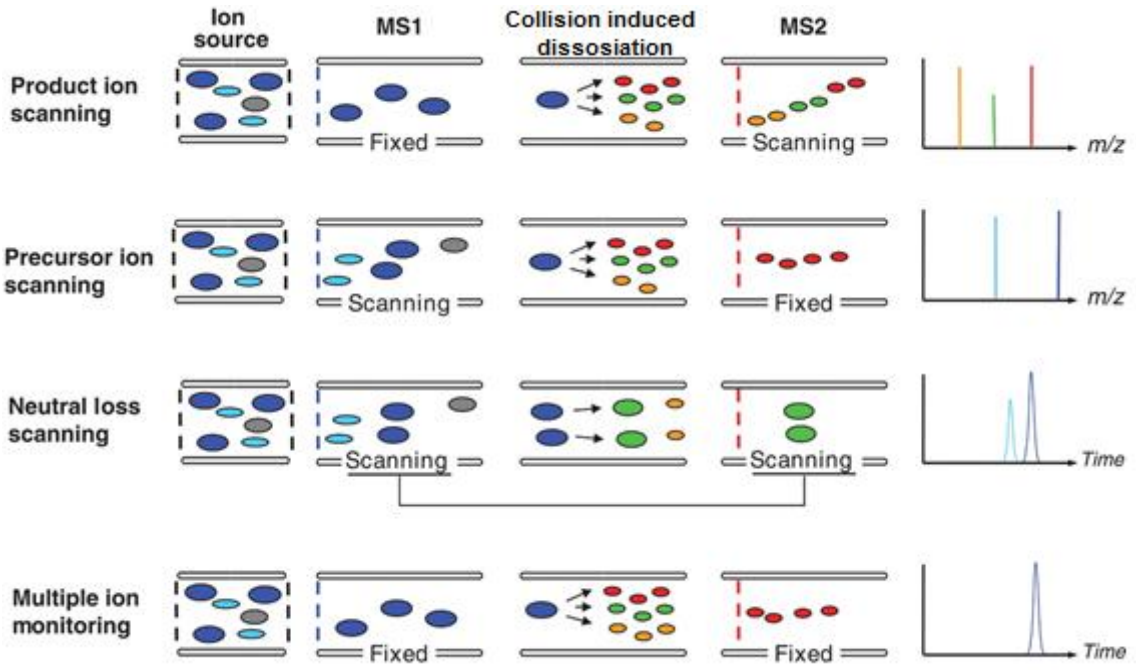


Figure 5. Different scanning modes in QqQ (modified from Domon and Aebersold[86]).

1.4.4 Multiple reaction monitoring

Multiple reaction monitoring (MRM) has been used as a quantitative technique for the analysis small molecules for over 30 years. Baty and Robinson were the first to report the monitoring of phenytoin and its metabolites in plasma by MRM in 1977[87]. In MRM, mass spectrometer scans only selected precursor-product ion pairs and excludes all other ions from the scan. This enhances specificity and sensitivity in targeted quantitative metabolite analysis.

In the MRM mode, the instrument scans though a list of selected transitions in an operation called the cycle time. If the cycle time is one second, the intensity value is recorded for each transition at one second intervals. Dwell time, for one, is the length of time in seconds when the highlighted mass is monitored[88]. The number of transitions-of-interest is a crucial factor in MRM. The amount of scanning points for each transition-of-interest within a cycle time defines the shape of the peak. Therefore, an analysis should allow at least 10-15 scanning points for each peak to ensure acceptable peak shape and adequate quantification. Cycle times of 1.1 s (Fig 6A) and 0.4 s (Fig 6B) result in different shapes of the peak and have an impact on the accuracy of the measurement of the metabolite concentration. Cycle times and the amount of scanning points in the peak depend on the number of transitions.

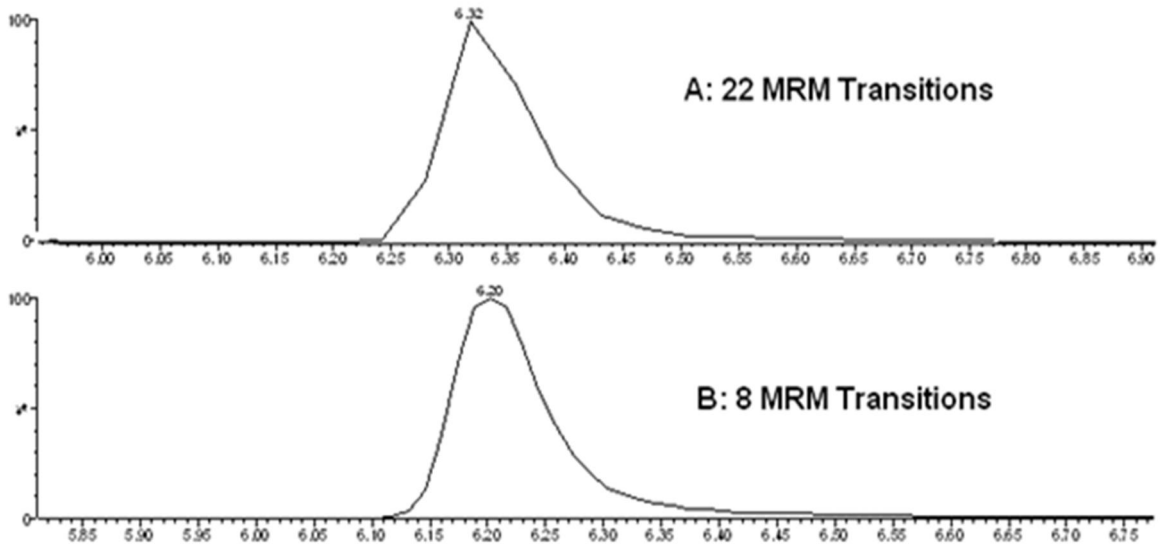


Figure 6. Impact of the cycle time on the peak shape in MRM. (A) 22 MRM transitions, cycle time 1.1 s. (B) 8 MRM transitions, cycle time 0.4 s.

1.4.5 Matrix effect

Matrix effect is a phenomenon known to influence the accuracy of MS analyses[89]. Matrix effects have been demonstrated mainly in biological matrices like plasma and urine[90,91]. The explanation of the mechanism is that the analyte and the co-eluting sample matrix components compete for ionization in the ion source. Such a competition between molecules may cause ion suppression or ion enhancement of the analyte. Molecules with higher mass tend to suppress the signal of smaller molecules, and polar molecules are more prone to suppression[92]. Phospholipids, which constitute a major part of the lipid bi-layer in cell membrane, cause major ion suppression in MS[93]. The choice of ionization technique may play an important role in quantitative MS analysis. It has been shown for some compounds, that APCI is less prone to ion suppression than ESI[94,95].

Several attempts have been made to reduce matrix effects. Modifications of sample preparation or chromatographic conditions and standard addition method have proved to be powerful ways to compensate it[92,96-100]. A properly selected solvent composition and concentration[101] and the use of stable isotope labeled compounds as internal standards can be used to correct for the inaccuracy caused by matrix effects. Labeled compounds mimic the analytes of interest in the ionization process and thus provide a powerful tool to correct for the suppression related to a non-linear response. However, in some cases it is not possible to use labeled compounds as internal standards. These compounds may be very expensive or synthesis of labeled standards may be challenging. Furthermore, the internal standard method does not always work as expected. Wang et al. have shown that high level of matrix suppression affected ionization of the analyte and its deuterated internal standard differently in human plasma making the correction of analyte response unreliable[102]. In MS assay for testosterone, the use of ^{13}C labeled internal standard may underestimate the true concentration due to the natural 1.1% isotopic abundance of ^{13}C [103]. Therefore, the method to compensate for the matrix effect must be chosen with care.

1.5 Sample preparation in metabolite analysis by LC-MS/MS

Analyzing specific compounds from biological samples is challenging because the sample contains large amounts of different components (lipids, salts, proteins, cellular components etc.)[104]. In metabolite analysis, the sample usually contains many undesired biomolecules (e.g. proteins) with different size and concentration. Furthermore, proteins may form complexes among themselves or with other biomolecules[105]. These factors complicate the sample preparation and make it an extremely important part of the analysis. Without proper sample preparation the risk of instrument contamination and loss of sensitivity and specificity are possible.

1.5.1 Solid phase extraction

Solid phase extraction (SPE) is often used for sample pretreatment before analysis because of ease of use, specificity and selectivity[106]. The basic principle of SPE is adsorption of analytes into a chosen SPE sorbent material (reversed phase, normal phase, ion-exchange or covalent interaction)[107]. First, SPE sorbent is usually conditioned and equilibrated. Then, the sample is applied followed by washing steps and finally, the analytes are eluted from the sorbent. The advantages of SPE are selectivity, versatility, wide selection of sorbent materials and possibility of automation. Compared to precipitation techniques, SPE may be more laborious and more expensive to perform. SPE can be performed manually employing extraction cartridges, disks or microplates, or with commercial automation platforms (Hamilton, Tecan, Biotage Extrahera)[108,109]. In clinical chemistry laboratories, a 96 well microplate SPE is used e.g. in urinary metanephrines LC-MS/MS assay[110]. Thibeault et al.[111] have developed a faster on-line SPE method compared to liquid-liquid extraction (LLE) in D-vitamin LC-MS/MS assay. Microextraction by packed sorbent (MEPS) is a miniaturized SPE technique that can be connected on-line to LC or GC. MEPS works with small sample volumes and the solvent volume used for the elution of the analytes can be injected directly into the LC system[112]. It has been used for instance in determination of cyclophosphamide from human plasma in therapeutic drug monitoring[113].

1.5.2 Liquid-liquid extraction

Liquid-liquid extraction (LLE) is based on the partition of analytes and other compounds between an aqueous and an organic phase. Factors affecting the separation are analyte solubility, pKa, solution pH and ionic strength[114]. LLE has been used for the preparation of samples especially in environmental field[106]. In clinical chemistry laboratories LLE is used mostly for the preparation of steroid and vitamin samples[115,116]. LLE is a powerful sample preparation method, but more laborious to perform than SPE or precipitation. Without automated liquid handling LLE requires a lot of challenging manual pipetting of solvents.

1.5.3 Protein precipitation

Proteins can be precipitated by adding a denaturating organic solvent into the sample. Methanol, acetone and acetonitrile are used the most often in metabolite analyses. In addition, acid, salt or metal ions have been used as a denaturing agent[117]. Protein-metabolite interactions are eliminated in the denaturation process. Phospholipids cause commonly ion suppression in MS assays[118]. Simultaneous protein precipitation and phospholipid removal can be performed in a specific commercial plate which allows

filtration of the precipitated samples[119,120]. For example, protein precipitation is used for immunosuppressants before LC-MS/MS assay[121].

1.5.4 On-line methods

The development of on-line methods answers the needs for minimizing laboratory work and high-throughput assays. Furthermore, an interest for continuous monitoring and collecting data from biological processes requires on-line methodologies. The use of HPLC and LC-MS/MS on-line methods has increased in pharmaceutical industry and metabolite research[122,123]. On-line methodologies provide faster analyses, decrease laboratory work and enable continuous collecting of the data. The biggest challenge of on-line methods is to ensure proper functionality of the automatic multi-step assay. Especially when developing quantitative assays, the possible interferences must be taken into consideration to ensure reliable quantification results. The above described chemistries can be utilized to on-line sample pretreatment. The sample preparation in on-line methods is often based on the automated 96-well format for solid-phase extraction (SPE) or liquid–liquid extraction (LLE)[124,125]. Furthermore, direct injection from sample vials is also commonly used. For example, hemoglobin A1c and its variants have been measured directly from whole blood by HPLC including the hemolysis procedure[126]. On-line HPLC is used for continuous monitoring of compounds in fermentation processes[127] and from waste water[128]. Membrane introduction mass spectrometry (MIMS) is also an effective technique for monitoring of metabolites. It has been used in continuous monitoring of metabolites from fermentation broths with 3-min sampling intervals[129]. There is a wide selection of membrane types in MIMS and the analysis time is short. However, it is most useful for small and non-polar compounds.

1.6 Assay validation

All analytical assays must undergo precise and systematic validation before implementation into routine use[130]. Validation determines the functionality of the assay, the validity of the results and whether the analytical method is suitable for the intended purpose. The importance of validation cannot be overestimated especially when analysing clinical or forensic samples. The forensic or doping results have to be reliable in the court. Furthermore, unreliable clinical results may lead to wrong diagnosis or treatment of the patient. Full validation is important when developing and implementing a new analytical method. Partial validation is accepted when an existing method is modified[131]. Clinical and forensic laboratories follow quality management and accreditation procedures according to international standards[130]. Also, the requirements of assay validation for studies to be accepted for publication in scientific journals are strict[132].

Despite the robustness of LC-MS/MS methods there are several factors that influence the reliability of the quantitative analysis of metabolite concentrations in biological samples. A few were already mentioned earlier. In addition, sample loss during sample preparation and instrument specific “crosstalk” may cause unreliable quantification results. Usually, crosstalk may take place if several mass transitions with identical product ions are acquired[133]. Incorrect signals can be recorded if the collision cell is not emptied completely during the very short dwell time between different transitions. Crosstalk can also occur in transitions without similar product ions. It has been shown that plasma metanephrines affected the concentration of 3-methoxytyramine (3MT). Metanephrine calibration material was found to produce a measurable 3MT peak corresponding nearly 2% of the actual injected concentration. A likely explanation was that metanephrines may fragment within the ion source into ions mimicking 3MT[134]. Adequate scanning time of compounds in MS can be a crucial factor for reliable quantification[135]. Isotopically labeled internal standards are generally used to correct for loss of sample recovery during pretreatment. Especially in quantitative metabolomics, the objective is to quantify more and more metabolites in one analysis. This fact sets enormous challenges to achieve reliable metabolite quantification. In quantitative high-throughput analysis a substantial amount of compounds requires several internal standards. Only one or few internal standards do not fit a batch of several different compounds which have diverse chemical properties. Accordingly, these facts make appropriate internal standard selection problematic particularly if there is no possibility to use labeled standards.

1.6.1 Analytical validation

Analytical validation of a method includes tests to confirm assay specificity, sensitivity, precision, accuracy, recovery, linearity, limit of detection (LOD) and limit of quantification (LOQ). Each of these parameters should be investigated carefully before implementation of the assay[131,136,137].

Analytical specificity and sensitivity

Specificity is the capability of an assay to separate and quantify an analyte from the sample. Sensitivity is the capability of the assay to discriminate small differences in the concentration of the analyte[136].

Accuracy and precision

Accuracy means the closeness of the measured analyte concentration to absolute concentration when the assay is performed in several repeats. Accuracy can be determined by spiking a standard into the sample matrix and calculating the recovery. The precision describes the difference in results between separate analyses[136]. The intra- and inter-assay precision should be determined separately. According to US Food and Drug Administration (FDA) bioanalytical method validation guideline assay inaccuracy and imprecision should be <15%[131].

Recovery and linearity

Recovery is a measure of yield after sample preparation. Recovery can be estimated by adding a known amount of the analyte of interest to the sample and calculating the recovery after sample preparation. With well optimized assays recovery is usually more than 70%. The use of an internal standard can correct for sample loss. Linearity verifies that two quantities (e.g. concentration and peak area) are directly proportional within a given range. The liner range of an assay can be determined by preparing and analyzing calibrators in different concentrations (covering 50% to 150% of the normal analyte concentration) during several days. Calibrators should be prepared and analysed at least three times[137].

LOD and LOQ

LOD is the lowest concentration of an analyte in a sample that can be detected. LOD is sometimes confused with the sensitivity of the method. LOD can be determined as the average + 3 standard deviations of ten to fifteen blank samples. LOQ is the lowest concentration that can be quantified with inaccuracy and imprecision less than 20%[131]. The LOQ can be divided to the lower limit of quantitation (LLOQ) and the upper limit of quantitation (ULOQ) and they are the highest and lowest standard curve points, respectively, that can be used for quantification.

1.6.2 Preanalytical validation

Preanalytical validation includes all crucial steps, which may influence result reliability before the performance of the assay. Factors related to sample collecting, handling and storage before analysis need to be studied (Table 2). Sample stability may be affected for example by repeated cycles of freezing and thawing or long-term storage. There are several studies reporting that steroid hormones have significant diurnal variation[138,139]. Male testosterone concentrations are at the highest level in the morning[140]. Renin-aldosterone ratio is used for the diagnosis of primary aldosteronism. Medication, dietary

sodium, posture and time of day affect renin and aldosterone concentrations[141]. Thus, it is crucial to eliminate any preanalytical factors affecting the test results. In order to do so, lucid instructions for sample donors and adequate training of the laboratory staff are the most important things to keep in mind.

Table 2. Preanalytical validation parameters.

Step	**Parameter**
Collecting	Sample matrix (serum, plasma, urine, saliva etc.)
	Sampling device
	Postprandial effect
	Diurnal variation
	Effect of diet
	Effect of medication
	Effect of exercise
	Effect of stress
	Posture (lying/sitting position)
Handling and transport	Handling time of sample (immediately, delayed)
	Delivery of samples (at room temperature, on ice, frozen)
	Way of transport (pneumatic mail, by car etc.)
Storing	Storing temperature (room temperature, + 4^{o}C, - 20^{o}C, - 80^{o}C)
	Storing time (length of time at different temperatures)
	Freeze-thawing (amount of times)

1.6.3 Clinical validation of diagnostic biomarkers

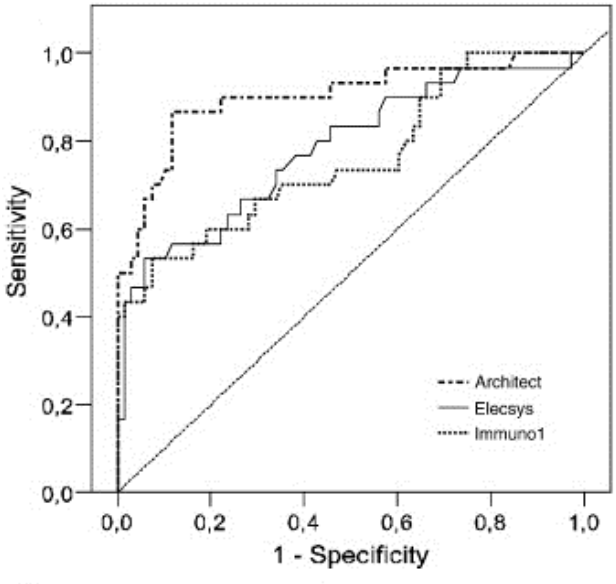
Reference values

Gräsbeck and Saris introduced the concept of reference values in 1969[142]. Reference values are for describing the normal levels of the analyte in healthy individuals. The reference interval is determined with an upper and lower reference limit and includes population-based reference intervals usually consisting of 95% of healthy individuals. The selection of reference individuals is crucial in the determination of reference values. A representative sample is a group including at least 120 reference individuals from different age groups and both genders. Two statistical methods, a nonparametric and a parametric, are generally used for determining the reference limits[143]. However, determination of reference intervals is sometimes challenging and expensive. Therefore, laboratories also adopt carefully verified reference intervals from other laboratories. Analytical performance

of an assay and an analytical system employed may affect to reference values. This must be taken into consideration in the transference of reference values[143].

Ability of the assay to discriminate between healthy individuals and patients – ROC analysis

The performance of a clinical assay is described by sensitivity, specificity, efficiency, usefulness and value of the test. Many terms can describe the clinical performance but the main idea is diagnostic accuracy of the assay. The most important point is how well a test performs clinically and discriminates between false negatives and positives from true negatives and positives. Receiver operating characteristic (ROC) is an adequate way to describe the diagnostic accuracy of a clinical assay[144]. ROC methodology is based on statistical decision theory and it is a practical tool to define the ability of an assay to discriminate between healthy and diseased individuals[145]. The area under the curve (AUC) is a commonly used summary of the ROC curve. The basic principle of ROC is that the closer the AUC is to value of 1, the better the assay discriminates between healthy individuals and diseased ones (Fig. 7). The ROC analysis also summarizes the sensitivity and specificity of an assay. Sensitivity determines the part of actual positives (i.e. a patient has a disease) which are identified correctly, and is also called the true positive rate. Specificity (sometimes called the true negative rate) determinates the part of negatives (i.e. a patient does not have a disease) which are identified correctly. For example, when comparing three different immunological assays of carbohydrate antigen 19-9 (CA19-9) in gastrointestinal cancer patients, the Architect CA 19-9XR assay provided the best discrimination by ROC between benign and malignant disease[146].



AUC	
Architect	0.90
Elecsys	0.78
Immuno1	0.76

Figure 7. ROC analysis and AUCs of CA 19-9 immunological assays for differentiation between benign GI disease and pancreatic cancer (modified from Hotakainen et al.[146]).

1.7 Neuroendocrine tumors

Neuroendocrine tumors (NETs) are heterogeneous due to their diverse anatomical and cellular origins[147]. Classification of NETs was made by the World Health Organisation (WHO) in 2000[148,149]. NETs originate mainly from entrochromaffin and Kulchitsky cells and are slow-growing tumors with hypersecretory symptoms[150]. A small but significant proportion of NETs are malignant and difficult to manage. NETs can secrete various bioactive substances[151]. A tumor which secretes specific hormones and forms liver metastases, leads usually to a carsinoid syndrome. The diagnosis of NETs is based on symptoms, biomarker assays, radiological and nuclear imaging and pathology[152].

1.7.1 Classification of the tumor

Classification of NETs is complex according to International Classification of Disease for Oncology (ICD-O-3)[153] and they are divided to three different grades (G1-G3)[154]. NETs are categorized according to their origin from different embryonic division of the gut into tumors of foregut (lungs, bronchi, stomach, pancreas, duodenum, thymys), midgut (small intestine, appendix and proximal large bowel) and hindgut (distal colon and rectum)[155]. Tumors of adrenal glands are called pheochromocytomas[156]. Paragangliomas are catecholamine secreting tumors outside of the adrenal gland[157]. Catecholamine secreting neuroblastomas are the most common malignant extracranial tumors of childhood[158]. NETs may originate from almost any organ but around 95% of them are derived from the appendix, rectum and small intestine[159-161]. The biological and clinical characteristics of NETs may vary considerably. Therefore, a classification system takes into account also tumor differentiation and hormone production[155,162]. Some NETs are named by the secreted hormone; e.g. insulin – insulinoma.

1.7.2 Symptoms and prevalence

NETs are often indolent asymptomatic tumors and definitive diagnosis can be difficult to make. The symptoms are caused by overproduction of hormones and other biologically active substances. Episodic flushing, diarrhea, wheezing, sweating, eventual right-sided valvular heart disease are general symptoms of NETs[163,164]. The incidence of NETs is approximately 3.7/100 000 cases[165]. The number has increased during the past decades[166,167]. The 5-year survival rate for all NETs is 70-80%[167,168]. The stage of the NET affects the prognosis and the poorest survival rate is in patients with distant metastatic NET[166]. In 75% of the small intestine NET patients the disease will recur in 15 years[169].

1.7.3 NET markers

NET markers are hormones and amines that are secreted by NETs derived from the enterochromaffin cells. There is no ideal marker for NETs because these tumors may secrete varying amounts of serotonin, tachykinins, prostaglandins, catecholamines and histamine[170,171]. Urinary serotonin metabolite 5-HIAA is universally the most often used marker for NETs. It is a good indicator especially for midgut tumors, which are the most common type of NETs[172,173]. Serotonin is synthesized from an amino acid called L-tryptophan. However, the major part of dietary tryptophan is exploited for protein synthesis and only 1–3% is metabolized to serotonin[174]. Approximately half of plasma serotonin is taken up by platelets by a transport mechanism[175]. Ninety-nine percent of serotonin is metabolized to 5-HIAA by monoamine oxidase (MAO)[176] (Fig. 8B). Other tumor markers like chromoganin A (CgA) are used side by side with the 5-HIAA assay. Welin et al.[177] showed that CgA is an important marker with radically operated midgut NETs. However, CgA is ineffective in first-line diagnostics of NETs[178]. Furthermore, tachykinins neurokinin A and substance P are used as biomarkers for midgut carcinoid tumors[179]. Pancreatic polypeptide (PP) levels are increased in 80% of the patients with pancreatic tumors and in 50% of the patients with neuroendocrine tumors[180,181].

VMA and metanephrines are used as markers for catecholamine-secreting tumors e.g. neuroblastoma and pheochromocytoma. Three catecholamines; norepinephrine, epinephrine and dopamine are known to occur *in vivo* and NETs may secrete all or only one of them[182,183]. Dopamine is first metabolized to norepinephrine and VMA is the end-product of catecholamine metabolism. In Figure 8A the biosynthesis route of VMA is described. The final enzymatic steps take place in the liver by MAO and catechol-O-methyltransferase (COMT).

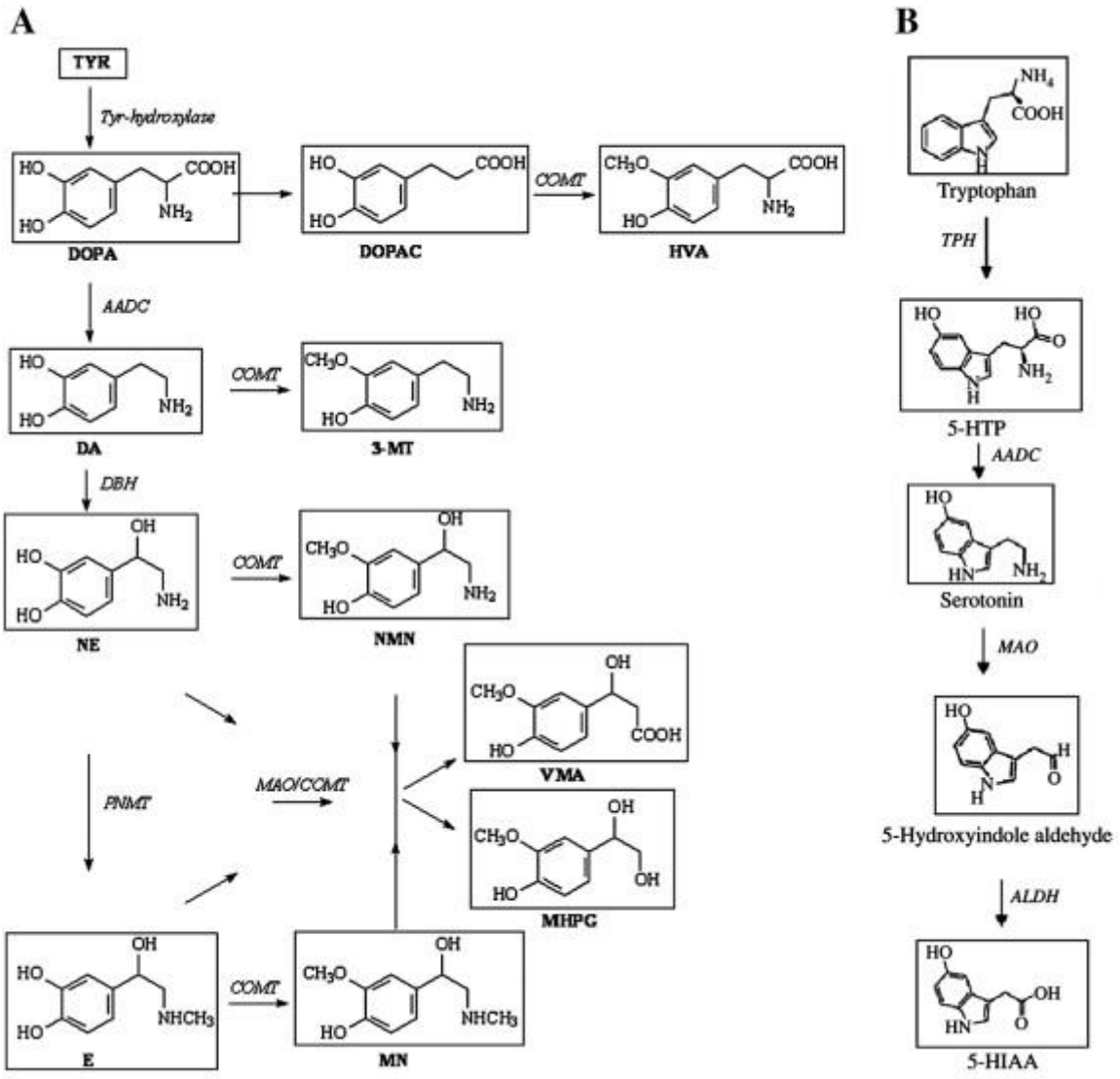


Figure 8. Metabolism of catecholamines (A) and serotonin (B) (modified from de Jong et al.[184]). Abbreviations: TYR: tyrosine, DOPA: 3,4-dihydroxyphenylalanine, DOPAC: 3,4-dihydroxyphenyl-acetic acid, HVA: homovanillic acid, DA: dopamine, 3-MT: 3-methoxytyramine, NE: norepinephrine, NMN: normetanephrine, VMA: vanillylmandelic acid, MHPG: 3-Methoxy-4-hydroxyphenylglycol, E: epinephrine, MN: metanephrine, COMT: catechol-O-methyltransferase, AADC: aromatic acid decarboxylase, PNMT: phenylethanolamine N-methyltransferase, MAO: monamine oxidase, TPH: tryptophan hydroxylase; 5-HTP: 5-hydroxytryptophan; ALDH: aldehyde dehydrogenase; ALDR: aldehyde reductase; 5-HIAA: 5-hydroxyindole acetic acid.

Different NET markers, tumor sites and analysis methods are presented in Table 3.

Table 3. Common NET markers, sites and general assays (modified from Lloyd[185]).

Tumor site	Tumor type	Marker	Specificity	General assay
Ileum	Midgut tumor	5-HIAA, Serotonin	High	HPLC
Colon and Rectum	Hindgut tumor	Peptide YY, Somatostatin	Intermediate	Immunometric
Thymys	Foregut tumor	Adrenocorticotropic hormone (ACTH)	Intermediate	Immunometric
Bronchus	Foregut tumor	ACTH, 5-HIAA, Serotonin	Intermediate	Immunometric, HPLC
Stomach	Foregut tumor, Gastrinoma, Ghrelinoma	Histamine, Gastrin, Ghrelin	Intermediate Low	Immunometric
Pancreas	Gastrinoma, Insulinoma	Gastrin, Insulin	High	Immunometric
Duodenum	Gastrinoma, Somatostatinoma	Somatostatin, Gastrin	High	Immunometric
Adrenal gland	Pheocromocytoma, Paraganlioma, Neuroblastoma	VMA, Metanephrines	High	HPLC, LC-MS/MS

1.7.4 Treatment and follow-up

The objective of NET treatment is removal or reducing of tumor mass by surgery, alleviation of symptoms and extension of the patient's lifespan[186]. The main issues in the follow-up are the symptoms experienced by the patient, the analysis of the tumor markers and imaging studies[187]. The follow-up is generally lifelong and for an asymptomatic patient a follow-up interval of 6-12 months is adequate[188]. The 24-h urine collections for NET marker analysis are troublesome to perform. All 24-h urine should be collected and the sample should be kept in the refrigerator during the collection period[189]. Serotonin and catecholamine-containing foods may increase the urinary excretion of 5-HIAA and VMA, respectively, and are advised to be avoided for 3 days prior to urine collection[172,190]. Furthermore, coffee and tea stimulate catecholamine and thus VMA secretion and some medications decrease it[189,191,192]. For the patient, the relatively frequent laboratory tests are a burden and for the laboratory, the conventional HPLC assays are laborious to perform and prone to interferes[184]. Sample preparation in conventional HPLC assay is a multistep and time-consuming procedure. Furthermore, some medications can cause chromatographic interference and may affect quantification results. Therefore, alternatives

for urinary HPLC assays have been developed[193,194]. Improved assays are still needed, and that was the main goal of our study. In the diagnosis of neuroblastoma, point measurement of urinary VMA has been shown to be as good as that from 24-h urine collection[195]. Therefore, point measurement of serum VMA and 5-HIAA is a notable alternative for the diagnosis of NETs.

2 Aims of the study

The aim of this study was to develop sensitive and specific assays, exploiting LC and MS techniques, for the quantification of relevant metabolites from biological samples and accomplish marked benefit with these new methods in biomedical research and clinical diagnostics. The main goal was to design new methods which would be useful in research and clinical practice.

The more detailed aims of the research papers (I–IV) were:

- To develop on-line LC and off-line LC-MS/MS methods for the analysis of extra- and intracellular metabolites directly from cell cultivations to be used in biotechnology (I).

- To develop and validate quantitative LC-MS/MS methods for the analysis of NET biomarkers from human serum to be used in clinical practice (II, III, IV).

- To study the effect of serotonin containing foodstuffs to serum NET biomarker 5-HIAA concentrations and to review the diet restriction protocol before the 5-HIAA LC-MS/MS assay (III).

3 Materials and methods

The materials and methods are described briefly in the next chapters. More detailed information can be found in the original publications or the supplementary material.

3.1 Reagents

α-Ketoglutarate (AKG), malate (MAL), citrate/isocitrate (CIT/ICIT) and glucose-1-phosphate (G1P), glucose 6-phosphate (G6P), fructose 1,6-bisphosphate (F16P), bisphosphoglycerate (BPG), phosphoenolpyruvate (PEP), 5-HIAA and VMA were purchased from Sigma Aldrich (St. Louis, MO). Deuterium labeled 5-HIAA-D_2 and VMA-D_2 were from Medical isotopes Inc. (Pelham, NH). The 50% sodium hydroxide (NaOH), sodium chloride (NaCl), methanol for quenching the metabolism, MS-grade methanol, MS-grade acetonitrile (ACN), formic acid and ammonium formate were from Fluka (Sigma-Aldrich Co.). All reagents were of the highest analytical grade.

3.2 Cell cultivations (I)

The yeast strain used was *Saccharomyces cerevisiae* Yeast Strain CEN.PK113-7D from Euroscarf (Frankfurt, Germany). Yeast cultivation was performed by a Braun Biostat CT5-DCU 3 bioreactor (B. Braun Biotech International GmbH, Meisungen, Germany). The bioreactor was equipped with automated sampling and measurement of the optical density (OD) of the cells. The parameters of cultivation were adjusted to a temperature of +30 ^{o}C, pH 5, aeration 2.5 L/min and agitation 1000 revolutions per minute (rpm).
In on-line HPLC analysis, a software-controlled sequence automatically pumped the sample from the sample collector through the filter and injected 10 µL of the filtrate into the separation column. On-line HPLC samples were taken at 5-min intervals. Quantification software automatically detected peaks of glucose, glycerol, acetate, and ethanol from the chromatogram. Samples for off-line HPLC and manual OD measurements were obtained from the manual valve at the bottom of the bioreactor at 1-h intervals.

Samples for intracellular metabolite analysis were collected automatically at 1-hour intervals into plastic tubes containing 70% methanol placed in a sampling carousel submerged into ethanol-filled Lauda RE120 cold bath (Lauda, Lauda-Königshofen, Germany) at −35 °C. Rapid sampling to cold methanol was used to guarantee the quenching of all metabolic reactions. The methanol-containing samples were centrifuged

at −10 °C and the cell pellets were stored at −80 °C until sample preparation. Schema of the on-line system is presented in Figure 9.

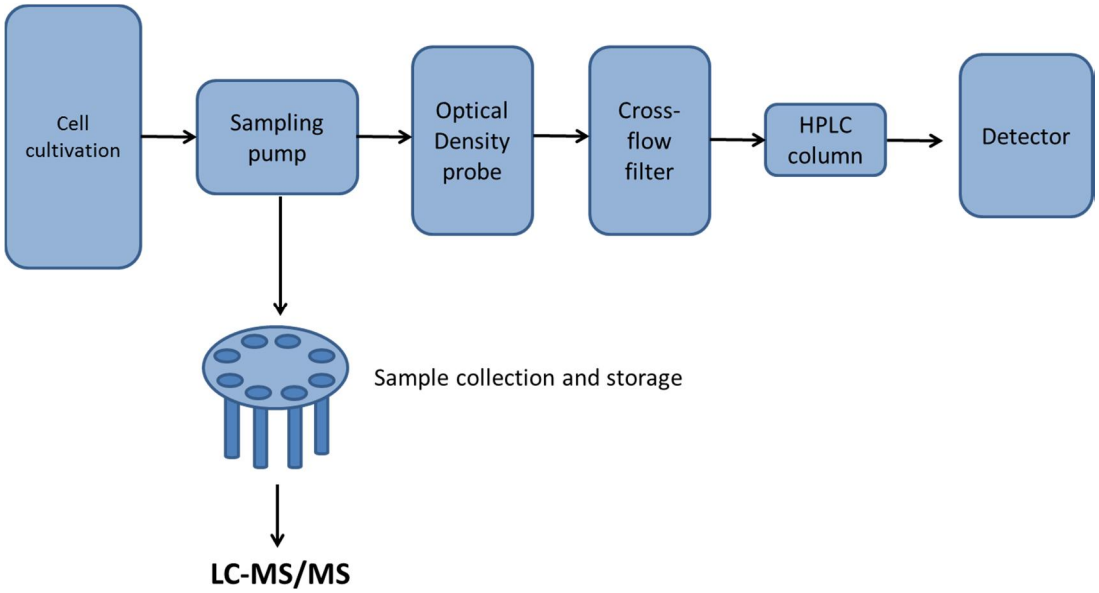


Figure 9. Schema of the on-line system.

3.3 Patient samples (II, III, IV)

Serum samples were obtained from healthy volunteers participating in the Nordic Reference Interval Project (NORIP) [196] and from our laboratory staff. For method validation and comparison, we also used urine, serum and lithium-heparin plasma samples from healthy volunteers, and from patients who were in suspicion of or followed for NET (Study II, III and IV). The patient samples were collected during June 2010 and August 2013. Informed consent was obtained from all healthy individuals. Patient samples were analyzed as part of their normal diagnostic process or follow-up of NET at Helsinki University Central Hospital. This study was approved by the Ethical Committee of Helsinki University Central Hospital, Helsinki, Finland (permission number 211/13/03/00/14).

3.4 Sample preparation

Manually collected samples for off-line HPLC analysis (study I) were centrifuged, and the supernatants were frozen and stored in HPLC vials at −20°C until analysis.

The intracellular metabolite samples (study I) were automatically collected into cold methanol, manually extracted with boiling ethanol, centrifuged and the remaining

supernatants, containing the metabolite fraction, were dried (SPD Speed Vac, Thermo Savant, Waltham, MA), dissolved into IS (500 µmol/L piperine acid) and analysed by LC-MS/MS.

Serum samples and calibrators (study II, III and IV) were pipetted into the wells of a 96-well microtiter plate along with IS working solution. A µElution SPE plate was conditioned with methanol and water. Samples and standards with IS were transferred into the µElution wells followed by washing. Finally, the analytes were eluted into 96-well plates and analyzed by LC-MS/MS.

3.5 Preanalytical validation

To study the stability of serum 5-HIAA and VMA (study II and IV), freshly drawn serum samples from healthy individuals were divided into aliquots and stored at room temperature, +4 °C and −20 °C for various time periods. Samples were allowed to reach room temperature before LC-MS/MS analysis. Blood samples from 18 healthy volunteers were collected into plain serum tubes, serum catalyzator tubes (CAT), serum gel tubes (SST™ II Advance, all from BD Vacutainer, Plymouth, UK) and lithium-heparin tubes (Venosafe 60 USP U Lithium Heparin, Terumo, Leuven, Belgium) to compare the effect of the sampling device (study II and IV). The diurnal variation of serum 5-HIAA and VMA concentrations (study II and IV) was studied in 7 volunteers. The samples were collected at 8 a.m., 12 a.m. and 4 p.m. and frozen immediately until LC-MS/MS analysis. When studying the of effect of breakfast (study II and IV), blood samples were collected before 10 a.m. during one week before and after a regular Finnish breakfast that consists of some of these: coffee, tea, milk, juice, bread, cheese, ham, porridge, cereals or yogurt.

The effect of serotonin-containing foodstuffs (study III) was studied in 35 healthy volunteers (31 women and 4 men). After avoiding serotonin-containing foods for three days, a blood sample was drawn between 8–9 a.m. into a plain serum tube. The subjects then ate either banana, pineapple, tomatoes, walnuts or kiwi-fruit during the next 30 min or freely during the first day. Additional samples were drawn at 10 a.m., 12 a.m., 14 p.m. and following three mornings at 8 a.m. The samples were centrifuged and kept at –20 ^{o}C until LC-MS/MS analysis.

Reference intervals for serum 5-HIAA and VMA (study II and IV) were established using 111 serum samples from healthy volunteers. Reference ranges were calculated according to the guidelines of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC). To study the stability of 5-HIAA and VMA in the NORIP samples, that

had been kept frozen at –70 °C for 10–12 years, we compared the 5-HIAA and VMA concentrations in the NORIP sample and in freshly drawn samples from the laboratory staff in the different age groups.

3.6 Analytical methods

Automated on-line HPLC system (study I) consisted of a sample collector, a cross-flow filter, an injection valve, a separation column (Aminex Fast Acid 100 × 7.8 mm, Bio-Rad, Hercules, CA), a peristaltic pump, an HPLC pump and a refractive index (RI) detector (Knauer WellChrom K-2301, Berlin, Germany).

Off-line HPLC analysis (study I) was performed with a Waters HPLC system (Waters, Milford, MA) containing a 717 autosampler, a 600S controller, a 626 pump, a degasser and a 2414 RI detector. Chromatographic separation was carried out using a Rezex RHM-monosaccharide analytical column (150 × 7.80 mm) (Phenomenex, Torrance, CA).

Intracellular metabolite analysis (study I) was performed with the Alliance HPLC system (Waters) connected to triple quadrupole Quattro Micro mass spectrometer (Mircomass, Manchester, UK). We used a Dionex IonPac AS11 (2 x 250 mm) anion exchange column connected to a Dionex IonPac AG11 (2 x 50 mm) guard column. The mass spectrometer was equipped with an electrospray ionization (ESI) interface.

LC-MS/MS analyses (study II, III and IV) were performed with an Agilent 1200 liquid chromatograph (Agilent Technologies, Santa Clara, CA) and a 4000 QTRAP mass spectrometer (AB Sciex, Toronto, Canada) equipped with a Turbo-V electrospray ion source. The analytical column was an Atlantis HILIC 50 x 2.10 mm 2.6 µm from Waters.

Gel filtration chromatography (study III and IV) was carried out with the ÄKTApurifier system (GE Healthcare Bio-Sciences AB, Uppsala, Sweden, www.gelifesciences.com) using a Superdex™ 200 10/300 GL column (GE Healthcare Biosciences). The column was equilibrated with phosphate buffered saline (PBS) and absorbance at 280 nm was monitored.

Urinary 5-HIAA and VMA HPLC analyses (study II and IV) were performed with the Agilent 1200 system connected to Antec Leyden Intro electrochemical detector (Boston, MA). Chromatographic separation was carried out using a ZORBAX Eclipse XDB-C18 5 µm (150 × 4.60 mm) (Agilent Technologies).

Plasma CgA samples (study II) were analysed by a commercial radioimmunoassay (EURIA-Chromogranin A, Euro Diagnostiga, Malmö, Sweden). The samples were collected to lithium-heparin tubes, separated by centrifugation at +4^{o}C and stored at -20^{o}C. The samples were diluted 1:10 with the assay diluent. The calibrators, controls and samples were pipetted along with assay reagents and incubated. The radioactivity of the pellets was counted in a gamma counter.

Serum normetanephrine (Nor), metanephrine (Met) and 3-methoxytyramine (3MT) (study IV) were analyzed by a LC-MS/MS consisting of an Atlantis HILIC Silica 50x2.10 mm column (3 µm, Waters). The mobile phases were ACN and 100 mmol/L ammonium formate, pH 3. The samples were extracted using Oasis® WCX µElution plate (Waters, Milford, MA, USA). To each eluate, 100 µL of 95% ACN – 5% 100 mmol/L NH4-formiate, pH 3.0 was added.

3.7 MS data analysis

The MS data was acquired and processed by the QuanLynx software (Waters) in the study I and by the Analyst software (Ver. 1.5, AB Sciex) in studies II, III and IV.

3.8 Statistical methods

All statistical tests were performed by Analyse-it software for Microsoft Excel 2010 (Ver. 2, Analyse-it software Ltd., Leeds, UK, http://www.analyse-it.com).

4 Results

The main results are described below. More specific details can be found in the original publications or the supplementary material.

4.1 Study I. On-line high performance liquid chromatography measurements of extracellular metabolites in an aerobic batch yeast (*Saccharomyces cerevisiae*) culture

The on-line HPLC assay of cell culture medium was linear up to 50 g/L for all metabolites and the LOQ was 0.08 g/L for glucose, 0.1 g/L for glycerol, 0.2 g/L for acetate and 0.25 g/L for ethanol. The intra and inter assay precision were 5.5% and 2.8% (averages for all metabolites), respectively. The accuracy was 7% for glycerol and 9.5% for glucose.

The on-line HPLC measurements of extracellular metabolites in three different cell cultivations were in line. In all cell cultivations, glucose was consumed within the first 7.5 hours. The non-fermentable carbon sources were consumed within 11 hours (acetate), 14.5 hours (glycerol) and 16 hours (ethanol) (Fig. 10).

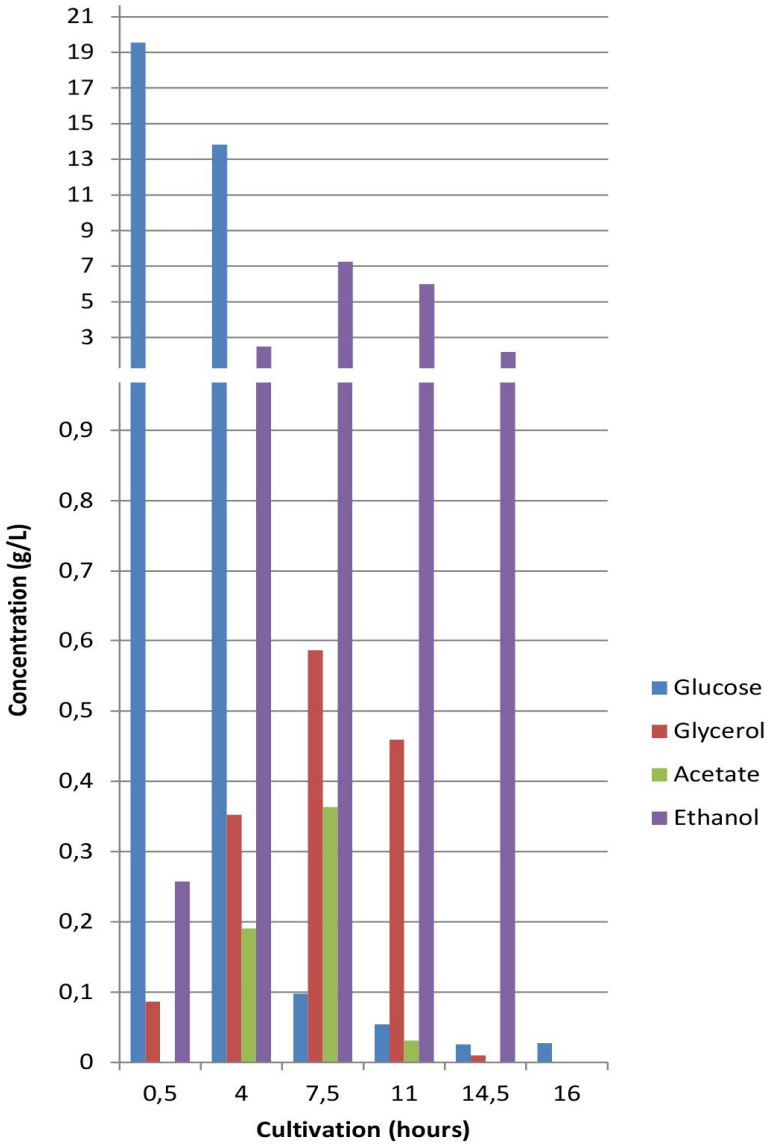


Figure 10. Average concentrations (n=3) of extracellular metabolites during cell cultivations.

The on-line HPLC assay was compared to the off-line HPLC assay with manual sample preparation (n=35). On-line and off-line results of extracellular metabolites correlated according to formula y=0.97x + 0.04 for glucose (r^2 =0.99), y=0.85x + 0.15 for ethanol (r^2=0.97) and y=0.92x + 0.04 for glycerol (r^2=0.96) and y=0.98x + 0.02 for acetate (r^2=0.94). The on-line and off-line OD measurements were comparable.

Quantification of intracellular metabolites revealed that the concentrations of G6P and F16P were at the highest level during the first eight hours of cultivation (Fig. 11). The CIT/ICIT ratio was the highest when the cells were consuming mainly acetate. After acetate consumption ceased, the CIT/ICIT ratio also decreased. Intracellular

concentrations of PEP peaked after ethanol and hence all the major carbon sources were consumed.

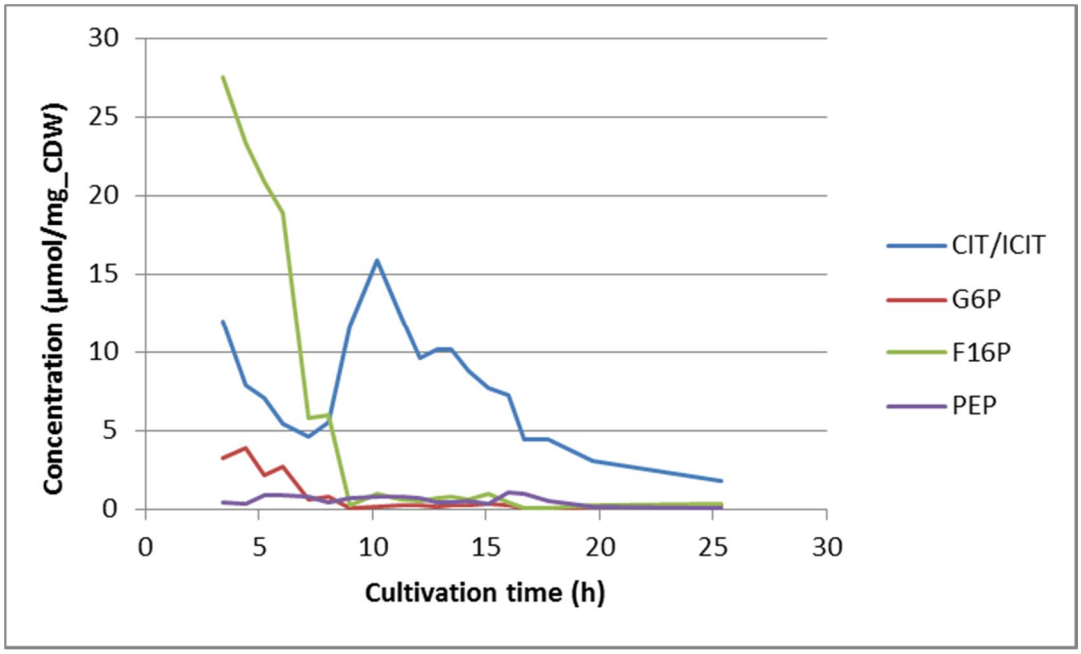


Figure 11. Averages of intracellular metabolite concentrations of G6P, F16P, PEP and CIT/ICIT during three different cell cultivations.

4.2 **Study II.** Analytical and preanalytical validation of a new mass spectrometric serum 5-hydroxyindoleacetic acid assay as neuroendocrine tumor marker

The developed LC-MS/MS assay for serum 5-HIAA is sensitive (LOQ 5 nmol/L) and has a wide linear range (5–10000 nmol/L). The inter-assay and intra-assay variation were 5.3−8.0% and 2.7−7.1%, respectively. The recovery of added 5-HIAA was 98−101% in three serum samples. Slight ion suppression (13%) of 5-HIAA was detected. 5-HIAA in serum was stable for several days at various temperatures and during five freeze-thaw cycles. There was a significant difference between serum samples drawn into gel tubes and plain tubes (Fig. 12). No differences were observed between the other sampling devices.

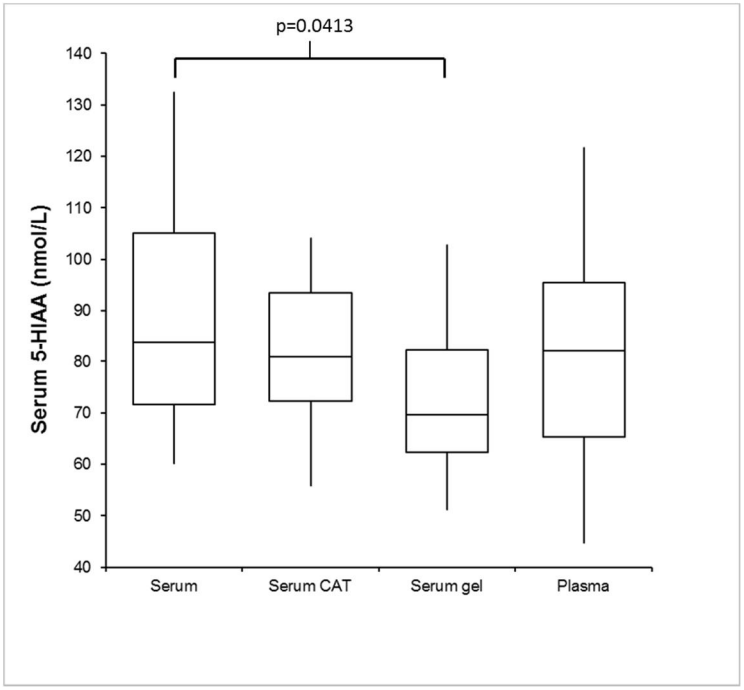


Figure 12. Serum 5-HIAA concentrations when using different sampling devices.

We found no diurnal variation (p≥0.20) and a typical Finnish breakfast meal had no effect on serum 5-HIAA (p=0.89). A reference range of 35−123 nmol/L was established for combined age groups and genders because there was no significant difference between them (p≥0.27) (Fig. 13). The upper reference limit (123 nmol/L) was suggested as clinical cut-off value into NET diagnostics.

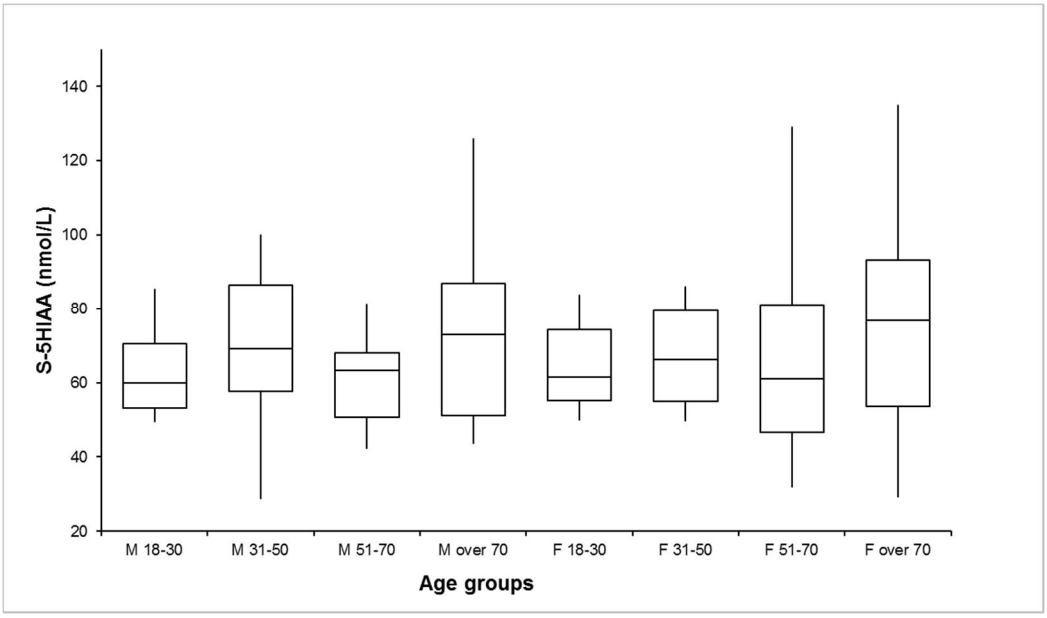


Figure 13. Serum 5-HIAA concentrations in men (M) and women (F) in different age groups (p≥0.27).

Our LC-MS/MS assay for serum 5-HIAA was compared to urinary 5-HIAA HPLC and plasma chromogranin A (CgA) assays using samples from healthy individuals (n=8) and NET patients (n=129). The LC-MS/MS assay correlated well with both urine 5-HIAA HPLC (Fig. 14) and plasma CgA assays. The correlation was determined by Deming regression and the serum LC-MS/MS assay correlated with the urinary HPLC and plasma CgA assays according to formulas y (LC-MS/MS) = 5.81 × (HPLC) − 122.02 (Sy|x = 165.34, n = 137) and y (LC-MS/MS) = 25.92 × (CgA) − 129.63 (Sy|x = 475.69, n = 120), respectively.

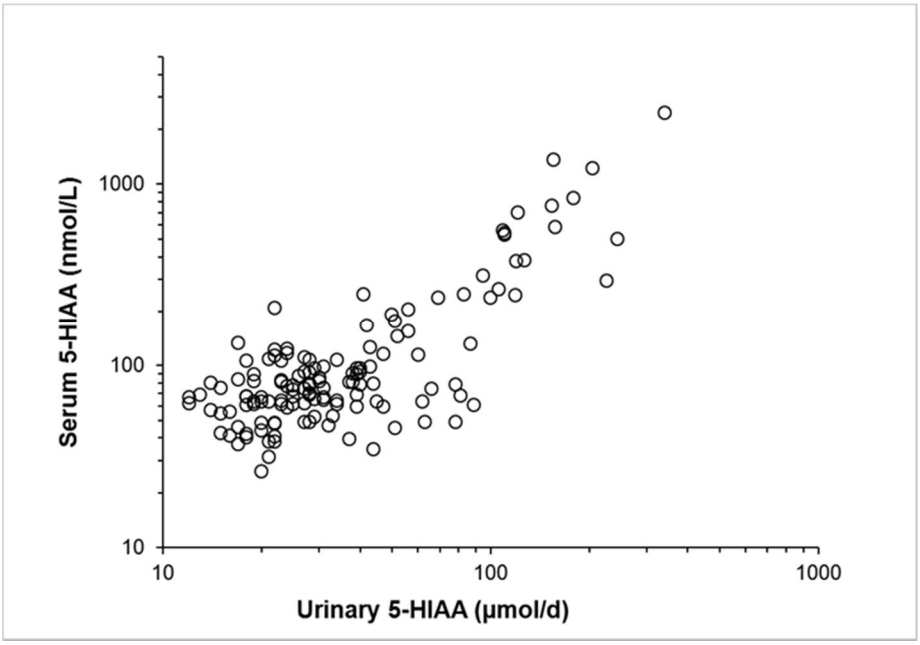


Figure 14. Correlation between concentrations of 5-HIAA by serum LC-MS/MS and urinary HPLC assays.

In ROC analysis of 46 NET patients and 29 healthy individuals the AUC was 0.83 for urinary 5-HIAA, 0.81 for serum 5-HIAA and 0.76 for plasma CgA assay (Fig. 15). There was no significant difference between the assays (p≥0.17). The sensitivity and specificity was 57% and 95%, respectively, for serum 5-HIAA LC-MS/MS assay.

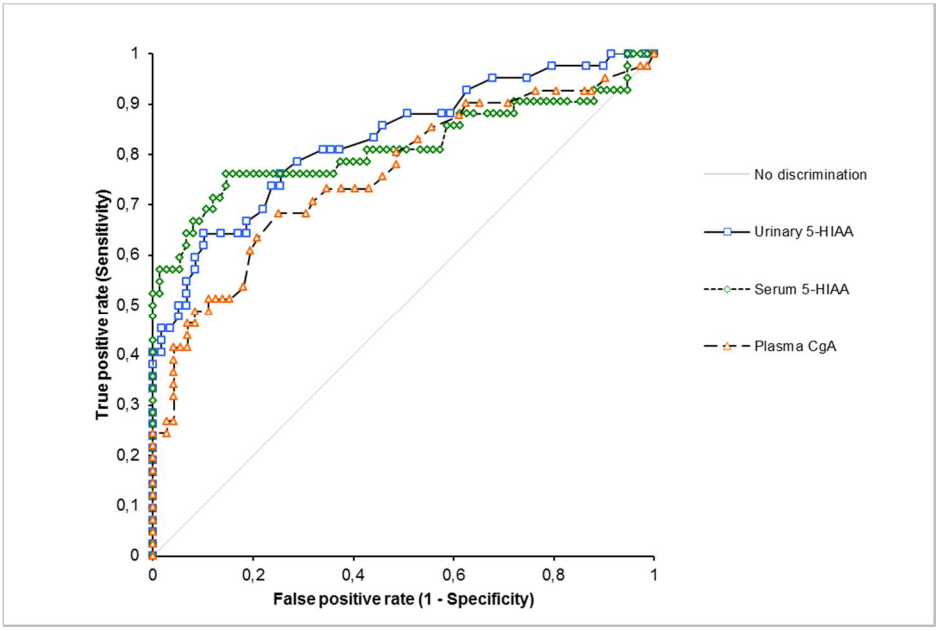


Figure 15. ROC-analysis of serum 5-HIAA LC-MS/MS, urinary 5-HIAA HPLC and plasma CgA immunological assays.

4.3 **Study III.** Transient elevation of serum 5-HIAA by dietary serotonin and distribution of 5-HIAA to plasma protein fractions

Dietary serotonin (1.2–28.4 mg) had a significant but transient effect to serum 5-HIAA concentration ($p \leq 0.001$). Serum 5-HIAA concentration increased within 2 hours after ingestion of serotonin containing food and was the highest (average 1797 nmol/L, n=3) in samples from individuals who had eaten walnuts (Fig. 16). A decrease in serum 5-HIAA was seen within 4 hours after ingestion and concentration reached the basal level after 24 hours in all individuals. The calculated half-life of 5-HIAA in circulation was 1.3 hours.

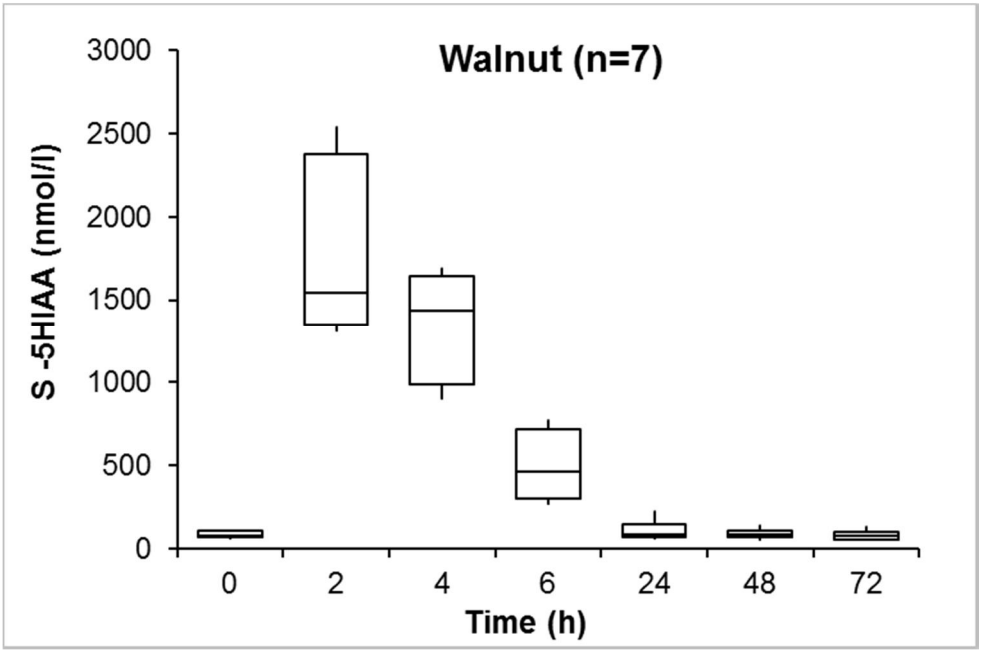


Figure 16. Serum 5-HIAA concentrations in test individuals (n=7) who has eaten walnuts.

Analysis of 5-HIAA in gel filtration fractionated serum samples revealed that the peaks eluting at 1.1 min, 1.5 min, 2.3 min and 3.1 min are derived from the background, from free 5-HIAA, and from α2-globulin and albumin fractions, respectively (Fig. 17). Only the peak from background eluting at 1.1 min was detected in every gel filtration fraction.

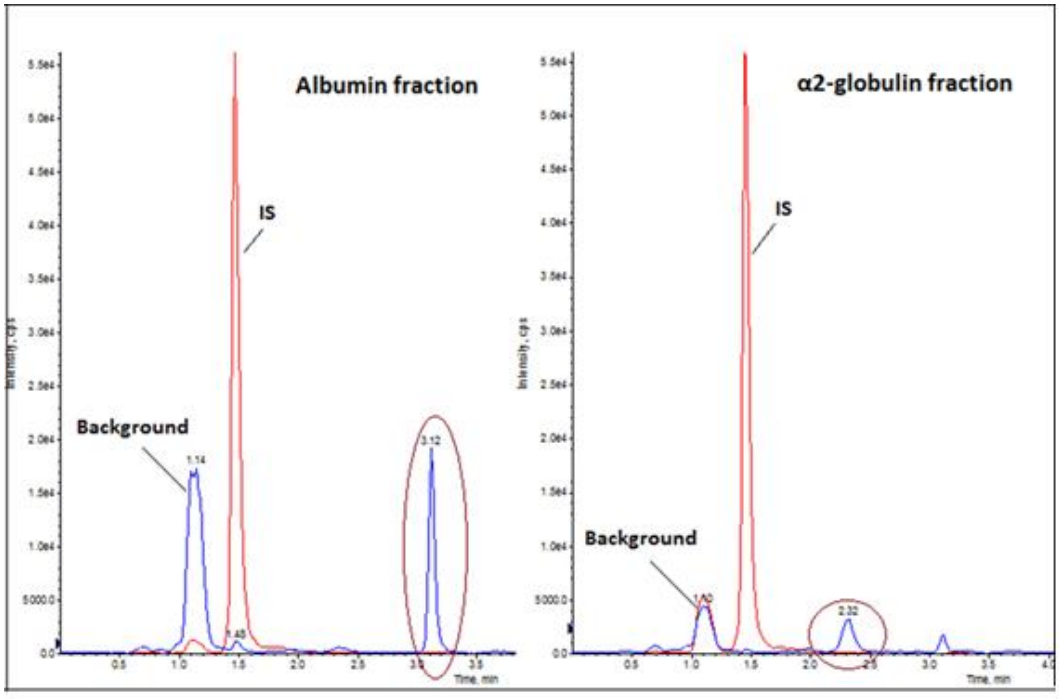


Figure 17. Chromatograms of serum α2-globulin and albumin fractions by the 5-HIAA assay.

Our study revealed that in serum from a NET patient with elevated serum 5-HIAA and from a diet test individual with a transient increase of 5-HIAA, the majority of 5-HIAA (84% and 65%, respectively) was found in the free 5-HIAA fraction and 11% and 29%, respectively, presumably in the albumin fraction. In serum from a healthy individual only 5% of 5-HIAA was free and 83% was presumably in the albumin fraction. Furthermore, in all samples ≤11% of 5-HIAA was found probably in the α2-globulin fraction.

4.4 **Study IV.** Preanalytical validation and reference values of mass spectrometric assay of serum vanillylmandelic acid for diagnosis of catecholamine secreting neuroendocrine tumors

Our LC-MS/MS assay for serum VMA was linear over the concentration range of 1.25–10000 nmol/L. The LOQ was 1.25 nmol/L and the intra- and inter-assay variations were 2.7–6.0% and 5.4–6.4% at 60 nmol/L and 610 nmol/L, respectively. The recovery of added VMA was 97–99%. Minor matrix effect (average 3%) was detected. Serum VMA was stable for two days at +4 ^{o}C and at least for seven days at room temperature, during two freeze-thaw cycles and for at least 98 days at -20 ^{o}C (Fig. 18). There were no significant differences (p≥0.45) between VMA concentrations in samples drawn into plain tubes, gel tubes, Li-heparin tubes and in clotting activator tubes.

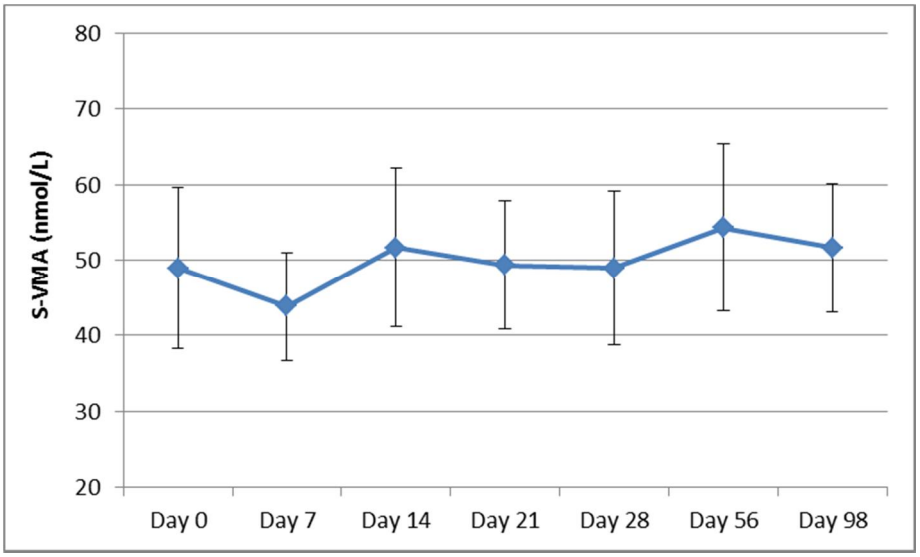


Figure 18. Average serum VMA concentrations in samples (n=9) kept at -20^{o}C.

No diurnal variation of serum VMA concentrations was observed. However, there was a significant difference in serum VMA concentrations between samples drawn after breakfast and 12-h fasting (p=0.0031). We found no effect of catecholamine rich foodstuffs to serum VMA concentrations (p≥0.18). There were significant differences in serum VMA concentrations between the various age groups (p≤0.0001), but not between

the genders within the same age group (p≥0.1445). We suggest cut-off values of 62 nmol/L, 80 nmol/L and 108 nmol/L for combined genders in age groups 18−50 yrs (n=53), 51−70 yrs (n=33) and >70 yrs (n=24), respectively. In gel filtration fractions of serum from a healthy individual, VMA was only found in the fraction corresponding to free VMA by our LC-MS/MS assay, i.e. our assay determines serum free VMA.

Our serum VMA LC-MS/MS assay and urinary VMA HPLC assay correlated according to the formula y(LC-MS/MS) = 3,906x(HPLC) + 30,07 (n=17) by Deming regression (Fig. 19). We analyzed samples (<16 yrs) from active neuroblastoma patients (n=4) compared to healthy individuals (n=9) and patients in remission from neuroblastoma (n=4). Serum VMA was elevated in three out of four samples from active neuroblastoma patients. However, urinary VMA and serum normetanephrine and 3MT were elevated only in two out of four samples from active neuroblastoma patients. In analysis of samples of study subjects over 16 years of age (one paraganglioma patient, one adenoma patient and 25 healthy individuals), serum VMA was slightly elevated (147 nmol/L, mean of healthy individuals 59 nmol/L) in a sample from the paraganlioma patient. In these samples the concentrations of urinary (23.3 µmol, mean of healthy individuals 1.3 µmol) and serum (9.7 µmol, mean of healthy individuals 0.9 µmol) normetanephrine were also elevated. Only serum VMA was slightly elevated in a sample from the adenoma patient.

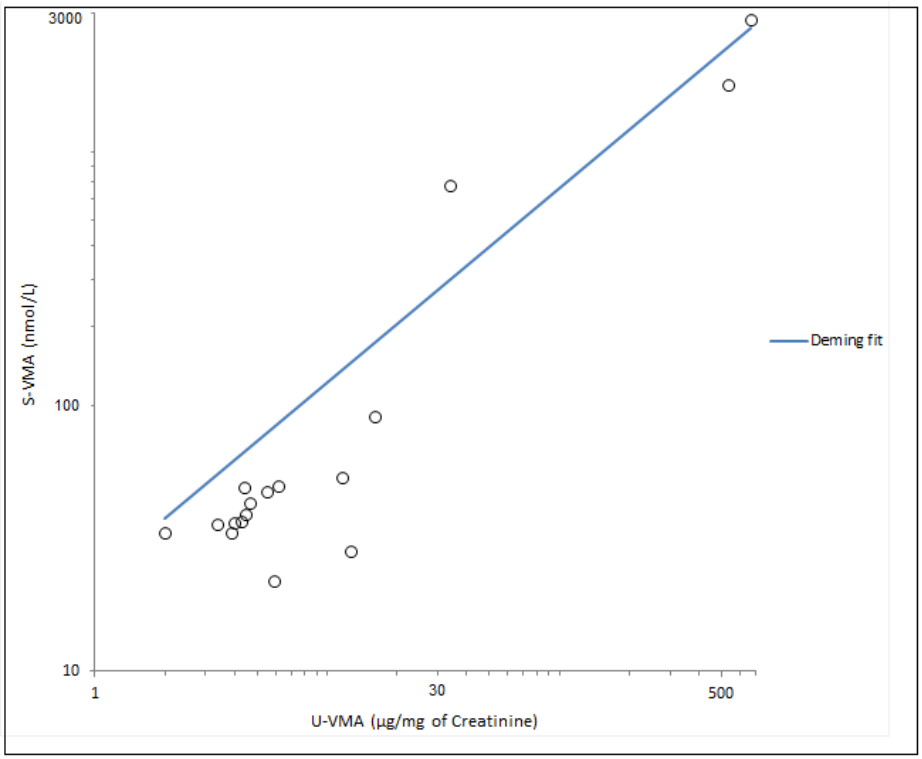


Figure 19. Correlation between concentrations of VMA by serum LC-MS/MS and urinary HPLC assays (n=17).

5 Discussion

5.1 Method development

In this study, a need for improved methods was the main objective in all original research papers. HPLC combined to mass spectrometric detection is a very powerful technique for developing highly sensitive assays of metabolites. Urinary NET markers 5-HIAA, VMA and the metanephrines are generally analyzed by HPLC with electrochemical or fluorometric detection[197,198]. These assays may suffer from analytical interferences like drugs or other interfering molecules. Furthermore, the collection of 24-hour urine is prone to errors, and HPLC assays may be laborious to perform because of multistep sample preparation before the analysis[172]. Our mass spectrometric assays for measuring 5-HIAA and VMA from human serum improve analytical specificity and minimize the laboratory work.

An on-line HPLC was constructed and developed for monitoring of extracellular metabolites on time during cell cultivation with frequent automatized sampling of intracellular metabolites. This enables continuous quantitation data of cultivation and observation of physiological processes of cells. The on-line system also enables automated optical density and gas exhaust measurements and sample storage. Sample preparation methods were improved in all assays. In on-line HPLC, sample preparation is totally automatized by using specific filtration before chromatographic column. On-line measurement decreases the laboratory work significantly compared to off-line measurements[199]. In 5-HIAA and VMA assays, a 96-well plate SPE protocol is fast and efficient when compared to urinary HPLC sample preparation in separate tubes and cartidges[200]. Serum sample collection is well controlled and easier to perform than 24-h urine collection.

To verify the performance and functionality of the newly developed assays they were compared with the existing assays. Serum 5-HIAA LC-MS/MS assay correlated well with urinary HPLC and plasma CgA assays. According to preliminary results, serum VMA LC-MS/MS showed good correlation with urinary VMA HPLC and plasma metanephrine assays. However, the clinical performance of the VMA LC-MS/MS assay needs further clinical studies with larger patient sample material. On-line measurements were performed with three different cell cultivations to examine the reproducibility of the automated sampling and measurement system. On-line HPLC results of extracellular metabolites correlated well with those measured by off-line HPLC. The co-efficient of determination (r^2) was 0.96–0.99 for all four metabolites. Automated OD measurements showed also good correlation compared to manual OD measurements.

5.2 Analytical and preanalytical validation

All developed assays had a wide linear range. Our LC-MS/MS assays for 5-HIAA and VMA were linear at least from 5 to 10 000 nmol/L and our on-line HPLC assay for extracellular metabolites was linear up to 50 g/L. Sadilkova et al.[194] reported recently a linear range of 2−1000 ng/mL (10−5000 nmol/L) for VMA UPLC-MS/MS assay. LOQs and linear ranges of our 5-HIAA and VMA assays were lower or similar when compared to previous reports[193,194]. In our on-line HPLC assay, the LOQ of glucose was 0.08 g/L. Glucose is the limiting nutrient in the medium and its concentration can affect cell growth[201]. Sufficiently low LOQ enables the monitoring of the consumption of the final glucose concentrations in cell cultivation.

We observed a slight matrix effect (–13%) in our serum 5-HIAA LC-MS/MS assay. Miller et al.[193] reported also minor ion suppression in their study. However, the recovery of 5-HIAA in our assay was 98−101%. Thus, use of IS corrects the ion suppression completely. Minor ion suppression (3%) was observed with our VMA LC-MS/MS assay. Fang et al.[202] reported 60% ion suppression in plasma VMA in LC-MS/MS assay, but they were able to reduce the matrix effect by changing the mobile phase conditions.

5-HIAA serum sample concentrations were significantly lower in serum gel tubes compared to plain serum tubes, but no such differences were observed between VMA samples drawn into different kinds of tubes. In our experience, plasma samples may block the μelution SPE matrix. Therefore, we suggest plain or CAT serum tubes for sampling of 5-HIAA and VMA assays. We found that both 5-HIAA and VMA are stable at -20 ^{o}C for at least 98 days. Furthermore, 5-HIAA is unaffected by five and VMA by two freeze-thaw cycles, and both were stable for at least seven days at room temperature. Therefore, serum samples from outpatient clinics can be transported to the analytical laboratory at room temperature which is convenient and cost-effective when compared to cooled or frozen samples.

No diurnal variation was observed for serum VMA and 5-HIAA concentrations. We found that a typical Finnish breakfast had no effect on serum 5-HIAA concentrations and concluded that a meal without serotonin-containing foodstuffs before sampling does not affect the results. However, it has been indicated that serotonin-containing food increases serum and urinary 5-HIAA concentrations significantly, but the duration of the increase in serum had not been studied[203-205]. We wanted to study the effect and duration serotonin-containing food to serum 5-HIAA concentrations. Our findings showed that dietary

serotonin causes a significant but transient dose-dependent increase in serum 5-HIAA. Urinary 5-HIAA collection guidelines[172,190] advise to avoid serotonin-containing food for three days before sampling. We showed that a diet restriction for several days before blood collection is not necessary for the diagnosis of NET. On the other hand, we observed that breakfast increased, but cathecholamine-containing food as such had no effect on serum VMA concentrations. It is known that typical breakfast drinks, coffee and tea stimulate catecholamine secretion[191,192] and this is likely to explain our finding. Therefore, we suggest sampling for serum VMA after an overnight fast.

5.3 On-line analysis

On-line HPLC assays can be used in different applications in the research, industry and clinical laboratories. These systems have often been in-house-built equipment and used for the production of engineered proteins[206], monitoring of azo dye degradation processes[207] and production of monoclonal antibodies in cell culture[208].

Our software controlled on-line HPLC was able to collect samples from cell cultivation within 5 min intervals for quantification. Extracellular metabolites; ethanol, acetate, glucose and glycerol were directly analyzed from the supernatant and the quantification results were immediately available. This sampling equipment also collected samples automatically at 1-hour intervals for intracellular metabolite MS analysis. Combined on-line HPLC and intracellular metabolite data indicated two phases of diuaxic shift in cell cultivation. The cultivated yeast cells consumed primarily glucose for nutrition. By frequent sampling it was possible to show the turning point when the consumption of glucose was switched to consumption of acetate, glycerol and ethanol. The concentrations of intracellular metabolites were at the lowest level during the consumption of the last glucose residues. After acetate had been consumed, but the cells were still consuming glycerol and ethanol, the levels of CIT/ICIT and MAL decreased. Intracellular concentrations of PEP and G1P peaked when ethanol and all the major carbon sources were consumed. To our knowledge, this was the first study to show the two phases in a diauxic shift in yeast cell cultivation. Gene expression during the diauxic shift has been studied widely[209-211]. A 5-minute interval for sampling of four metabolites in our study is frequent. Usually, the sampling interval in multiple metabolite HPLC assays is approximately 20 to 35 minutes[207,212]. Due to the frequent sampling interval, our HPLC device is applicable for detailed physiological characterization of the cells.

Our on-line HPLC system has been commercialized by On-line HPLC Ltd. (Helsinki, Finland)[213]. The system has been used for monitoring of the production of glycolic acid in

Saccharomyces cerevisiae and *Kluyveromyces lactis* cell cultivations[214]. Glycolic acid is used widely in medical industry, for example as starting material in packing products or drug delivery[215,216]. The on-line HPLC is also well suited for monitoring of mammalian cell cultures (Chinese hamster ovarian cells, Tohmola et al. unpublished data).

The main benefit of the on-line HPLC is the possibility of simultaneous monitoring and collecting the quantification data. Processing of biofuel by micro-organisms is a growing field in the biotechnological research. Accurate and frequent monitoring of metabolite concentrations in biofuel process is important and made possible by on-line sampling[217]. Our newly developed on-line HPLC enables automated and scheduled sampling without sample preparation and monitoring of multiple bioreactors with one HPLC. As a result, the analysis becomes more cost-effective when the hours of manual laboratory work are decreased. On-line assays are likely to increase in the industry and research in the future.

5.4 NET marker analysis

We found that serum VMA concentrations in apparently healthy individuals increased with age, but there were no differences between the genders. However, no such differences were seen for serum 5-HIAA. Therefore, we suggest a cut-off value of 123 nmol/L for 5-HIAA, and 62 nmol/L, 80 nmol/L and 108 nmol/L for age groups 18−30 yrs, 51−70 yrs and >70 yrs, respectively, for VMA. These reference values are in line with previously reported studies. Tellez et al.[218] and Carling et al.[219] reported cut-off values of 115 nmol/L and 118 nmol/L for 5-HIAA, respectively. Neuroblastoma occurs usually in the childhood. Due to low incidence, our sample material was limited and the reference individuals were ≥18 yrs. However, Sadilkova et al.[194] calculated a plasma VMA cut-off value of 100 nmol/L for children <16 yrs and our cut-off value of 62 nmol/L for the age of group 18-30 yrs is in accordance with this. In future studies, true pediatric reference values for our VMA assay remain to be established. Urinary VMA concentrations have been reported to be similar in boys and girls[220] and to show age-dependent elevation[221]. Therefore, our results on serum VMA are similar to the findings on urinary VMA.

In the clinical diagnosis of NET, our serum 5-HIAA LC-MS/MS assay was comparable with urinary HPLC 5-HIAA and plasma CgA assays. The ROC analysis revealed that serum 5-HIAA (AUC = 0.81) and urinary 5-HIAA (AUC = 0.83) assays discriminated better between NET patients and healthy individuals than the CgA assay (AUC = 0.76), but the difference was not statistically significant ($p \geq 0.17$). The diagnostic sensitivity and specificity of our 5-HIAA assay were 57% and 95%, respectively, with a cut-off value of 123 nmol/L. The sensitivity is lower compared to urinary 5-HIAA assay (67%), but the difference is not significant. The serum assay is a point measurement compared to 24-h urine collection

and this might explain the difference in the results. According to our preliminary data, VMA LC-MS/MS assay showed good discrimination with active NET patients (3 neuroblastomas and one paraganglioma). In the case of our adenoma patient, only serum VMA concentration was increased compared to urinary and plasma metanephrines and urinary VMA. Blood sampling is well controlled and convenient for the patient when compared to 24-h urine collection.

In 5-HIAA LC-MS/MS assay, we observed additional peaks with the same transitions as 5-HIAA in the chromatograms. Therefore, we wanted to study the distribution of 5-HIAA and VMA in serum fractions and to find out whether our LC-MS/MS assay determines free or protein-bound 5-HIAA and VMA. 5-HIAA and VMA were found in gel filtration fractions corresponding to free 5-HIAA and VMA. In addition, a possible 5-HIAA peak was also found in the albumin and alpha-2-globulin fractions. This may explain the additional late-eluting peaks seen in the 5-HIAA chromatograms. Itkonen et al.[222] have reported a late-eluting hepcidin peak in the LC-MS/MS chromatogram of their hepcidin assay. After similar gel filtration studies, the late-eluting peak was found in the albumin fraction in which the presence of albumin was confirmed with matrix assisted laser desorption ionization (MALDI) mass spectrometry. The majority of assumed 5-HIAA was found in the albumin fraction and only 5% of 5-HIAA was free in the serum of a healthy individual. The distribution of 5-HIAA in plasma has not been studied earlier. Further studies are needed to confirm the binding or association of 5-HIAA to the proteins in the albumin and alpha-2-globulin fractions. In conclusion, 5-HIAA is likely to bind to albumin and proteins in the alpha-2-globulin fraction, but our assays determine serum free 5-HIAA and VMA.

Our LC-MS/MS assays for serum 5-HIAA and VMA are well suited for use in clinical laboratories and for diagnosis of NETs. Serum 5-HIAA LC-MS/MS assay has been used in routine diagnostics in HUSLAB in Helsinki University Central Hospital since January 2013. Serum VMA LC-MS/MS assay will be introduced to the test panel after training of the personnel.

6 Conclusions and future prospects

The main findings of this study are:

- We developed and validated an on-line HPLC equipment for direct quantification of extracellular metabolites from cell cultivation. The equipment was connected to automatic sampling for intracellular metabolites. The HPLC equipment has been commercialized by On-line HPLC Ltd, Helsinki, Finland.

- We developed and validated LC-MS/MS assays for NET markers 5-HIAA and VMA from serum. 5-HIAA LC-MS/MS assay is routinely used for the analysis of NET patient samples in the HUSLAB, Helsinki University Central Hospital. VMA LC-MS/MS assay will be in routine use in the near future after training of the personnel.

- We observed that a diet restriction for 3 days before serum 5-HIAA assay is not necessary. Instead, one day restriction before sampling is sufficient because the half-life of 5-HIAA is only 1.3 hours. The diet restriction protocol for patients was reassessed according to these findings.

There is a need for more specific and straightforward assays in research and industrial laboratories. The use of MS technology is likely to increase in clinical laboratories and manufactures are making efforts to simplify analysis protocols. Like all other assays, LC-MS/MS assays, too, should undergo systematic validation before introduction into patient care. There are some pitfalls in MS analyses and the users should be aware of potential limitations of the methodology.

The methods developed in this study are sensitive and well suited for the purpose of demand. Furthermore, compared to the existing methods all assays developed in this study improved the methodology. The 5-HIAA assay has been in clinical use for two years with expected performance and reliability. Awareness of the new assay among clinicians is increasing and the monthly sample amounts are constantly growing (Fig. 20). The patient sample material in study IV was limited due to low incidence and time constrains. There is a plan for collection of lager amounts of patient samples in the near future. After collecting of the sample material, we will establish pediatric reference intervals for serum VMA. Furthermore, some new assays for tumor markers or other metabolites will be developed.

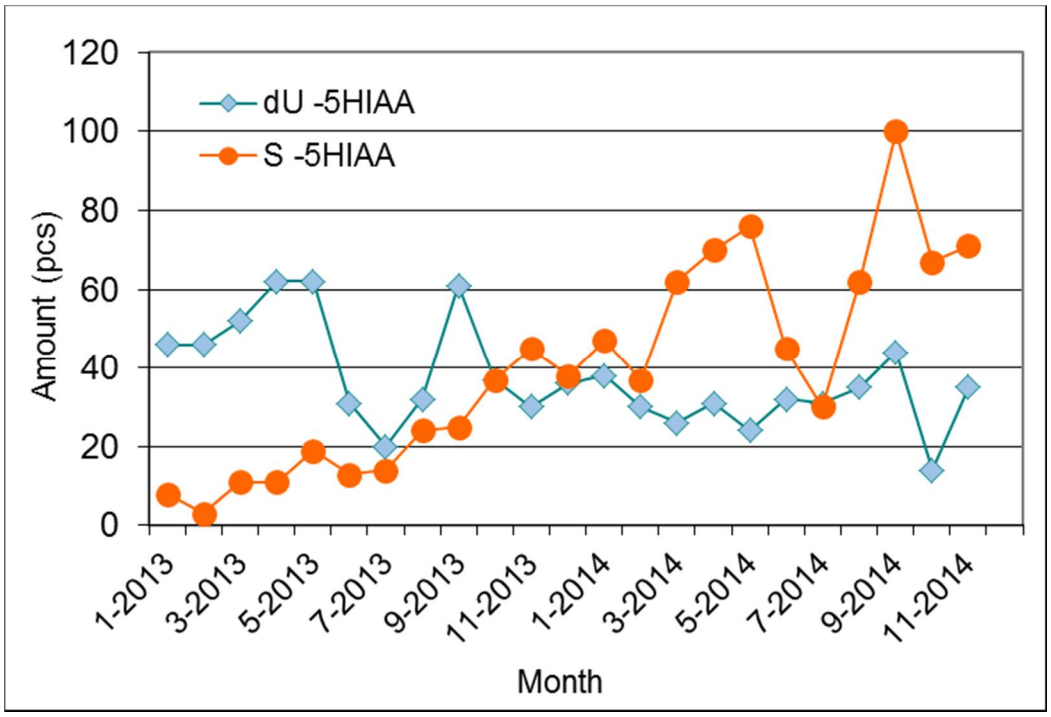


Figure 20. Monthly sample amounts of serum (S -5HIAA) and urinary (dU -5HIAA) 5-HIAA assays during 2013-2014.

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Helsinki, March 2015

Niina

References

1. Roux A, Lison D, Junot C, et al. Applications of liquid chromatography coupled to mass spectrometry-based metabolomics in clinical chemistry and toxicology: A review. *Clin Biochem*. 2011;44(1):119-135.

2. Oliver SG, Winson MK, Kell DB, et al. Systematic functional analysis of the yeast genome. *Trends Biotechnol*. 1998;16(9):373-378.

3. Goodacre R, Vaidyanathan S, Dunn WB, et al. Metabolomics by numbers: Acquiring and understanding global metabolite data. *Trends Biotechnol*. 2004;22(5):245-252.

4. Förster J, Famili I, Fu P, Palsson BØ, et al. Genome-scale reconstruction of the saccharomyces cerevisiae metabolic network. *Genome Res*. 2003;13(2):244-253.

5. Http://www.hmdb.ca/.

6. Raamsdonk LM, Teusink B, Broadhurst D, et al. A functional genomics strategy that uses metabolome data to reveal the phenotype of silent mutations. *Nat Biotechnol*. 2001;19(1):45-50.

7. Allen J, Davey HM, Broadhurst D, et al. High-throughput classification of yeast mutants for functional genomics using metabolic footprinting. *Nat Biotechnol*. 2003;21(6):692-696.

8. Woo HM, Kim KM, Choi MH, et al. Mass spectrometry based metabolomic approaches in urinary biomarker study of women's cancers. *Clin Chim Acta*. 2009;400(1):63-69.

9. Asiago VM, Alvarado LZ, Shanaiah N, et al. Early detection of recurrent breast cancer using metabolite profiling. *Cancer Res*. 2010;70(21):8309-8318.

10. Sabatine MS, Liu E, Morrow DA, et al. Metabolomic identification of novel biomarkers of myocardial ischemia. *Circulation*. 2005;112(25):3868-3875.

11. Nicholls AW, Holmes E, Lindon JC, et al. Metabonomic investigations into hydrazine toxicity in the rat. *Chem Res Toxicol*. 2001;14(8):975-987.

12. German JB, Roberts MA, Watkins SM. Personal metabolomics as a next generation nutritional assessment. *J Nutr*. 2003;133(12):4260-4266.

13. Brindle JT, Antti H, Holmes E, et al. Rapid and noninvasive diagnosis of the presence and severity of coronary heart disease using 1H-NMR-based metabonomics. *Nat Med*. 2002;8(12):1439-1445.

14. Bro C, Nielsen J. Impact of 'ome'analyses on inverse metabolic engineering. *Metab Eng*. 2004;6(3):204-211.

15. Kell DB. Metabolomics and systems biology: Making sense of the soup. *Curr Opin Microbiol*. 2004;7(3):296-307.

16. Biomedical F. NIH definition of biomarker. *Clin Pharmacol Ther*. 2001;69:89-95.

17. Khleif SN, Doroshow JH, Hait WN. AACR-FDA-NCI cancer biomarkers collaborative consensus report: Advancing the use of biomarkers in cancer drug development. *Clin Cancer Res*. 2010;16(13):3299-3318.

18. Matsumoto I, Kuhara T. A new chemical diagnostic method for inborn errors of metabolism by mass spectrometry—rapid, practical, and simultaneous urinary metabolites analysis. *Mass Spectrom Rev*. 1996;15(1):43-57.

19. Millington D, Kodo N, Norwood D, et al. Tandem mass spectrometry: A new method for acylcarnitine profiling with potential for neonatal screening for inborn errors of metabolism. *J Inherit Metab Dis*. 1990;13(3):321-324.

20. Botelho JC, Shacklady C, Cooper HC, et al. Isotope-dilution liquid chromatography-tandem mass spectrometry candidate reference method for total testosterone in human serum. *Clin Chem*. 2013;59(2):372-380.

21. Nordström A, Lewensohn R. Metabolomics: Moving to the clinic. *J Neuroimmune Pharm*. 2010;5(1):4-17.

22. Rifai N, Gillette MA, Carr SA. Protein biomarker discovery and validation: The long and uncertain path to clinical utility. *Nat Biotechnol*. 2006;24(8):971-983.

23. Burtis C, Ashwood E. *TIETZ TEXTBOOK OF clinical chemistry.* 2nd edition. United States of America, W.B. Saunders Company, 1994.

24. Spencer CA, Takeuchi M, Kazarosyan M. Current status and performance goals for serum thyrotropin (TSH) assays. *Clin Chem*. 1996;42(1):140-145.

25. Schalin-Jäntti C, Tanner P, Välimäki MJ, et al. Serum TSH reference interval in healthy finnish adults using the abbott architect 2000i analyzer. *Scan J Clin Lab Inv*. 2011;71(4):344-349.

26. Janse F, Eijkemans MJ, Goverde AJ, et al. Assessment of androgen concentration in women: Liquid chromatography-tandem mass spectrometry and extraction RIA show comparable results. *Eur J Endocrinol*. 2011;165(6):925-933.

27. Handelsman D, Wartofsky L. Requirement for mass spectrometry sex steroid assays in the journal of clinical endocrinology and metabolism. *J Clin Endocr Metab*. 2013;98(10):3971-3973.

28. Holt DW, Mandelbrot DA, Tortorici MA, et al. Long-term evaluation of analytical methods used in sirolimus therapeutic drug monitoring. *Clin Transplant*. 2014;28(2):243-251.

29. Grebe SK, Singh RJ. LC-MS/MS in the clinical laboratory - where to from here? *Clin Biochem Rev*. 2011;32(1):5-31.

30. Guilhaumou R, Lacarelle B, Sampol-Manos E. A rapid, simple and sensitive liquid chromatography-tandem mass spectrometry method for routine clinical monitoring of tacrolimus with the waters masstrak immmunosuppressant kit. *Methods Find Exp Clin Pharmacol*. 2010;32(10):737-743.

31. Koal T, Schmiederer D, Pham-Tuan H, et al. Standardized LC–MS/MS based steroid hormone profile-analysis. *J Steroid Biochem Mol Biol*. 2012;129(3):129-138.

32. Nicholson JK, Lindon JC. Systems biology: Metabonomics. *Nature*. 2008;455(7216):1054-1056.

33. Lin S, Liu N, Yang Z, et al. GC/MS-based metabolomics reveals fatty acid biosynthesis and cholesterol metabolism in cell lines infected with influenza A virus. *Talanta*. 2010;83(1):262-268.

34. Jonsson P, Johansson AI, Gullberg J, et al. High-throughput data analysis for detecting and identifying differences between samples in GC/MS-based metabolomic analyses. *Anal Chem*. 2005;77(17):5635-5642.

35. Ceglarek U, Leichtle A, Bruegel M, et al. Challenges and developments in tandem mass spectrometry based clinical metabolomics. *Mol Cell Endocrinol*. 2009;301(1):266-271.

36. Bajad SU, Lu W, Kimball EH, et al. Separation and quantitation of water soluble cellular metabolites by hydrophilic interaction chromatography-tandem mass spectrometry. *J Chromatogr A*. 2006;1125(1):76-88.

37. Soga T, Ohashi Y, Ueno Y, et al. Quantitative metabolome analysis using capillary electrophoresis mass spectrometry. *J Proteome Res*. 2003;2(5):488-494.

38. Ramautar R, Somsen GW, de Jong GJ. CE-MS for metabolomics: Developments and applications in the period 2010–2012. *Electrophoresis*. 2013;34(1):86-98.

39. Krishnan P, Kruger NJ, Ratcliffe RG. Metabolite fingerprinting and profiling in plants using NMR. *J Exp Bot*. 2005;56(410):255-265.

40. Lei Z, Huhman DV, Sumner LW. Mass spectrometry strategies in metabolomics. *J Biol Chem*. 2011;286(29):25435-25442.

41. Xiao JF, Zhou B, Ressom HW. Metabolite identification and quantitation in LC-MS/MS-based metabolomics. *TrAC Trend Anal Chem*. 2012;32:1-14.

42. Claessens HA. Trends and progress in the characterization of stationary phases for reversed-phase liquid chromatography. *TrAC Trend Anal Chem*. 2001;20(10):563-583.

43. Cabrera K. Applications of silica-based monolithic HPLC columns. *J Sep Sci*. 2004;27(10-11):843-852.

44. Trivedi DK, Iles RK. Do not just do it, do it right: Urinary metabolomics–establishing clinically relevant baselines. *Biomed Chromatogr*. 2014;28:(11):1491-1501.

45. Plumb R, Castro-Perez J, Granger J, et al. Ultra-performance liquid chromatography coupled to quadrupole-orthogonal time-of-flight mass spectrometry. *Rapid Commun Mass Sp*. 2004;18(19):2331-2337.

46. Johnson KA, Plumb R. Investigating the human metabolism of acetaminophen using UPLC and exact mass oa-TOF MS. *J Pharm Biomed Anal*. 2005;39(3):805-810.

47. Swartz ME. UPLC™: An introduction and review. *J Liq Chromatogr Rel Technol*. 2005;28(7-8):1253-1263.

48. Want EJ, Wilson ID, Gika H, et al. Global metabolic profiling procedures for urine using UPLC–MS. *Nature protocols*. 2010;5(6):1005-1018.

49. www.ncbi.nlm.nih.gov/pubmed.

50. Strege MA. Hydrophilic interaction chromatography-electrospray mass spectrometry analysis of polar compounds for natural product drug discovery. *Anal Chem*. 1998;70(13):2439-2445.

51. Linden JC, Lawhead CL. Liquid chromatography of saccharides. *J Chromatogr A*. 1975;105(1):125-133.

52. Alpert AJ. Hydrophilic-interaction chromatography for the separation of peptides, nucleic acids and other polar compounds. *J Chromatogr A*. 1990;499:177-196.

53. Guo Y, Gaiki S. Retention behavior of small polar compounds on polar stationary phases in hydrophilic interaction chromatography. *J Chromatogr A*. 2005;1074(1):71-80.

54. Paek IB, Moon Y, Ji HY, et al. Hydrophilic interaction liquid chromatography–tandem mass spectrometry for the determination of levosulpiride in human plasma. *J Chromatogr B*. 2004;809(2):345-350.

55. Zhang X, Rauch A, Lee H, et al. Capillary hydrophilic interaction chromatography/mass spectrometry for simultaneous determination of multiple neurotransmitters in primate cerebral cortex. *Rapid Commun Mass Sp*. 2007;21(22):3621-3628.

56. Thomson JJ. Rays of postive electricity and their applications to chemical analysis. *J Röntg Soc.* 1914; 10:(39):41.

57. El-Aneed A, Cohen A, Banoub J. Mass spectrometry, review of the basics: Electrospray, MALDI, and commonly used mass analyzers. *Appl Spectrosc Rev*. 2009;44(3):210-230.

58. Dooley KC. Tandem mass spectrometry in the clinical chemistry laboratory. *Clin Biochem*. 2003;36(6):471-481.

59. van den Ouweland, Johannes MW, Kema IP. The role of liquid chromatography–tandem mass spectrometry in the clinical laboratory. *J Chromatogr B*. 2012;883:18-32.

60. Farrell CJ, Martin S, McWhinney B, et al. State-of-the-art vitamin D assays: A comparison of automated immunoassays with liquid chromatography-tandem mass spectrometry methods. *Clin Chem*. 2012;58(3):531-542.

61. Singh RJ. Quantitation of 25-OH-vitamin D (25OHD) using liquid tandem mass spectrometry (LC-MS-MS). In: *Clinical applications of mass spectrometry.* Springer; 2010:509-517.

62. Nelson RE, Grebe SK, OKane DJ, et al. Liquid chromatography-tandem mass spectrometry assay for simultaneous measurement of estradiol and estrone in human plasma. *Clin Chem*. 2004;50(2):373-384.

63. Juutilainen A, Savolainen K, Romppanen J, et al. Combination of LC–MS/MS aldosterone and automated direct renin in screening for primary aldosteronism. *Clin Chim Acta*. 2014;433:209-215.

64. Taylor PJ, Cooper DP, Gordon RD, et al. Measurement of aldosterone in human plasma by semiautomated HPLC-tandem mass spectrometry. *Clin Chem*. 2009;55(6):1155-1162.

65. Sallustio BC, Noll BD, Morris RG. Comparison of blood sirolimus, tacrolimus and everolimus concentrations measured by LC-MS/MS, HPLC-UV and immunoassay methods. *Clin Biochem*. 2011;44(2):231-236.

66. Marinova M, Artusi C, Brugnolo L, et al. Immunosuppressant therapeutic drug monitoring by LC-MS/MS: Workflow optimization through automated processing of whole blood samples. *Clin Biochem*. 2013;46(16):1723-1727.

67. Ahonen L, Maire FB, Savolainen M, et al. Analysis of oxysterols and vitamin D metabolites in mouse brain and cell line samples by ultra-high-performance liquid chromatography-atmospheric pressure photoionization–mass spectrometry. *J Chromatogr A*. 2014;1364:214-222.

68. Jiang X, Sidhu R, Porter FD, et al. A sensitive and specific LC-MS/MS method for rapid diagnosis of niemann-pick C1 disease from human plasma. *J Lipid Res*. 2011;52(7):1435-1445.

69. Wilm M. Principles of electrospray ionization. *Mol Cell Proteomics*. 2011;10(7):M111.009407.

70. Fenn JB, Mann M, Meng CK, et al. Electrospray ionization for mass spectrometry of large biomolecules. *Science*. 1989;246(4926):64-71.

71. Ketola R, Kostiainen R, Kotiaho T, Vainiptalo P. *Massaspektrometrian perusteet.* Helsinki: Hakapaino; 2010:273.

72. Cole RB. Some tenets pertaining to electrospray ionization mass spectrometry. *J Mass Spectrom*. 2000;35(7):763-772.

73. Kebarle P. A brief overview of the present status of the mechanisms involved in electrospray mass spectrometry. *J Mass Spectrom*. 2000;35(7):804-817.

74. www.lamondlab.com/MSResource/LCMS/MassSpectrometry/electrosprayIonisation.

75. John H, Walden M, Schäfer S, et al. Analytical procedures for quantification of peptides in pharmaceutical research by liquid chromatography–mass spectrometry. *Anal Bioanal Chem*. 2004;378(4):883-897.

76. Straub RF, Voyksner RD. Negative ion formation in electrospray mass spectrometry. *J Am Soc Mass Spectrom*. 1993;4(7):578-587.

77. Constantopoulos TL, Jackson GS, Enke CG. Effects of salt concentration on analyte response using electrospray ionization mass spectrometry. *J Am Soc Mass Spectrom*. 1999;10(7):625-634.

78. Schmidt A, Karas M, Dülcks T. Effect of different solution flow rates on analyte ion signals in nano-ESI MS, or: When does ESI turn into nano-ESI? *J Am Soc Mass Spectrom*. 2003;14(5):492-500.

79. Makarov A, Scigelova M. Coupling liquid chromatography to orbitrap mass spectrometry. *J Chromatogr A*. 2010;1217(25):3938-3945.

80. Yost R, Enke C. Triple quadrupole mass spectrometry for direct mixture analysis and structure elucidation. *Anal Chem*. 1979;51(12):1251-1264.

81. Dawson P. Quadrupole mass analyzers: Performance, design and some recent applications. *Mass Spectrom Rev*. 1986;5(1):1-37.

82. Jennings KR. Collision-induced decompositions of aromatic molecular ions. *Int J Mass Spectrom Ion Phys*. 1968;1(3):227-235.

83. Kelleher NL, Zubarev RA, Bush K, et al. Localization of labile posttranslational modifications by electron capture dissociation: The case of γ-carboxyglutamic acid. *Anal Chem*. 1999;71(19):4250-4253.

84. Dongre AR, Somogyi A, Wysocki VH. Surface-induced dissociation: An effective tool to probe structure, energetics and fragmentation mechanisms of protonated peptides. *J Mass Spectrom*. 1996;31(4):339-350.

85. Baldwin MA. Mass spectrometers for the analysis of biomolecules. *Meth Enzymol*. 2005;402(0):3-48.

86. Domon B, Aebersold R. Mass spectrometry and protein analysis. *Science*. 2006;312(5771):212-217.

87. Baty J, Robinson P. Single and multiple ion recording techniques for the analysis of diphenylhydantoin and its major metabolite in plasma. *Biol Mass Spectrom*. 1977;4(1):36-41.

88. Unwin RD, Griffiths JR, Leverentz MK, et al. Multiple reaction monitoring to identify sites of protein phosphorylation with high sensitivity. *Mol Cell Proteomics*. 2005;4(8):1134-1144.

89. Kebarle P, Tang L. From ions in solution to ions in the gas phase-the mechanism of electrospray mass spectrometry. *Anal Chem*. 1993;65(22):972A-986A.

90. Cappiello A, Famiglini G, Palma P, et al. Overcoming matrix effects in liquid chromatography− mass spectrometry. *Anal Chem*. 2008;80(23):9343-9348.

91. Buhrman DL, Price PI, Rudewiczcor PJ. Quantitation of SR 27417 in human plasma using electrospray liquid chromatography-tandem mass spectrometry: A study of ion suppression. *J Am Soc Mass Spectrom*. 1996;7(11):1099-1105.

92. Bonfiglio R, King RC, Olah TV, et al. The effects of sample preparation methods on the variability of the electrospray ionization response for model drug compounds. *Rapid Commun Mass Sp*. 1999;13(12):1175-1185.

93. Little JL, Wempe MF, Buchanan CM. Liquid chromatography–mass spectrometry/mass spectrometry method development for drug metabolism studies: Examining lipid matrix ionization effects in plasma. *J Chromatogr B*. 2006;833(2):219-230.

94. Dams R, Huestis MA, Lambert WE, et al. Matrix effect in bio-analysis of illicit drugs with LC-MS/MS: Influence of ionization type, sample preparation, and biofluid. *J Am Soc Mass Spectrom*. 2003;14(11):1290-1294.

95. Liang H, Foltz R, Meng M, et al. Ionization enhancement in atmospheric pressure chemical ionization and suppression in electrospray ionization between target drugs and stable-isotope-labeled internal standards in quantitative liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Sp*. 2003;17(24):2815-2821.

96. Chambers E, Wagrowski-Diehl DM, Lu Z, et al. Systematic and comprehensive strategy for reducing matrix effects in LC/MS/MS analyses. *J Chromatogr B*. 2007;852(1):22-34.

97. Matuszewski BK, Constanzer ML, Chavez-Eng CM. Strategies for the assessment of matrix effect in quantitative bioanalytical methods based on HPLC−MS/MS. *Anal Chem*. 2003;75:(13)3019-3030.

98. Hernandez F, Sancho J, Pozo O. Critical review of the application of liquid chromatography/mass spectrometry to the determination of pesticide residues in biological samples. *Anal Bioanal Chem*. 2005;382(4):934-946.

99. Villagrasa M, Guillamón M, Eljarrat E, et al. Matrix effect in liquid chromatography–electrospray ionization mass spectrometry analysis of benzoxazinoid derivatives in plant material. *J Chromatogr A*. 2007;1157(1):108-114.

100. Basilicata P, Miraglia N, Pieri M, et al. Application of the standard addition approach for the quantification of urinary benzene. *J Chromatogr B*. 2005;818(2):293-299.

101. Zhang G, Terry AV, Bartlett MG. Determination of the lipophilic antipsychotic drug ziprasidone in rat plasma and brain tissue using liquid chromatography tandem mass spectrometry. *Biomed Chromatogr*. 2008;22(7):770-778.

102. Wang S, Cyronak M, Yang E. Does a stable isotopically labeled internal standard always correct analyte response?: A matrix effect study on a LC/MS/MS method for the determination of carvedilol enantiomers in human plasma. *J Pharm Biomed Anal*. 2007;43(2):701-707.

103. Rappold BA, Grant RP. Isotopic abundance yields bias in the assessment of testosterone in a new reference method procedure. *Clin Chem*. 2013;59(7):1129-1130.

104. Anderson NL, Polanski M, Pieper R, et al. The human plasma proteome: A nonredundant list developed by combination of four separate sources. *Mol Cell Proteomics*. 2004;3(4):311-326.

105. Link AJ, Eng J, Schieltz DM, et al. Direct analysis of protein complexes using mass spectrometry. *Nat Biotechnol*. 1999;17(7):676-682.

106. Hennion M. Solid-phase extraction: Method development, sorbents, and coupling with liquid chromatography. *J Chromatogr A*. 1999;856(1):3-54.

107. Xu RN, Fan L, Rieser MJ, et al. Recent advances in high-throughput quantitative bioanalysis by LC–MS/MS. *J Pharm Biomed Anal*. 2007;44(2):342-355.

108. Yang AY, Sun L, Musson DG, et al. Determination of M 4 stable isotope labeled cortisone and cortisol in human plasma by µElution solid-phase extraction and liquid chromatography/tandem mass spectrometry. *Rapid Commun Mass Sp*. 2006;20(2):233-240.

109. Hull CK, Martin PD, Warwick MJ, et al. Quantification of the N-desmethyl metabolite of rosuvastatin in human plasma by automated SPE followed by HPLC with tandem MS detection. *J Pharm Biomed Anal*. 2004;35(3):609-614.

110. Peitzsch M, Pelzel D, Glöckner S, et al. Simultaneous liquid chromatography tandem mass spectrometric determination of urinary free metanephrines and catecholamines, with comparisons of free and deconjugated metabolites. *Clin Chim Acta*. 2013;418:50-58.

111. Thibeault D, Caron N, Djiana R, et al. Development and optimization of simplified LC–MS/MS quantification of 25-hydroxyvitamin D using protein precipitation combined with on-line solid phase extraction (SPE). *J Chromatogr B*. 2012;883:120-127.

112. Abdel-Rehim M. Microextraction by packed sorbent (MEPS): A tutorial. *Anal Chim Acta*. 2011;701(2):119-128.

113. Said R, Hassan Z, Hassan M, et al. Rapid and sensitive method for determination of cyclophosphamide in patients plasma samples utilizing microextraction by packed sorbent online with liquid Chromatography-Tandem mass spectrometry (MEPS-LC-MS/MS). *J Liq Chromatogr Rel Technol*. 2008;31(5):683-694.

114. Henion J, Brewer E, Rule G. Peer reviewed: Sample preparation for LC/MS/MS: Analyzing biological and environmental samples. *Anal Chem*. 1998;70(19):650A-656A.

115. Yamashita K, Okuyama M, Nakagawa R, et al. Development of sensitive derivatization method for aldosterone in liquid chromatography–electrospray ionization tandem mass spectrometry of corticosteroids. *J Chromatogr A*. 2008;1200(2):114-121.

116. Højskov CS, Heickendorff L, Møller HJ. High-throughput liquid–liquid extraction and LC-MS/MS assay for determination of circulating 25 (OH) vitamin D3 and D2 in the routine clinical laboratory. *Clin Chim Acta*. 2010;411(1):114-116.

117. Polson C, Sarkar P, Incledon B, et al. Optimization of protein precipitation based upon effectiveness of protein removal and ionization effect in liquid chromatography–tandem mass spectrometry. *J Chromatogr B*. 2003;785(2):263-275.

118. Ismaiel OA, Zhang T, Jenkins RG, et al. Investigation of endogenous blood plasma phospholipids, cholesterol and glycerides that contribute to matrix effects in bioanalysis by liquid chromatography/mass spectrometry. *J Chromatogr B*. 2010;878(31):3303-3316.

119. Van Damme T, Lachová M, Lynen F, et al. Solid-phase extraction based on hydrophilic interaction liquid chromatography with acetone as eluent for eliminating matrix effects in the analysis of biological fluids by LC-MS. *Anal Bioanal Chem*. 2014;406(2):401-407.

120. Williams MG, Palandra J, Shobe EM. Rapid determination of rat plasma uridine levels by HPLC-ESI-MS utilizing the captiva™ filter plates for sample preparation. *Biomed Chromatogr*. 2003;17(4):215-218.

121. Keevil BG, McCann SJ, Cooper DP, et al. Evaluation of a rapid micro-scale assay for tacrolimus by liquid chromatography-tandem mass spectrometry. *Ann Clin Biochem*. 2002;39(Pt 5):487-492.

122. Shaner RL, Kaplan P, Hamelin EI, et al. Comparison of two automated solid phase extractions for the detection of ten fentanyl analogs and metabolites in human urine using liquid chromatography tandem mass spectrometry. *J Chromatogr B*. 2014;962:52-58.

123. Bouzas NF, Dresen S, Munz B, et al. Determination of basic drugs of abuse in human serum by online extraction and LC–MS/MS. *Anal Bioanal Chem*. 2009;395(8):2499-2507.

124. Schütze D, Boss B, Schmid J. Liquid chromatographic–tandem mass spectrometric method for the analysis of a neurokinin-1 antagonist and its metabolite using automated solid-phase sample preparation and automated data handling and reporting. *J Chromatogr B*. 2000;748(1):55-64.

125. Zhang N, Yang A, Rogers JD, et al. Quantitative analysis of simvastatin and its β-hydroxy acid in human plasma using automated liquid–liquid extraction based on 96-well plate format and liquid chromatography-tandem mass spectrometry. *J Pharm Biomed Anal*. 2004;34(1):175-187.

126. Badiou S, Guillot J, Kuster N, et al. Comparison of arkray/ELITech ADAMS HA-8180V® with Bio-Rad variant, TMII Turbo2. 0® and tosoh bioscience HLC®-723G8 for HbA1c determination. *J Clin Lab Anal*. 2014;28:(6):428-434.

127. Zumbusch Pv, Meyer-Jens T, Brunner G, et al. On-line monitoring of organic substances with high-pressure liquid chromatography (HPLC) during the anaerobic fermentation of waste-water. *Appl Microbiol Biotechnol*. 1994;42(1):140-146.

128. Bouzige M, Machtalere G, Legeay P, et al. New methodology for a selective on-line monitoring of some polar priority industrial chemicals in waste water. *Waste Manage*. 1999;19(2):171-180.

129. Johnson RC, Srinivasan N, Cooks RG, et al. Membrane introduction mass spectrometry in a pilot plant: On-line monitoring of fermentation broths. *Rapid Commun Mass Sp*. 1997;11(4):363-367.

130. Suomen Standardisoimisliitto. ISO STANDARDI 15189, 2012: Lääketieteelliset laboratoriot. Laatua ja pätevyyttä koskevat vaatimukset.

131. U.S. Deparment of Health and Human Services Food and Drug Administration. Guidance for industry: Bioanalytical method validation. 2013.

132. Peters FT, Maurer HH. Bioanalytical method validation and its implications for forensic and clinical toxicology—a review. *Validation in chemical measurement. Accured Qual Assur.* 2002;7:441-449.

133. Vogeser M, Seger C. Pitfalls associated with the use of liquid chromatography-tandem mass spectrometry in the clinical laboratory. *Clin Chem*. 2010;56(8):1234-1244.

134. Twentyman JM, Cradic KW, Singh RJ, et al. Ionic cross talk can lead to overestimation of 3-methoxytyramine during quantification of metanephrines by mass spectrometry. *Clin Chem*. 2012;58(7):1156-1158.

135. Lange V, Picotti P, Domon B, et al. Selected reaction monitoring for quantitative proteomics: A tutorial. *Mol Syst Biol*. 2008;4:222.

136. Riley C, Rosanske T. *Development and validation of analytical methods.* 3th edition. Great Britain, Elsevier Science Ltd, 1996:352.

137. Green JM. Peer reviewed: A practical guide to analytical method validation. *Anal Chem*. 1996;68(9):305A-309A.

138. Rose RM, Kreuz LE, Holaday JW, et al. Diurnal variation of plasma testosterone and cortisol. *J Endocrinol*. 1972;54(1):177-178.

139. Resko JA, Eik-Nes KB. Diurnal testosterone levels in peripheral plasma of human male subjects. *J Clin Endocr Metab*. 1966;26(5):573-576.

140. Brambilla DJ, Matsumoto AM, Araujo AB, et al. The effect of diurnal variation on clinical measurement of serum testosterone and other sex hormone levels in men. *J Clin Endocr Metab*. 2009;94(3):907-913.

141. Ahmed AH, Gordon RD, Taylor P, et al. Effect of atenolol on aldosterone/renin ratio calculated by both plasma renin activity and direct renin concentration in healthy male volunteers. *J Clini Endocr Metab*. 2010;95(7):3201-3206.

142. Gräsbeck R, Saris NE. Establisment and use of normal values. *Scand J Clin Lab Invest*. 1969;26:62-63.

143. Clinical and Laboratory Standards Institute. How to define and determine reference intervals in the clinical laboratory: Approved guideline. 2nd edition. 2000.

144. Zweig MH, Campbell G. Receiver-operating characteristic (ROC) plots: A fundamental evaluation tool in clinical medicine. *Clin Chem*. 1993;39(4):561-577.

145. Metz CE. ROC methodology in radiologic imaging. *Invest Radiol*. 1986;21(9):720-733.

146. Hotakainen K, Tanner P, Alfthan H, et al. Comparison of three immunoassays for CA 19-9. *Clin Chim Acta*. 2009;400(1):123-127.

147. Modlin IM, Oberg K, Taylor A, et al. Neuroendocrine tumor biomarkers: Current status and perspectives. *Neuroendocrinology*. 2014;100:(4):1-13.

148. Klöppel G, Perren A, Heitz PU. The gastroenteropancreatic neuroendocrine cell system and its tumors: The WHO classification. *Ann N Y Acad Sci*. 2004;1014(1):13-27.

149. Faggiano A, Mansueto G, Ferolla P, et al. Diagnostic and prognostic implications of the world health organization classification of neuroendocrine tumors. *J Endocrinol Invest*. 2008;31(3):216-223.

150. Solcia E, Capella C, Buffa R, et al. Endocrine cells of the digestive system. *Phys Gastroint Trac*. 1987;1:11-29.

151. de Herder WW. Biochemistry of neuroendocrine tumours. *Best Pract Res Clin En*. 2007;21(1):33-41.

152. Kaltsas GA, Besser GM, Grossman AB. The diagnosis and medical management of advanced neuroendocrine tumors. *Endocr Rev*. 2004;25(3):458-511.

153. Fritz AG. *International classification of diseases for oncology: ICD-O.* World Health Organization; 3th edition, Geneva. 2000.

154. Klimstra DS, Modlin IR, Coppola D, et al. The pathologic classification of neuroendocrine tumors: A review of nomenclature, grading, and staging systems. *Pancreas*. 2010;39(6):707-712.

155. Williams E, Sandler M. The classification of carcinoid tumors. *Lancet*. 1963;1:238-239.

156. Bryant J, Farmer J, Kessler LJ, et al. Pheochromocytoma: The expanding genetic differential diagnosis. *J Natl Cancer Inst*. 2003;95(16):1196-1204.

157. Amar L, Bertherat J, Baudin E, et al. Genetic testing in pheochromocytoma or functional paraganglioma. *J Clin Oncol*. 2005;23(34):8812-8818.

158. Brodeur GM. Neuroblastoma: Biological insights into a clinical enigma. *Nat Rev Cancer*. 2003;3(3):203-216.

159. Modlin IM, Champaneria MC, Chan AK, et al. A three-decade analysis of 3,911 small intestinal neuroendocrine tumors: The rapid pace of no progress. *Am J Gastroenterol*. 2007;102(7):1464-1473.

160. Stinner B, Rothmund M. Neuroendocrine tumours (carcinoids) of the appendix. *Best Pract Cl Ga*. 2005;19(5):729-738.

161. Moertel CG. An odyssey in the land of small tumors. *J Clin Oncol*. 1987;5(10):1503-1522.

162. Janson ET, Sørbye H, Welin S, et al. Nordic guidelines 2010 for diagnosis and treatment of gastroenteropancreatic neuroendocrine tumours. *Acta Oncol*. 2010;49(6):740-756.

163. Kulke MH, Siu LL, Tepper JE, et al. Future directions in the treatment of neuroendocrine tumors: Consensus report of the national cancer institute neuroendocrine tumor clinical trials planning meeting. *J Clin Oncol*. 2011;29(7):934-943.

164. Vinik AI, Woltering EA, Warner RR, et al. NANETS consensus guidelines for the diagnosis of neuroendocrine tumor. *Pancreas*. 2010;39(6):713-734.

165. Lawrence B, Gustafsson BI, Chan A, et al. The epidemiology of gastroenteropancreatic neuroendocrine tumors. *Endocrinol Metab Clin North Am*. 2011;40(1):1-18.

166. Yao JC, Hassan M, Phan A, et al. One hundred years after "carcinoid": Epidemiology of and prognostic factors for neuroendocrine tumors in 35,825 cases in the united states. *J Clin Oncol*. 2008;26(18):3063-3072.

167. Modlin IM, Lye KD, Kidd M. A 5-decade analysis of 13,715 carcinoid tumors. *Cancer*. 2003;97(4):934-959.

168. Hauso O, Gustafsson BI, Kidd M, et al. Neuroendocrine tumor epidemiology. *Cancer*. 2008;113(10):2655-2664.

169. Norlén O, Stålberg P, Öberg K, et al. Long-term results of surgery for small intestinal neuroendocrine tumors at a tertiary referral center. *World J Surg*. 2012;36(6):1419-1431.

170. Caplin ME, Buscombe JR, Hilson AJ, et al. Carcinoid tumour. *The Lancet*. 1998;352(9130):799-805.

171. Kulke MH, Mayer RJ. Carcinoid tumors. *N Engl J Med*. 1999;340(11):858-868.

172. O'Toole D, Grossman A, Gross D, et al. ENETS consensus guidelines for the standards of care in neuroendocrine tumors: Biochemical markers. *Neuroendocrinology*. 2009;90(2):194-202.

173. Janson ET, Holmberg L, Stridsberg M, et al. Carcinoid tumors: Analysis of prognostic factors and survival in 301 patients from a referral center. *Ann Oncol*. 1997;8(7):685-690.

174. Hallin ML, Mahmoud K, Viswanath A, et al. 'Sweet dreams', 'happy days' and elevated 24-h urine 5-hydroxyindoleacetic acid excretion. *Ann Clin Biochem*. 2013;50(Pt 1):80-82. .

175. Hourani SM, Cusack NJ. Pharmacological receptors on blood platelets. *Pharmacol Rev*. 1991;43(3):243-298.

176. Kema IP, de Vries EG, Muskiet FA. Clinical chemistry of serotonin and metabolites. *J Chromatogr B*. 2000;747(1):33-48.

177. Welin S, Stridsberg M, Cunningham J, et al. Elevated plasma chromogranin A is the first indication of recurrence in radically operated midgut carcinoid tumors. *Neuroendocrinology*. 2009;89(3):302-307.

178. Marotta V, Nuzzo V, Ferrara T, et al. Limitations of chromogranin A in clinical practice. *Biomarkers*. 2012;17(2):186-191.

179. Turner GB, Johnston BT, McCance DR, et al. Circulating markers of prognosis and response to treatment in patients with midgut carcinoid tumours. *Gut*. 2006;55(11):1586-1591.

180. Eriksson B, Öberg K, Stridsberg M. Tumor markers in neuroendocrine tumors. *Digestion*. 2000;62(Suppl. 1):33-38.

181. Panzuto F, Severi C, Cannizzaro R, et al. Utility of combined use of plasma levels of chromogranin A and pancreatic polypeptide in the diagnosis of gastrointestinal and pancreatic endocrine tumors. *J Endocrinol Invest*. 2004;27(1):6-11.

182. Tsunoda M. Recent advances in methods for the analysis of catecholamines and their metabolites. *Anal Bioanal Chem*. 2006;386(3):506-514.

183. Eisenhofer G, Huynh T, Hiroi M, et al. Understanding catecholamine metabolism as a guide to the biochemical diagnosis of pheochromocytoma. *Revi Endocr Metab Disord*. 2001;2(3):297-311.

184. de Jong WH, de Vries EG, Kema IP. Current status and future developments of LC-MS/MS in clinical chemistry for quantification of biogenic amines. *Clin Biochem*. 2011;44(1):95-103.

185. Lloyd RV. *Endocrine pathology: Differential diagnosis and molecular advances.* New York, Springer, 2010.

186. Ramage J, Davies A, Ardill J, et al. Guidelines for the management of gastroenteropancreatic neuroendocrine (including carcinoid) tumours. *Gut*. 2005;54(Suppl 4):1-16.

187. Arnold R, Chen YJ, Costa F, et al. ENETS consensus guidelines for the standards of care in neuroendocrine tumors: Follow-up and documentation. *Neuroendocrinology*. 2009;90(2):227-233.

188. Janson ET, Sorbye H, Welin S, et al. Nordic guidelines 2014 for diagnosis and treatment of gastroenteropancreatic neuroendocrine neoplasms. *Acta Oncol*. 2014;53(10):1284-1297.

189. Rosano TG, Swift TA, Hayes LW. Advances in catecholamine and metabolite measurements for diagnosis of pheochromocytoma. *Clin Chem*. 1991;37(10 Pt 2):1854-1867.

190. Http://Huslab.net/ohjekirja/index.html.

191. Gitlow SE, Mendlowitz M, Kruk E, Khassis S. Diagnosis of pheochromocytoma by assay of catecholamine metabolites. *Circ Res*. 1961;9:746-754.

192. Bellet S, Roman L, DeCastro O, Kim KE, et al. Effect of coffee ingestion on catecholamine release. *Metab Clin Exp*. 1969;18(4):288-291.

193. Miller AG, Brown H, Degg T, et al. Measurement of plasma 5-hydroxyindole acetic acid by liquid chromatography tandem mass spectrometry—Comparison with HPLC methodology. *J Chromatogr B*. 2010;878(7):695-699.

194. Sadilkova K, Dugaw K, Benjamin D, et al. Analysis of vanillylmandelic acid and homovanillic acid by UPLC–MS/MS in serum for diagnostic testing for neuroblastoma. *Clin Chim Acta*. 2013;424:253-257.

195. Cangemi G, Barco S, Reggiardo G, et al. Interchangeability between 24-hour collection and single spot urines for vanillylmandelic and homovanillic acid levels in the diagnosis of neuroblastoma. *Pediat Blood Cancer*. 2013;60(12):E170-E172.

196. Felding P, Rustad P, Mårtensson A, et al. Reference individuals, blood collection, treatment of samples and descriptive data from the questionnaire in the nordic reference interval project 2000. *Scan J Clin Lab Inv*. 2004;64(4):327-342.

197. Mulder EJ, Oosterloo-Duinkerken A, Anderson GM, et al. Automated on-line solid-phase extraction coupled with HPLC for measurement of 5-hydroxyindole-3-acetic acid in urine. *Clin Chem*. 2005;51(9):1698-1703.

198. Mercolini L. Fast analysis of catecholamine metabolites MHPG and VMA in human plasma by HPLC with fluorescence detection and a novel SPE procedure. *Talanta*. 2009;78(1):150.

199. Liu C, Hofstadler SA, Bresson JA, et al. On-line dual microdialysis with ESI-MS for direct analysis of complex biological samples and microorganism lysates. *Anal Chem*. 1998;70(9):1797-1801.

200. Odink J, Korthals H, Knijff J. Simultaneous determination of the major acidic metabolites of catecholamines and serotonin in urine by liquid chromatography with electrochemical detection after a one-step sample clean-up on sephadex G-10; influence of vanilla and banana ingestion. *J Chromatogr B*. 1988;424:273-283.

201. Ozturk S, Thrift J, Blackie J, et al. Real-time monitoring and control of glucose and lactate concentrations in a mammalian cell perfusion reactor. *Biotechnol Bioeng*. 1997;53(4):372-378.

202. Fang L, Lv Y, Sheng X, et al. Sensitive, rapid and easy analysis of three catecholamine metabolites in human urine and serum by liquid chromatography tandem mass spectrometry. *J Chromatogr Sci*. 2012;50(5):450-456.

203. Kema IP, Schellings AM, Meiborg G, et al. Influence of a serotonin- and dopamine-rich diet on platelet serotonin content and urinary excretion of biogenic amines and their metabolites. *Clin Chem*. 1992;38(9):1730-1736.

204. Degg T, Allen K, Barth J. Measurement of plasma 5-hydroxyindoleacetic acid in carcinoid disease: An alternative to 24-h urine collections? *Ann Clin Biochem*. 2000;37:724-726.

205. Feldman JM, Lee EM. Serotonin content of foods: Effect on urinary excretion of 5-hydroxyindoleacetic acid. *Am J Clin Nutr*. 1985;42(4):639-643.

206. Turner C, Gregory ME, Thornhill NF. Closed-loop control of fed-batch cultures of recombinant escherichia coli using on-line HPLC. *Biotechnol Bioeng*. 1994;44(7):819-829.

207. Rehorek A, Urbig K, Meurer R, et al. Monitoring of azo dye degradation processes in a bioreactor by on-line high-performance liquid chromatography. *J Chromatogr A*. 2002;949(1):263-268.

208. Paliwal SK, Nadler TK, Wang DI, et al. Automated process monitoring of monoclonal antibody production. *Anal Chem*. 1993;65(23):3363-3367.

209. DeRisi JL, Iyer VR, Brown PO. Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science*. 1997;278(5338):680-686.

210. Törönen P, Kolehmainen M, Wong G, et al. Analysis of gene expression data using self-organizing maps. *FEBS Lett*. 1999;451(2):142-146.

211. Eisen MB, Spellman PT, Brown PO, Botstein D. Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA*. 1998;95(25):14863-14868.

212. Liu Y, Wang F, Lee W. On-line monitoring and controlling system for fermentation processes. *Biochem Eng J*. 2001;7(1):17-25.

213. Http://www.onlinehplc.com.

214. Koivistoinen OM, Kuivanen J, Barth D, et al. Glycolic acid production in the engineered yeasts saccharomyces cerevisiae and kluyveromyces lactis. *Microb Cell Fact*. 2013;12:82-2859-12-82.

215. Fredenberg S, Wahlgren M, Reslow M, et al. The mechanisms of drug release in poly (lactic-co-glycolic acid)-based drug delivery systems—a review. *Int J Pharm*. 2011;415(1):34-52.

216. Makadia HK, Siegel SJ. Poly lactic-co-glycolic acid (PLGA) as biodegradable controlled drug delivery carrier. *Polymers*. 2011;3(3):1377-1397.

217. Yazdani SS, Gonzalez R. Anaerobic fermentation of glycerol: A path to economic viability for the biofuels industry. *Curr Opin Biotechnol*. 2007;18(3):213-219.

218. Tellez MR, Mamikunian G, O'Dorisio TM, et al. A single fasting plasma 5-HIAA value correlates with 24-hour urinary 5-HIAA values and other biomarkers in midgut neuroendocrine tumors (NETs). *Pancreas*. 2013;42:405-410.

219. Carling R, Degg T, Allen K, et al. Evaluation of whole blood serotonin and plasma and urine 5-hydroxyindole acetic acid in diagnosis of carcinoid disease. *Ann Clin Biochem*. 2002;39(Pt 6):577.

220. Pussard E, Neveux M, Guigueno N. Reference intervals for urinary catecholamines and metabolites from birth to adulthood. *Clin Biochem*. 2009;42(6):536-539.

221. Abd-Allah N, Hassan FH, Esmat AY, et al. Age dependence of the levels of plasma norepinephrine, aldosterone, renin activity and urinary vanillylmandelic acid in normal and essential hypertensives. *Biol Res*. 2004;37(1).

222. Itkonen O, Stenman UH, Parkkinen J, et al. Binding of hepcidin to plasma proteins. *Clin Chem*. 2012;58(7):1158-1160.