Ultra-high Performance Liquid Chromatography in Steroid Analysis

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Tiivistelmä – Referat – Abstract

The latest version of liquid chromatography is ultra-high performance (or pressure) chromatography (UHPLC). In the technique, short and narrow-bore columns with particle sizes below 3 µm are used. The extremely high pressure used results in very short analysis times, excellent separation, and good resolution. This makes UHPLC a good choice for steroidal analysis. Steroids are a highly interesting area of study; they can be recognized as biomarkers for several diseases and are a relevant topic in doping testing. In this thesis articles on the topic 'steroid analysis with UHPLC', published prior to April 2017, are reviewed.

UHPLC is always combined with mass spectrometry (MS) for steroid analysis. The MS utilized is usually of multi-dimension: quadrupole time of flight (QTOF) or triple quadrupole (QqQ). The instrumentation is suitable for both untargeted and targeted analysis. In untargeted studies, the study of changes in the human metabolome has been especially interesting. The articles on targeted studies are usually focused on doping control and quantification of identified biomarkers. The analysis with UHPLC-MS/MS usually provide reliable results with fast analysis time, without complicated sample preparation. Typically, the sample preparation processes can include only protein precipitation, liquid-liquid extraction or solid-phase extraction.

UHPLC is also a valuable tool in simple and routine analysis. The separation efficiency is increased by the small plate height and the analysis time can thus be reduced. In this thesis work the technique was utilized for the analysis of food additives. For validation of an UHPLC method the repeatability, trueness, bias, measurement uncertainty and other factors need to be assessed. The experimental part of the thesis is dedicated to describe the development and validation of a method for analysis of five food additives and caffeine.

The developed method was partly validated, with the aim to fulfil the needs of the Finnish Customs Laboratory. The optimized method comprised of an injection volume of 2 µL and a flow rate of 1.0 mL/min. The buffer was a phosphate buffer at pH of 4.0 and the gradient elution program was from 6 % to 30 % of acetonitrile in 1.6 minutes, then 1.6-1.7 minutes with 6% acetonitrile. The total run time was only 1.7 minutes. The limit of detection values was between 0.02 µg/mL and 1.73 µg/mL. The limit of quantitation values was between 0.054 µg/mL to 5.78 µg/mL, which should be sufficient for the Customs needs in the sense of checking if a product is over a certain limit. Expanded measurement uncertainties were around 20 %.

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List of abbreviations

A	Eddy diffusion
AAS	androgenic anabolic steroid
ACN	acetonitrile
API	atmospheric pressure ionization
ASE	accelerated solvent extraction system
As-K	acesulfame potassium
Asp	aspartame
B	longitudinal diffusion
BA	benzoic acid
C	diffusion caused by mass transfer
C18	octadecyl carbon chain bonded silica
DAD	diode array detector
DLLME	dispersive liquid-liquid microextraction
d_p	particle size distribution of stationary phase
dSPE	dispersive solid phase extraction
ESI	electrospray ionization
Fapas	Food Analysis Performance Assessment Scheme
FLD	fluorescence detector
GC	gas chromatography
GC/MS	gas chromatography combined to mass spectrometry
H	plate height

H295R	adrenal gland cells
HF-LPME	hollow fiber liquid-phase microextraction
HPLC	high pressure liquid chromatography
K_D	distribution coefficient
λ	describes the uniformness of the packing
LC	liquid chromatography
LC-MS quality	Quality, that is needed for liquid chromatography with a mass spectrometer as detector.
LE	liquid extraction
LLE	liquid-liquid extraction
MS/MS	tandem mass spectrometry
MSTFA	N,O-bis(trimethylsilyl)trifluoroacetamide
N	plate number
NH_4Ac	ammonium acetate buffer
OPLS	orthogonal projections to latent structures
PCA	principal component analysis
PFP	pentafluorophenyl column
PLS	partial least square regression
Q	quadropole mass spectrometer
QqQ	triple quadropole
QToF	Hybrid mass spectrometer with a quadrupole and time of flight combined.
QuEChERS	quick, easy, cheap, effective, rugged and safe sample preparation

RI	refractive index
ROC	receiver operating characteristic
RPLC	reversed phase liquid chromatography
R_s	resolution
SA	sorbic acid
Sac	saccharin
SDME	single-drop micro extraction
SIMCA	soft independent modelling of class analogies
SPE	solid phase extraction
SPME	solid phase micro extraction
ToF	time of flight mass spectrometer
t_r	retention time
u	mobile phase velocity
UHPLC	ultra-high pressure liquid chromatography
UV/Vis	ultraviolet and visible light detector
WADA	World Anti-Doping Agency

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I. Theoretical part

1. Introduction

There are many different chromatographic methods that can be utilized for the separation and analysis of compounds. The most common techniques are gas chromatography (GC) and liquid chromatography (LC). LC has developed a lot since the first available instruments. Tswett was the first to describe and utilize LC. He separated pigments from plants with the help of a glass tube filled with calcium carbonate as a stationary phase and petrol ether as a mobile phase. This was already in the beginning of the 20th century.[1] The latest version of the LC is ultra-high performance (or pressure) chromatography (UHPLC), which can be used with short and narrow columns with small particles and at an extremely high pressure to achieve short analysis time and high peak capacity.

LC is superior to GC in the analysis of high molecular weight compounds and other compounds of low volatility. The reason for this is that the higher the temperature in the GC oven, the higher is the risk for degradation of the sample compounds. This is especially important to take into consideration when working with biological matrixes.[2] However, GC has the advantage of having larger resolving power compared to regular LC. This has pushed the development of LC in the direction of smaller particle columns, which reduces the plate height and increases the resolution.[3]

The UHPLC instrument was first introduced in 2004 by Waters and was trademarked as UPLC®.[4] Other manufacturers have after this launched similar instruments by the name UHPLC. The UHPLC is an instrument that can operate in pressures above 1000 bars. This enables the usage of particle sizes below 2 µm with flowrates high enough to achieve minimum band broadening.[5]

The UHPLC instrument can be connected to a variety of detectors. Common detectors are the UV/Vis detector and the mass spectrometer. The workhorse UV/Vis detector can be selective enough for simple mixtures and is commonly utilized in routine analysis. The true efficiency of UHPLC can though be seen when it is connected to a tandem mass spectrometer (MS/MS). At its best UHPLC-MS/MS provides an efficient setup for analysis of steroids and steroid metabolites. It can for example efficiently separate up to 124 metabolites, which can be detected and identified by MS/MS.[6]

Steroids are a highly interesting area of study; they can be recognized as biomarkers for several diseases and are a relevant topic in doping testing. Steroids also provide a challenging task for the separation scientist, as they are structurally very similar. UHPLC offers high peak capacity and short

analysis time, as it can be utilized with sub 2 µm solid phase particles.[7] This means that it suits well for the challenging task to resolve steroids.

UHPLC is also a valuable tool in simple and routine analysis. With the small plate height, the analysis time can be reduced and the separation efficiency increased. The technique can be utilized for the analysis of food additives. For the validation of an UHPLC method several factors, like the repeatability, trueness, bias, measurement uncertainty etc. need to be assessed. The experimental part of the thesis is dedicated to describe the development and validation of a method for the analysis of five food additives and caffeine.

2. Analysis of steroids

Steroids play a large role throughout the body and can therefore be found in any matrix taken from the human body. Typically, they are analyzed from blood or urine samples, which are both aqueous matrixes that can easily be prepared for LC analyses. However, such samples are also routinely analyzed by GC and immunoassays.[7,8]

The reason why steroids are of great interest in analysis is that they are relevant in detection of doping drugs and as possible disease biomarkers. They have been linked to Alzheimer's disease, cancers, and diabetes.[9,10,11,12] Generally, physiological steroid levels are low and vary from individual to individual, depending on age, sex, stress level, etc.[13] This makes it hard to compare results between individuals.

The basic structure of steroids consists of a perhydro-1,2-cyclopentanophenanthrene ring system (figure 1). They can be classified per the amount of carbons or by their function. The division by function divides the steroids into four main groups: androgens, estrogens, glucocorticoids, and mineralocorticoids. All steroids are lipophilic.[7]

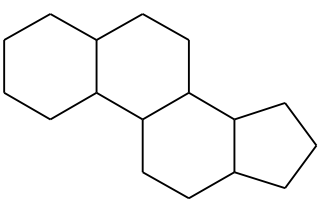

FIGURE 1: BASIC STRUCTURE OF STEROIDS.

GC/MS derivatization is needed for thermos-labile compounds with low volatility and high polarity, which includes estrogens.[14] This means that some of the interesting steroids cannot be analyzed by GC without derivatization. In some cases, the compound needs to be hydrolyzed to be volatile enough for GC, however, this makes it hard to identify the total of the original compound.[7] These factors make LC a very attractive method for analysis of steroids.

The steroidal structure easily metabolizes with both first and second phase metabolism (figure 2). The first phase metabolism is mainly reduction, hydroxylation, and oxidization by enzyme activity, whereas the second phase is the addition of a glucuronic acid or sulfate.[15] Without metabolism, it would be impossible to excrete compounds from the body. For a compound to be excreted via the urine it usually has to undergo first and second phase metabolism.[16] The downside to this is that, as steroids have many metabolic pathways it can be hard to recognize the parent compound, even without any sample pretreatment.[13] This needs to be taken into consideration when the sample type is chosen.

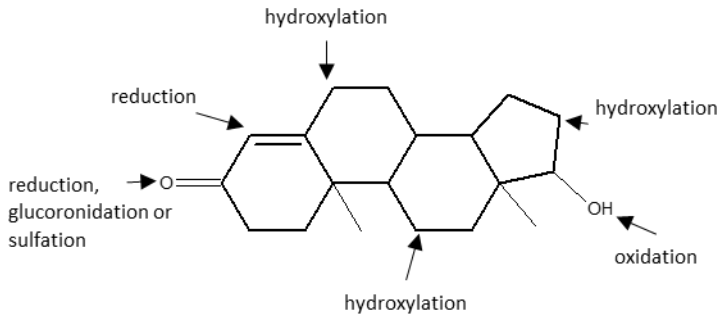

FIGURE 2: THE GENERAL METABOLISM PATHWAYS FOR TESTOSTERONE.[17]

The MS detector is crucial as the steroids are very alike in their structures and are thus hard to separate and identify reliably with other detectors. Especially in metabolite studies, the MS makes it possible to identify compounds for which there are no standards available in the laboratory.[2] With the information provided by the MS, a complete metabolome can be harnessed to find patterns for different diseases, without the knowledge of which all the compounds are.[18]

Immunoassay is a completely different method, which is based on binding an antigen or antibody to the compound. This causes a measurable change, for example by making the compound fluorescent.[19] Immunoassays are sensitive, but are prone to antibody cross reactivity, which gives

false positives.[20] This is especially problematic, when analyzing congenital adrenal hyperplasia in newborn children or other serious diseases.[21]

GC-MS/MS methods are more selective than immunoassays, but also demand more elaborate sample preparation. The samples need to be hydrolyzed and/ or derivatized prior to analysis. Kotronoululas *et al.* utilized the sample preparation procedure in the guidelines of World Anti-Doping Agency (WADA).[22] The sample was first enzymatically hydrolyzed with β-glucuronidase, then incubated for an hour, and further derivatized with N-methyl-N-(trimethylsilyl) trifluoroacetamide. The problem of this is that not all steroid metabolites undergo enzymatic hydrolysis, which can give too low values in doping control for some compounds.[22] The derivatization step and hydrolysis step also take a lot of time compared to the very simple sample preparation methods required before UHPLC analysis. The sample preparation prior to UHPLC can be as simple as dilution, but usually some further preparation is done.

As can be seen from this, it is not easy to study steroids on a general level. The aim to recognize patterns is hard, not only because of the differences from individual to individual – but also since very few samples can be analyzed directly. The sample preparation, the capability of the instrument to separate the compounds, and the data processing are all sources of error.

At its best UHPLC-MS analysis provides a short and simple way to measure selectively and sensitively, with low limits of detection (LODs), metabolites that can be of interest in doping control or as biomarkers of diseases.

3. Theory of ultra-high performance chromatography

The separation mechanisms of LC are based on the separation power of the mobile phase and solid phase partitioning. These are mainly affected by the choice of column and mobile phase(s). The quality of the column for a certain analysis can be estimated by calculation of plate height, resolution, separation factors, and retention factors. The required properties of a column depend on the compounds of interest. It is, however, hard to estimate the separation of different compounds in a column. The separation depends on molecular interactions: dipole-dipole bonding, hydrogen bonding, π-bonding, and acid-base interactions.[2]

The small particle sizes utilized in UHPLC is the result of careful optimization of solid phases based on the theory described by Van Deemter.[23] According to him band broadening is caused by three

mofactors: Eddy diffusion (A), longitudinal diffusion (B), and mass transfer (C). The plate height (H) is utilized for estimation of column efficiency based on these three factors. The mass transfer and longitudinal diffusion are both dependent on the linear mobile phase velocity (u).[23] At low velocities, the longitudinal diffusion is dominant and as the velocity increases its effect is reduced; the compounds just do not have time to diffuse longitudinally. On the other hand, the input of mass transfer grows as the velocity increases.

$$H = A + \frac{B}{u} + Cu \quad (1)$$

The A term is the one that accounts for most of the advantages of UHPLC compared to regular high performance liquid chromatograph (HPLC). As the column packing gets smaller and smaller the paths of the molecules have smaller differences in the routes they travel through the packing. This means that the A term converges towards zero as the particle size (d_p) is reduced, as it is in open tubular columns (equation 2).

$$A = \lambda_g d_p, \quad (2)$$

where λ describes the quality and uniformness of the packing.[24] This means that small uniform packing has real potential. Also, the C variable is reduced as the column packings get smaller, especially for core shell particles, and the compounds cannot diffuse as far into the stationary phase, which reduces C. The effects can clearly be seen in a study done by Szabolks Fekete *et al.* 2013 (figure 3).[3] From the figure it can be seen at which velocities the A, B, and C terms dominate. Interestingly the A factor seem to be the same for all the sub 3 µm column packings.

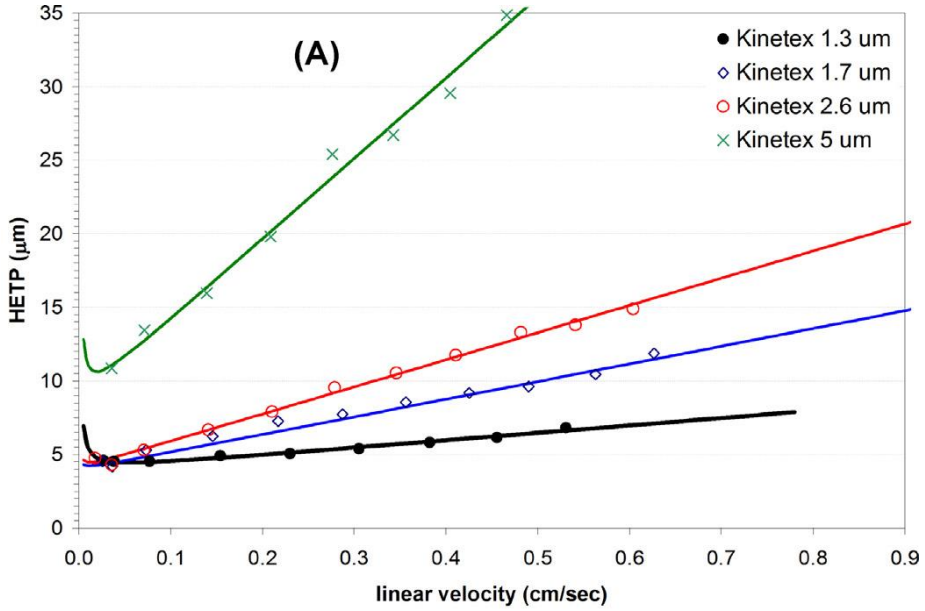

FIGURE 3: PLATE HEIGHT (HETP) OF DIFFERENTLY PACKED COLUMNS. [PUBLISHED WITH PERMISSION FROM ELSEVIER].[3]

The flipside of small packing particle sizes is that they result in high back pressure, which regular HPLC cannot handle. Every time the particle size is reduced by a factor of two the backpressure increases by four, without any change in the other paramters.[5] The pressure is caused by the resistance of the liquid going through the narrow paths between and in the stationary phase. The UHPLC systems can handle pressures up to 1300 bars.[25] This makes it possible to use sub 3 µm particles without reducing the flowrate. Another method to reduce the differences in routes is to use core-shell particles. The solid core reduces the diffusion into the particles, and hence reduces the time a compound needs to equilibrate between the phases.[2] Normally the particles are fully porous.

As the diffusion factors are minimized in the packed particles, very narrow peaks are formed. The resolution describes the separation power of a system. The resolution (R_s) can be expressed by the retention times (t_r) and the peak widths (w_b).[24]

$$R_s = \frac{t_{r1} - t_{r2}}{1/2(w_{b1} + w_{b2})} \quad (3)$$

Narrow peaks are directly connected to higher resolution. Low diffusion also generates a high plate number, as it is also connected to the peak width. The N is another measure on column efficiency (equation 4). The advantage of it is that only one compound is needed for determination of the column efficiency. N can be further converted to the plate height H, which gives the efficiency of a specific column (equation 5).

$$N = 16 \times \left(\frac{t_r}{w_b}\right)^2 \quad (4)$$

$$H = L/N \quad (5)$$

From the equations it can be seen how strongly the peak width affect both the resolution and the plate height. Many factors that play part in the achieved resolution can thus be optimized by minimizing the diffusion. This combined with the large surface area of the small particles makes UHPLC a powerful tool for the separation of compounds.

The resolution is not the only important factor in chromatography, also the sensitivity is of high importance. As the peak width is reduced the peak height is increased, which means that the sensitivity is better than in regular LC. How sensitive the method is, depends a lot on the analyzed compounds and the used detector.

As shown by the topics discussed in this section, UHPLC offers one answer to today's demands. It is more and more important to achieve faster and more sensitive analysis. With the superior pressure withstanding properties of UHPLC, it can be utilized with columns having extremely small packings and of short length. This leads to shorter analysis time without loss in resolution.

4. Parameters and apparatus for ultra-high pressure chromatography

The major components of a liquid chromatograph are several pumps, an injector, a degasser, a detector, and a column. These provide the base for the instrument. The pumps push the mobile phase through the column, the injector introduces the sample to the column, the column separates the components into narrow bands, and lastly the detector gives a response to the compounds present. The degasser does not take part in the analysis directly, but has the important function of removing dissolved gases from the mobile phases. Air bubbles can ruin the column and disturb the detector. There are two types of degassers available on the market: vacuum and helium.[2]

It is critical to have as steady flow as possible. Unsteady flow is not good for the filled columns and creates uneven signals in mass flow detectors. To achieve a steady flow there are often two or more pumps. The idea is when the other pump is filling up, the other one is maintaining the flow. A typical pump in LC systems is the reciprocating pump, but there are also pneumatic pumps and syringe

pumps.[24] The pumps for UHPLC need to be extremely efficient and reliable, as they need to be able to function at extremely high pressures.

There are quite a few parameters that can be adjusted in the UHPLC instrument. The main ones are injection volume, flow rate, choice of column, and temperature. Injection volumes can vary a lot depending on the required LOD. Generally, in biological samples very low LODs are desired. To achieve low LODs injection volumes of even 35 µL can be utilized.[26] To use large injection volumes are not problem free since one can easily overload the detector and column. Normally 2-10 µL injections are utilized in UHPLC applications. The small injection volumes are one of the parameters that are different in HPLC and UHPLC. With smaller columns, also smaller samples are needed. The injection is done by introducing the sample into a rotary sample loop injector, from which the sample is introduced by rotating the valve so that the mobile phase pushes the sample with it.[2]

The flow rate depends on which detector is connected to the LC. If the detector is a MS, lower flow rates need to be used not to overflow the detector, compared to a UV/Vis detector. The reason for this is that all the eluent needs to be vaporized for the detector.[2] In LC-MS flow rates between 0.15-0.45 mL/min are common, whereas in LC-UV/Vis even 2 mL/min can be utilized. In general, less solvent is needed compared to HPLC in total.

In UHPLC short columns are generally utilized. The columns are between 50 – 150 mm long, 2 mm wide and have stationary phases with particles smaller than 3 µm in diameter. The most popular stationary phase is octadecyl carbon chain bonded silica (C18) for reversed phase liquid chromatography (RPLC).[2] It provides good retention for a variety of compounds from food additives to steroids. RPLC is of course not the only operating mode, but is the most widely used mode.[2] In RPLC the stationary phase of the column is non-polar and the mobile phase is polar.

There are different kinds of manufacturing methods for silica.[27] From this follows that C18 columns from different providers can vary in selectivity. The column chosen should have as few free silanol groups as possible. The free silanol groups can adsorb or increase the retention of polar compounds in the analysis. Another reversed phase column which can be utilized for separation of steroids is a penta fluorophenyl column (PFP).[28]

To get repeatable results it is important to have a stable temperature in the column every day. This means that the column temperatures are usually at least five degrees over room temperature. The

change in temperature affects the mobile phase viscosity and the solubility of buffer salts and compounds.[24] The maximum temperature for the column depends on the column.

The mobile phases utilized vary depending on the detector and compounds. In RPLC, the main solvent is aqueous and the added part of organic solvent is considered an organic modifier.[2] The ratio of these can change during the run (gradient run) or stay constant (isocratic run). The amount can be easily changed in modern instruments as they have mixing compartments for combining solvents from several flasks. Gradient runs are often preferred to reduce the band broadening for late eluting compounds. As the amount of the organic modifier grows, the less organic compounds are retained by the column. For non-MS detectors phosphate buffers and acetic acid buffers in combination with acetonitrile, propanol, or methanol are commonly used.[24] In MS applications, very dilute formic acid is added to the solutions, as it can increase signal intensities.[29]

5. Detection modes

Common detectors utilized in UHPLC are UV/Vis, fluorescence (FLD), refractive index (RI), and mass spectrometers (MS). Many factors need to be considered when the detector is chosen: does the study require a universal or selective detector, non-destructive or destructive, how large working range, and sensitivity is needed. Optimally the detector is fast, requires small sample volumes, has minimal noise and give a constant mass to signal ratio. Detectors measure either concentration or mass-flow.[2]

Spectrophotometry is based on the absorption of a specific wavelength. Molecules absorb energy on many levels and everything from the core to the spin of the nuclei absorb at different wavelengths. Electron transitions can be detected in the ultraviolet and visible region of the spectrum and can be quantified. The UV/Vis detectors can be utilized for detecting compounds that have double or triple bonds, or nonbonding electrons. This means in practice that UV/VIS spectrometry is a powerful tool for detection and quantification of organic compounds. As previously mentioned the method is not universal, however in many applications this limitation does not matter and UV/vis spectrometers are widely considered as workhorse detectors.[2]

A typical type of UV/Vis detector is the diode array detector (DAD). The DAD can simultaneously record the complete UV/Vis spectrum and all wavelengths produced by the lamp can be detected simultaneously. The apparatus consists of a light source which is directed towards the sample. After

the light beam, has passed the sample the light is divided into its components by a grating. The grating separates the different wavelengths are to the different diodes. The diodes respond to light. The greatest downside of the detector is the risk of cross reference due to the possibility of other compounds absorbing similarly and having the same retention time as the compound of interest. The FLD is similar to the DAD, but is more selective as compounds need to be fluorescent in order to be detected or undergo derivatization to achieve that. The great advantage of FLD is that it is much more sensitive than UV/Vis due to low background noise levels, which are due to less scattered light.[2]

Mass spectrometers are very useful detectors for LC and they are widely applied in for example pharmaceutical sciences and biochemical applications.[30] MS instruments give more information about the structure than a DAD. The response of the instrument is relative to the amount of compound present. It can thus be utilized for both quantification, qualitative analysis, and identification of compounds. However, it is not trivial to connect LC to MS. UHPLC operate at extremely high pressure and MS do the opposite and mainly operate in vacuum. To be able to combine two so different instruments atmospheric pressure ionization interfaces (API) have been developed. The most common API is the electrospray (ESI) interface.[31] Other common APIs are atmospheric chemical ionization and atmospheric photon ionization.[32]

For one to be able to analyze samples by MS, the ions need to be in gas phase. In ESI the sample is nebulized by a strong electrical field induced by a corona needle (3-5kV) the evaporation of the sample is further enhanced by a warm stream nitrogen gas. As the solvent evaporates the charges on the droplets surface get closer to each other, a field-induced electrohydrodynamic droplet disintegration occurs, which is known as Coulomb explosion.[30] The microdroplets then further disintegrate to gas phase ions. There are two different ways this can happen; 1) by emission from the droplets surface, which is called the ion-evaporation model, or 2) by soft desolvation, the so-called charge-residue model.[31] The two different ionization models are presented in figure 4. ESI is a very soft ionization method, where multiply charged ions are commonly formed.[33]

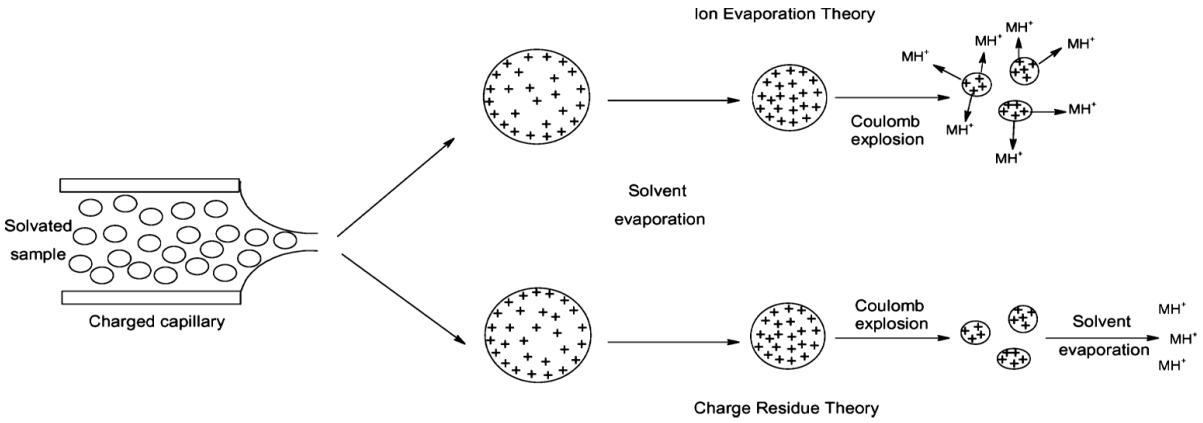

FIGURE 4: THE FORMATION OF IONS DURING ELECTROSPRAY IONIZATION. REPRINTED WITH PERMISSION, COPYRIGHT © 2009 TAYLOR & FRANCIS [33]

In MS ions are separated by their energy, mobility, or velocity. Two of these properties need to be known for the calculation of mass to charge ratio of the ion.[24] There are many types of mass analyzers on the market that can be interfaced with LC. Common mass analyzers are the quadrupole (Q), quadrupole ion trap, time of flight (ToF) and Fourier transform ion cyclotrone resonance.[33] From these the Q and ToF are widely utilized for steroidal samples and will be further discussed in this thesis.

The Q is the most common detector and is often used in tandem mode. Especially in bioanalysis the triple quadrupole (QqQ) is very common.[30] The detector separates ions by the means of an electric field, which varies by time. The electric field is built up from an oscillating radiofrequency and direct currents that induce an alternating current. Only ions with specific mass to charge ratios will be able to pass through this field and reach the detector.[2] This means that masses should be preselected. It is very advantageous to couple Q instruments into QqQ. The major reason for this is that the matrix effects can cause major disturbances and possible co-eluting peaks.[34] There are four different ways to do analysis by QqQ: scanning of parent ions, neutral losses, daughter ions or multiple reaction monitoring.[35] The second Q is commonly utilized as a filter or collision cell, which makes all of the previously mentioned phenomena possible.

The ToF is operationally very different to the Q. In ToF all the ions are accelerated to the same velocity and directed to a high vacuum flight tube, where the ions fly freely. The speed of the ions is relative to the square root of their mass. This means that the heavy molecules will reach the detector last.[24] The ToF has the great advantage that no preselection of target masses is needed and it has better mass resolution giving accurate masses. This means that all data is recorded simultaneously and there are less issues with matrix effects.[34] Compared to the QqQ the ToF has a

smaller dynamic range, but is generally more sensitive.[35] In practice the QqQ is utilized for quantification and target analysis and the ToF for untarget analysis.

Another very common method is a hybrid model, where a quadrupole mass analyzer is combined with a time of flight detector. It has the scanning power of a Q and the resolving power of the ToF. It is very similar to the QqQ, but instead the last quadrupole is replaced by a ToF instrument. The QToF provides high sensitivity, resolution, and mass accuracy for the precursor ions and fragments.[35] The earlier generation QTOFs had issues with low dynamic ranges and were thus poor choices for quantification. In the newer models this have improved to a magnitude of 5 order along with better inter-day and intra-day repeatability. [36]

In steroidal analysis, the need for resolving power is great, which leaves UV/Vis, IR, or FLD out of consideration. The reason for the need of high resolving power is the very similar structures of steroids. In practice this makes MS the detector of choice. The complex matrixes from the human body further requires detectors with even higher resolving power than a single MS, which means that the QqQ and QToF are extremely popular for steroidal analysis. A summary of the detectors is presented in table 1.

TABLE 1: SUMMARY OF TYPICAL DETECTORS FOR UHPLC.

Detector	Advantages	Down sides
DAD	almost universal non-destructive cheap easy to operate fast	risk of cross reference compounds need to absorb UV/Vis region background noise
FLD	non-destructive cheap easy to operate sensitive fast	few compounds are naturally fluorescent
RI	Universal, detects everything that differs from the mobile phase	Not suitable for gradient use, sensitive to temperature changes and flow rate changes
MS	Universal Selective Extremely small risk of cross reference Identification of unknowns	Expensive Hard to operate

6. Sample preparation techniques

Very few samples can be analyzed without any sample preparation. The separation can be either chemical or physical. The sample needs to be in a form that fulfils the requirements of the instrument. In the case of LC, it means that the samples need to be in a liquid form, of high enough concentration, homogenous and without interfering compounds. Traditional selective sample preparation techniques for LC are liquid-liquid extraction (LLE), solid phase extraction (SPE), and QuEChERS (quick, easy, cheap. effective, rugged, and safe). All these methods are suitable for organic compounds as there is no need for strong acids or extremely high temperature and/or pressure in the sample preparation step. In addition to these, the sample preparation often can include filtration, particle removal by centrifugation and concentration or dilution. More invasive methods like dry ashing and combustion or acid dissolution and digestion can sometimes be utilized for inorganic compounds, these however, will not be further discussed in this thesis.

Steroid samples are usually serum or urine samples. From the serum samples the proteins need to be removed prior to LC analysis. This can be done with protein precipitation. A typical precipitation technique is to add an organic solvent for example methanol[10,37,38] or acetonitrile[6,39]. This process denatures the proteins and as they do so they precipitate. The supernatant can then be utilized directly in analysis[6] or can be dried and then reconstituted for analysis[37]. In addition to protein precipitation also LLE or SPE can be utilized.

An optimal sample preparation technique is simple, fast, has great recovery rates, is selective, and utilize small amounts or no toxic solvents. It is not trivial to fulfil all these requirements' and sample preparation is often considered a bottleneck for analysis. The largest errors contributed to a method are caused by sampling and sample preparation.

6.1 Liquid extraction methods

Liquid extraction methods (LE) are utilized for the extraction of organic compounds into a liquid phase. The theoretical basis of the methods lies in the partitioning of the studied compound between two immiscible phases.[2] One of the phases is liquid, as the method name suggests, but the other one can be solid, liquid, or theoretically even a gas. The compound is divided between the two phases depending on polarity and other properties. This phenomenon is described by the distribution coefficient, K_D, and the concentration of a compound A in phase 1 and phase 2 (equation 6).

$$K_D = \frac{[A]_1}{[A]_2} \quad (6)$$

If the two phases are both liquids, the method is called liquid-liquid extraction (LLE). At optimal conditions, the compound is concentrated in the extraction fluid and any interfering components stay in the other phase.[2] The amount of compound in each phase can be adjusted by changing the volume of the extractant, however, it is more efficient to extract several times with new solvents and small amounts.[2] In the case of steroids the other phase is the liquid sample (serum or urine) and the organic solvent is for example dichlormethane[12] or 1-chlorobuthane [40].

The mixing of the phases can be done in many ways. One of the most traditional methods is to put two immiscible liquids into a separation flask and manually shake the bottle and separate the phases. For small volumes LLE can also be carried out in a test-tube. For efficient extraction of large volumes, more elaborate methods have been developed, such as Soxhlet extraction and Accelerated Solvent Extraction (ASE®). In Soxhlet extraction the solvent is circulating to enhance the extraction, and in ASE an elevated pressure of between 70 bar and 140 bar is additionally used.[41]

The trend nowadays is to miniaturize methodologies, and this has also been done with LE. The reason for miniaturization of processes is to use less toxic solvents and save money and time. There are many methods for this: *e.g.,* single-drop microextraction (SDME), hollow fibre liquid-phase microextraction (HF-LPME), and dispersive liquid–liquid microextraction (DLLME).[42] These are all based on extractions that only require minute amounts of solvent.

6.2 Solid phase extraction methods

Solid phase extraction (SPE) is a popular method for quick concentration of compounds and removal of matrix components. It requires only five steps: activation of adsorbent, removal of the activation sorbent, application of sample (sorption), a washing step, and finally elution.[43] Five steps might sound like a lot, but in practice each step is very simple to do and takes little time.

SPE is based on the partitioning of a compound between a liquid phase sample and a solid stationary phase. The compounds must have stronger affinity towards the solid phase than to the liquid phase to be adsorbed. The compounds are then eluted from the solid phase with a solvent towards the compounds have greater affinity than to the solid phase. This is an easy and popular sample preparation technique for steroid samples. The compounds of interest are absorbed into a solid

polymer and the rest is washed out. The compounds are then extracted from the polymer material with a mixture of organic solvent and water, for example MeOH/ water.[18]

The advantage of this method is that even big amounts of liquid sample can be processed and eluted into a small volume quickly. Simultaneously it functions as a sample concentration method. On the other hand, also small sample volumes can be processed. A large number of matrix components are removed and the recovery rates are typically very high. Another great advantage of SPE compared to LLE is that very low amounts of solvents are needed, especially for large volume samples.

The sorbents utilized in SPE are mainly the same types that are utilized in LC as stationary phase materials. The major differences are that in SPE the aim is to have as large volume as possible, which means that more porous materials are better. The phases can also be tuned a bit differently to the stationary phases in columns. The free silanol groups can provide in C18 based SPE materials important interaction sites for polar compounds. This makes the C18 adsorbent almost universal, especially when there is little of the sample.[44]

There are readily different types of solid phases available: cross-linked copolymers, graphitized carbons, and different n-alkylsilicas.[44] The more selective the SPE step, the higher is the sensitivity of the method. The stationary phases are based on polar, non-polar, or ion-exchange interactions. The type of solid phase depends on which types of functional groups are bound to the silica or copolymer and what solvent is utilized.[43] Common bound phases are the same as utilized in LC columns: C18 (non-polar), C8 (non-polar), and cyano (polar).[2] The solid phase is typically placed in cartridges and disks, but there are a lot of other formats on the market as well. The typical particle size is 40-60 µm in particle size distribution (d_p).[44]

SPE can either be utilized online or offline. It can also be utilized for simultaneous derivatization and sample clean-up.[43] SPE is a powerful clean up method with lot of applications.

6.3 Quick, easy, cheap, effective, rugged, and safe sample preparation

QuEChERS (Quick, easy, cheap, effective, rugged, and safe) is, as the name suggests, a simple and effective sample preparation method suitable to a variety of matrixes prior to LC analysis. It was developed in 2003 by Anastassiades *et al.* for the analysis of pesticides in food matrixes.[45] In the method developed by Anastassiades the compounds where first extracted with acetonitrile with

added salts. An aliquot of the supernatant was then treated by dispersive solid phase extraction (dSPE). This means that the sorbent is added to the solution and then mixed. The solid phase utilized for the dSPE varies depending on the application, and in the work by Anastassiades *et al*. a polymer secondary amine (PSA) was utilized. The idea is opposite to SPE; in QuEChERS the interfering matrix is adsorbed to the sorbent and the compounds are left in the supernatant. The process is aided with salts. Salts utilized in QuEChERS can be magnesium sulfate and sodium acetate. These buffer the solution and dries the organic solvent.[2]

QuEChERS is a highly popular technique due to its versatility and it has been utilized in over 700 research papers prior to 2013.[46] It can be applied to any matrix, but the method should be chosen depending on the compounds present in the sample. For example, if the target compounds are very polar they will be adsorbed to the primary and secondary amine (PSA).[2] Some compounds might need a buffered solution if they are easily ionized to be reproducibly extracted.

7. Type of matrixes with steroids

There are plenty of different matrixes in the human body, and basically steroids can be analyzed from anything. The most commonly studied matrixes are urine and blood serum, but also for example tissue, saliva, hair, and nails can be utilized. Upon selecting the matrix several factors need to be considered. Optimally the matrix requires no or only little sample preparation, have high levels of compounds of interest, can be taken non-invasively, and gives trustworthy results.

The selection of the matrix highly affects the amounts of compounds, that can be found and in what time interval. In hair are drugs detectable after weeks. Hair can thus be utilized for the detection of repeated use of anabolic steroids.[47] Urine gives good short-term information of used drugs, but mainly through metabolites as the parent compounds are not efficiently excreted through urine. Serum gives an even shorter time interval compared to hair and urine, since it only reflects the amounts circulating in the blood. Blood serum (and plasma) has the advantage of giving the possibility to reliably analyze the unmodified compounds.[48] Saliva reflects well the circulating levels of free steroids, even better than serum, however some steroids can be present at so low concentration that they are hard to detect.[17]

Depending on the matrix there are different challenges in sample preparation. Most of the matrixes need to be cleaned to remove residues (for example nails and hair), solid particles, and proteins prior to analysis. After this procedure, a clean-up and concentration step is usually taken.

A completely different kind of approach to human matrix is to grow human cells and study the effect of different drugs the produced metabolites. For steroid studies, adrenal gland cells are utilized (H295R).[49] In this way, it is easy to simulate how drugs affect the steroid production.

The great advantage of UHPLC is that the compounds do not need to be derivatized as there is no need to reduce their boiling points. However, the derivatization can sometimes add sensitivity[26].

8. Chemometrics

The data processing can be highly demanding, especially for multidimensional MS applications. To resolve the data, several statistical tools need to be utilized. In metabolomic research one of the main aims is to classify individuals. This can be done considering the complete metabolome by multivariate data analysis.[50]

Principal component analysis (PCA) is the base for multivariate data analysis. With it you can simplify, reduce and model data, detect outliers, select variables, classify, and predict properties based on previously made models. To be able to use PCA only a matrix of data with a specific number of objects (or samples) and a determined number of variables are needed.[51]

The basic idea of PCA is that you find the variables that give the biggest variation in the multidimensional cloud and ignore the dimensions that do not influence much. The cloud is formed by building a unique axis for every variable, which is orthogonal to the previous. Then the length of the vectors in each direction are formed. The vectors that give the least input to the cloud are ignored. This makes it possible to reduce dimensions without losing the essence of the data. When this is done, eigenvectors can be formed. These eigenvectors give the dimensions of the new cloud, which has the most important factors (principal components) of the old cloud.[52] This cloud can be utilized to compare the results from new samples to determine if it is similar. PCA is needed in metabolite studies to be able to form models for urine or serum samples with hundreds of metabolites. Without PCA it would be hard to grasp, what the data shows us and which factors are relevant. There are also other similar methods, for example factor analysis.[52]

Another data handling method is partial least square (PLS) regression. It has the same structure as PCA, but instead of just treating the subjects as two datasets, it maximizes the covariance first to make one of the data sets predict a second set of data. This means that not necessarily the largest principal components are utilized, as their predictive properties can be limited. Orthogonal projections to latent structures (OPLS) combined with PLS makes the data more interpretable, by separating predictive and uncorrelated information.[52] In practice PCA is a good method to detect outliers in multivariate space, whereas OPLS is good for the determination of which factors are the predictive factors in the data.

Data can be categorized to different classes with the help of supervised pattern recognition. To do this, an independent set of data with known parameters need to be available as a training set. The training set could be metabolite data from urine samples from both people with ovary cancer and people, who do not have ovary cancer. With the data two different classes are formed to which uncategorized data is compared. How well the uncategorized data fits into one class can be evaluated by distribution tests.[52] Soft independent modelling of class analogies (SIMCA) is a pattern recognition method that enables the formation classes and the comparison of uncategorized data to given classes with PCA as a basis.[53]

These multivariate models need to be validated. This can be done with receiver operating characteristic (ROC). With ROC true positive and false positive rates can be calculated. Another method for validation is crossvalidation.[52] Crossvalidation needs three sets of data: validation, training and test. From the validation data set each sample is compared to the training set once. The error given by this comparison is a good estimation for the prediction error for new samples.[50]

These methods are extremely powerful and can give too optimistic results. The major problem with these statistical tools is that it is hard to get enough data to give a good base for them. Often there are more variables assessed then there are samples. This makes classification unreliable due to overfitting.[50] Basically, this means that the method works very well for the training set, but does not predict future samples well.

9. Applications

In table 2 all the articles published prior to April 2017 on steroid analysis by UHPLC-MS are listed.

TABLE 2: METHODS USED FOR ANALYSIS OF STEROIDS.

Year	Apparatus	Column	Matrix	Compounds	Sample preparation	Mobile phase	Comments	Ref.
2016	UHPLC-QTOF	C18 (BEH), 150x2.1 mm, 1.7 µm	Urine	Steroid related biomarkers	SPE (Oasis HLB) 96 cartridge wellplate	Methanol/ACN		[18]
2016	UHPLC-QqQ		Liver tissue, serum	Estrogen and estrogen related compounds			Method and other parameters from Gaikwad *et al*. 2013.[13]	[54]
2016	UHPLC-QqQ	C18 (Poroshell), 150x2.1 mm, 2.7 µm	Serum	7 steroids (glucocorticoids, androgens, progesterones, estrogens)	Addition of ethyl acetate, centrifugation derivatization with dansyl chloride	ACN/ 0.1% formic acid	Run time 8 min, injection volume 10 µL, LOD 0.0048-4.6880 ng/mL, column temperature 35 °C.	[55]
2016	UHPLC-QTOF	C18 (BEH), 100x2.1 mm, 1.7 µm	Serum	Metabolite study	Protein precipitation with can	ACN/ water 0.1% formic acid	Flowrate 0.3 mL/min, injection volume 2 µL, runtime 35 min and 100 µL sample utilized.	[56]

Year	Apparatus	Column	Matrix	Compounds	Sample preparation	Mobile phase	Comments	Ref.
2015	UHPLC-QTOF	C18 (BEH), 100×2.1 mm, 3.0 µm	Serum	Metabolite study	LLE can	ACN/ water 0.1% formic acid	100 µL of sample, 5 µL injection and run time 30 min.	[57]
2015	UHPLC-QqQ	C18 (BEH), 100x2.1 mm, 1.7 µm	Urine	Glucuronides	SPE, C18	Water/ MeOH with 0.01% HCOOH and 1 mM NH4COOH	LOD even 2ng/mL and 20 µL injection.	[58]
2015	UHPLC-QTOF	C18 (HSS-T3) 100 mm × 2.1 mm, 1.8 µm)	Urine	Metabolite study	Addition of trichloro acetic acid and centrifugation	ACN/ water with 0.1% formic acid	Utilized 600 µL sample	[59]
2015	UHPLC-QTOF	C18 (BEH), 100x2.1 mm, 1.7 µm	Plasma	Metabolite study	Protein precipitation MeOH	ACN/ water with 0.05% formic acid and NH_4HCO_3+ ACN	Utilized only 75 µL of plasma, 2 µL injection and flowrate 0.14 mL/min.	[10]
2015	UHPLC-QTrap	C18 (Kinetex) 150x3 mm, 2.6 µm	Serum	Testosterone, 5α-dihydrotestosterone, androstenedione,	SPE (HLB), cartridges	MeOH/ water with 0.1% formic acid	Flowrate 0.25 mL/min, 16 min runtime, 10 µL injection and LOD 0.003-0.2 ng/mL.	[60]

Year	Apparatus	Column	Matrix	Compounds	Sample preparation	Mobile phase	Comments	Ref.
				dutasteride and finasteride				
2015	UHPLC-tripleTOF	C18 (Kinetex) 50x2.1 mm, 2.6 µm	Urine	Metabolite study	Protein precipitation MeOH/ water	ACN/ water with 21.70 µmol/l formic acid	Flowrate 0.5 mL/min, 15 min runtime and 3 µL injection.	[61]
2015	UHPLC-tandemQp	C18 (HSS-T3) 100 mm × 2.1 mm, 1.8 µm)	Plasma	Oubain	SPE (MAX)	MeOH/ water	LoQ 1.7 pmol/L, flowrate 0.35 mL/min and runtime 12.5 min.	[62]
2014	UHPLC-QTOF	C18 (BEH), 100x2.1 mm, 1.7 µm	Urine	Steroid metabolite study	SPE (HLB)	Water/ ACN with 0.1% formic acid	Flowrate 0.3 mL/min, injection volume 10 µL	[63]
2014	UHPLC-QTOF	C18 (BEH), 100x2.1 mm, 1.7 µm	Urine	Metabolite study	SPE (HLB)	ACN/ water with 0.1% formic acid	10 µL injection, inhouse made dimensionality reduction.	[64]
2014	UHPLC-Qtrap	C18 Zorbax SB 2.1x50mm, 1.8µm and	Serum	Seven androgen and estrogen related compounds	LLE (1-chlorobutane), derivatization	MeOH/ water (0.2% formic acid)	LOD 1-500 pg/mL, 30 µL injection and utilized 0.5 mL of sample.	[40]

Year	Apparatus	Column	Matrix	Compounds	Sample preparation	Mobile phase	Comments	Ref.
		30x2.1 mm, 2.7 µm			with Dansyl chloride			
2014	UHPLC-HDMS (QTOF)	C18 (BEH), 50x2.1 mm, 1.7 µm	Serum	Metabolite study, quantified 10	Protein precipitation methanol/ ACN/ Acetone and LLE dichlormethane	ACN/ water (0.1% acetic acid)	Utilized machine learning in their data analysis. Progressive flow rate 0.25-0.30-0.45 mL/min.	[12]
2014	UHPLC-HDMS (QTOF)	C18 (HSS-T3) 100 mm × 2.1 mm, 1.8 µm)	Saliva	Metabolite study	Methanol precipitation x 2	ACN/ water with 0.1% formic acid	5 µL injection, flowrate 0.5 mL and runtime 13 min.	[65]
2014	UHPLC-QTOF	C18 (BEH), 100x2.1 mm, 1.7 µm	Venous blood samples	Metabolite study	Blood coagulation promoting gel tubes, MeOH, ACN (2:1) precipitation	ACN/ water with 0.1% formic acid	18 min runtime.	[66]

Year	Apparatus	Column	Matrix	Compounds	Sample preparation	Mobile phase	Comments	Ref.
2013	UHPLC-QTOF	C18 (BEH), 150x2.1 mm, 1.7 µm	Urine	12 conjugated steroids	SPE (HLB)	Water/ ACN with 0.1% formic acid	Flow rate 0.3 mL/min, injection 10 µL, runtime 28 min and LOD 0.5-1 ng/mL.	[36]
2013	UHPLC-LTQ	not mentioned	Plasma	Metabolite study	Methanol precipitation		100 µL of sample utilized.	[67]
2013	UHPLC-TQ	C18 (HSS-T3) 150 mm × 1 mm, 1.8 µm)	Human breast tissue	Metabolite study of 101 exogenous and endogenous steroids.	Precipitation by methanol and water (1:1)	Water/ ACN with 0.1% formic acid	LOD 0.003-15.7 pmol/L, flowrate 0.15 mL/min and runtime 12 min.	[13]
2013	UHPLC-TQS	Kinetex PFP, 100×2.1mm, 2.6 µm	Plasma	Aldosterone and d7-aldosterone	Zinksulphate and methanol precipitation, SPE (HLB)	MeOH/ water	Runtime 5 min, LOD 2.6 & 30 pmol/L. flowrate 0.45 mL/min.	[28]
2013	UHPLC-TQ	C18 (BEH), 150x2.1 mm, 1.7 µm	Urine	Targeted and non-targeted steroids and steroid-glucuronides	SPE (HLB)	50 mM NH_4Ac (pH 4.5) in both water (1:9, v/v) and methanol/ acetonitrile (1:3:6,v/v/v).	LOD 10 pg/mL-5ng/mL, 32 min runtime, 0.13 mL/min, 10 µL injection	[68]

Year	Apparatus	Column	Matrix	Compounds	Sample preparation	Mobile phase	Comments	Ref.
							and 50 °C column temperature.	
2013	UHPLC-QTOF	C18 (HSS-T3) 50 mm × 2.1 mm, 1.8 µm)	Urine	Deglucuronidated and desulfated steroids	Particle removal with centrifuge and dilution to 1/5.	MeOH/ water with 0.1% formic acid		[69]
2013	UHPLC-TQ	C18 (BEH), 100x2.1 mm, 1.7 µm	Plasma	Eight adrenal C-21 steroids	SPE (MAX)	ACN/ water with 0.1% formic acid	Runtime 5 min, 0.1 mL of sample, 20 µL injection, LOD 0.2 ng/mL and 50 °C column temperature.	[70]
2013	UHPLC-QTOF	C18 (BEH), 50x2.1 mm, 1.7 µm	Blood samples	Metabolite study	Serum separator tube, precipitation with Methanol	ACN/ water with 0.1% formic acid	2 µL injection and 0.35 mL/min flowrate.	[9]

Year	Apparatus	Column	Matrix	Compounds	Sample preparation	Mobile phase	Comments	Ref.
2013	UHPLC-QTOF/ UHPLC-QqQTOF	C18 (BEH), 100x2.1 mm, 1.7 µm	Urine and plasma	Metabolite study	Precipitation with methanol	ACN/ 0.1 % formic acid		[71]
2013	UHPLC-QTOF/ UHPLC-QqQTOF	C18 (BEH), 50x2.1 mm, 1.7 µm	Urine and blood	Metabolite study	Anticoagulation (sodium citrate), ACN precipitation	Supplementary data ACN/ 0.1 % formic acid	5 µL injection.	[39]
2012	UHPLC-QTOF	C18 (BEH), 100x2.1 mm, 1.7 µm	Plasma, liver tissue	Bile acids	Methanol precipitation	ACN/ Water with 0.1 % formic acid	Needs only 5 mg of tissue and 25 µL plasma, 4 µL injection, LoQ 2.5-20 nM, runtime 21 min and column temperature 65 °C.	[72]
2012	UHPLC-tandemQ (Waters Quattro	C18 (BEH), 100x2.1 mm, 1.7 µm	Blister fluids, plasma	Testosterone, dehydroepiandrosterone,	SPE (MAX)	ACN/ water with 0.1 % formic acid	Measures local hormone levels from the skin. LOD 0.1-0.5 nmol/L	[73]

Year	Apparatus	Column	Matrix	Compounds	Sample preparation	Mobile phase	Comments	Ref.
	Premier XE)			androstenedione and testosterone				
2012	UHPLC-orthogonal accelerea-tion TOF	C18 (BEH), 100x2.1 mm, 1.7 µm	Urine	Metabolite study	Methanol precipitation, dilution (1:3)	ACN/ water with 0.1 % formic acid	Runtime 23 min and a flowrate of 0.4 mL/min.	[74]
2012	UHPLC-TQ	C18 (HSS-SB) 100 mm × 2.1 mm, 1.8 µm)	Plasma	Ethylestradiol	LLE (hexane/ ethyl acetate), derivatization (dansyl chloride) and SPE (MCX)	Water/ ACN: isopropanol (80:20) with 0.1 % formic acid	A LOD of 1 pg/mL for ethylestradiol, 35 µL injection, flow rate 0.4 mL/min.	[26]
2011	UHPLC-UV	C18 (BEH), 50x2.1 mm, 1.7 µm	Urine	Cortisol, cortisone and 6β-hydroxycortisol	SPE (HLB)	MeOH/ water with 0.1 % formic acid	7 min runtime, 20 µL injection, LOD 3-5 ng/mL	[75]

Year	Apparatus	Column	Matrix	Compounds	Sample preparation	Mobile phase	Comments	Ref.
2011	UHPLC-QTOF	C18 (BEH), 150x2.1 mm, 1.7 µm	Urine	Eleven endogenous sulfo- or glucuroconjugated steroids	SPE (HLB)	ACN/ water with 0.1 % formic acid	28 min runtime, column temperature 25 °C, 20 µL injection, LOD 1-5 ng/mL.	[76]
2011	UHPLC-tandemQ (Waters Quattro Premier XE)	C18 (BEH), 100x2.1 mm, 1.7 µm	Nails	Cortisol, cortisone, dehydroepiandrosterone (DHEA) and DHEA sulfate	Incubation in Sorensen buffer, SPE (HLB)	ACN/ 0.1% formic acid	10 µL injection, 30 °C, runtime 9.5 min, flowrate 0.4 mL/min, LOD 5-20 pg/mg.	[77]
2011	UHPLC-QTOF	C18 (BEH), 150x2.1 mm, 1.7 µm	Urine	Testosterone, epitestosterone, androsterone, etiocholanolone, dehydroepiandrosterone, 5α- or 5β-androstane-	SPE (HLB)	ACN/ water with 0.1 % formic acid	Done with the same method as Badoud 2011.[76]	[78]

Year	Apparatus	Column	Matrix	Compounds	Sample preparation	Mobile phase	Comments	Ref.
				3α,17β-diol and dihydrotestosterone				
2011	UHPLC-QTOF/ QqQ	C18 (BEH), 100x2.1 mm, 1.7 µm	Urine	SARM-S4 and metabolites	LLE (TBME and $NaHCO_3$+Na_2CO_3) or LLE (ethyl acetate Na_2CO_3)	ACN/ water with 0.1 % formic acid	First study to find SARM-S4 utilized as doping, flow rate 0.4 mL/min and injection volume 10 µL.	[79]
2011	UHPLC-tandemQ (XEVO)	C18 (BEH), 50x2.1 mm, 1.7 µm	Dried blood spots	17α-hydroxyprogesterone, 21-deoxycortisol, 11-deoxycortisol, 11-deoxycorticosterone and cortisol	LE (ACN:Acetone)	ACN/ water with 0.1 % formic acid	Run time 1.25 min, flow rate 0.6 mL/min, injection volume 7.5 µL, column temperature 40 °C and LOD 0.9-1.3 nmol/L.	[21]
2011	UHPLC-IM (ion mobility ms)	C18 (BEH), 100 (/50)x2.1 mm, 1.7 µm	Urine	Testosterone and epitestosterone glucuronide epimers	LLE (ethyl acetate)	Water/ methanol with 1mM ammonium acetate and 0.1 % formic acid	LOD 99 ng/mL testosterone, 98 ng/mL eptestosterone, flow rate 0.75 mL/min,	[80]

Year	Apparatus	Column	Matrix	Compounds	Sample preparation	Mobile phase	Comments	Ref.
							column temperature 40 °C, injection volume 20 µL.	
2010	UHPLC-Quattro Primer tandem mass spectrometer	C18 (BEH), 50x2.1 mm, 1.7 µm	Plasma	Anandamide, ovarian sex steroids and gonadotrophins	Precipitation (acetone) + LLE (MeOH/Chloroform)	2mM ammonium acetate/ ACN with 0.1 % formic acid	7 µL injection.	[81]
2010	UHPLC-Quattro Primer tandem mass spectrometer	C18 (HSS-T3) 50 mm × 2.1 mm, 1.8 µm)	Plasma	Cortisol, 21-deoxycortisol, 11-deoxycortisol, 4-androstene-3,17-dione and 17-hydroxyprogesterone	LLE (MTBE)	Water/ methanol with 1mM ammonium acetate and 0.1 % formic acid	50 µL of serum, with 20 µL injection, column temperature 60 °C, 0.6 mL/min flow rate. Run time 5 min.	[82]

Year	Apparatus	Column	Matrix	Compounds	Sample preparation	Mobile phase	Comments	Ref.
2009	UHPLC-QqQ	C18 (BEH), 100x2.1 mm, 1.7 µm	Urine	Screening of diuretics, beta-blockers, selected stimulants and steroids	SPE (Varian Nexus)	MeOH/ 0.1 % formic acid	2 µL injection, 0.5 mL/min flow rate, LODs 7-15.8 ng/mL and column temperature 45 °C.	[83]
2009	UHPLC QTOF/ToF /QqQ	C18 (BEH), 50x2.1 mm, 1.7 µm	Urine	Stanozolol metabolites	LLE (diethylether+ $NaHCO_3$+Na_2CO_3) or SPE (MCX)	Water/ MeOH with ammonium acetate (1mM) and 0.001% acetic acid	Flow rate 0.3 mL/, injection 20 µL and LODs 3ng-10ng/mL.	[84]
2009	UHPLC-Quattro Primer tandem mass spectrometer	C18 (BEH), 50x2.1 mm, 1.7 µm	Dried blood spots	Steroid profiling	LE 80% ACN and 20% water	MeOH/ 0.05 % formic acid	14 µL injection, flow rate0.6 mL/min	[20]

Year	Apparatus	Column	Matrix	Compounds	Sample preparation	Mobile phase	Comments	Ref.
2008	UHPLC-TQ	C18 (BEH), 100x2.1 mm, 1.7 µm	Serum	Testosterone and 5α-dihydrotestosterone	LLE (MTBE), derivatization with 2,3-pyridinedicarboxylic anhydride, SPE (HLB)	Water/ 2mM ammonium acetate in acetonitrile:water (98/2).	Column temperature 50 °C, flow rate 0.5 mL/min, 5 µL injection.	[85]
2007	UHPLC-MS	C18 (BEH), 50x2.1 mm, 1.7 µm	Urine	Corticosteroids	LLE (Diethylether+ $NaSO_4$), enzymatic deconjugation	ACN/ water with 0.1 % formic acid	Analysis time 5.5 min, LOD (calf urine): 0.1-3.3 µg/L	[86]

Year	Appara tus	Column	Cells	CompoundCompounds	Sample prep-aration	Eluents	Comments	Ref

2015	UHPLC-QTOF	C18 (BEH) 150x2.1 mm, 1.7µm	H295R	Metabolite study, quantification of 11 steroids	Protein precipitation (MeOH, ZnSO4), SPE (HLB)	Water/ MeCN with 0.1 % formic acid	flowrate 0.06 mL/min	[49]
2014	UHPLC-QqQ	Kinetex PFP (2.1×100 mm, 2.6 µm) /C18 (BEH) 50x2.1 mm, 1.7µm	H295R/ COS-1	Metabolite study	LLE (dichloromethane)	1 % formic acid/ 49 %:49 %:2 % methanol:acetonitrile: isopropanol	Analysis method from previous paper	[87]
2012	UHPLC-TOF	C18 (BEH) 150x2.1 mm, 1.7µm	H295R	Nine natural steroids	Addition of sodium acetate, SPE (Phenomenex Strata x)	water/ ACN with 20 mM formic acid	Metabolite study of drugs that affect the steroidogenesis, 25 µL injection	[88]
2012	UHPLC-QqQ	Kinetex PFP (2.1×100 mm, 2.6 µm) / BEH (2.1x50 mm, 1.7 µm)	H295R	Steroid metabolite study and quantification	LLE (dichloromethane)	1 % formic acid/ 49 %:49 %:2 % MeOH:ACN: isopropanol or 1 % formic acid/can	flow rate 0.4 mL/min	[89]

As a summary of the reviewed articles steroids are of interest in metabolite studies and in doping assays. UHPLC is always combined with a MS instrument for steroid analysis. The MS utilized is usually of multi-dimension: QTOF or QqQ. The instrumentation is suitable for both untargeted and targeted analysis. In untargeted studies the study of changes in the human metabolome has been especially interesting. The articles with targeted studies are usually focused on doping control.

It has been found that diseases make small changes in the human metabolome. These differences can be detected to diagnose diseases earlier or with higher confidence level. The diseases studied within the frame of this work are congenital adrenal hyperplasia[20,21,82], chronic liver disease[90], polycystic ovary syndrome[91], isolated post-challenge diabetes[9], Alzheimer's disease[10], post hepatitis B cirrhosis[74], coronary artery disease[6], prostate cancer[12], primary liver cancer[11] and B-cell non-Hodgkin's lymphoma.[66] Metabolome studies are not only restricted to diseases. They can also be utilized for changes in the metabolome caused by dioxin exposure markers[18,64], menstrual cycle[81], pregnancy[68], sleep restriction[67], to support testosterone replacement therapy for hypogonadism[85], and for assessing drug response[26,60]. Metabolomics can be an efficient tool to detect diseases, even at early stages, but also to learn more about the processes in the human body.

In antidoping related studies the aim has been to profile and determine the metabolism and metabolites for drugs[63,79,80,84], find biomarkers and fragments of drugs[78,92], and to determine reliably prohibited compounds from different matrixes[17,22,83,86]. It is important to have good understanding of the metabolites and drug behavior in the body to be able to detect doping in individuals. If the metabolome of an individual is known, it is easier to find differences and hence detect forbidden drug use.

In the cell studies made by UHPLC-MS, the human H295R adrenocarcinoma cell line is utilized. The cells show how chemicals affect steroidogenesis. Tonoli *et al.* showed that untargeted steroidal footprinting can be done, by studying the effects of triclorcarban on H295R cells.[49] Rijk *et al*. studied the effect of 10 compounds that are known to affect stereoidogenesis.[88] Lastly Schloms *et al*. studied the effect of rooibos on the cells.[87,89]

UHPLC-MS/MS usually provides reliable results with fast analysis time and without complicated sample preparation. As seen from table 1, typically the sample preparation processes include only protein precipitation, LLE, or SPE. The most common SPE cartridge is

the Oasis® HLB cartridge. It was used in 11 out of 19 cases. For protein precipitation, the most common solvent is methanol (12 out of 15 cases). In liquid extraction or LLE a variety of solvents are utilized. In most of the cases the sample was concentrated by drying and then reconstituting in a suitable solvent. Basically, all the matrixes need to be pre-treated prior to analysis. This is not only because the MS instrument is very sensitive, but also due to the very narrow bore columns, which can easily get clogged. For urine samples SPE is the most utilized sample preparation method, for serum on the other hand LLE is the most typical method. Derivatization can be utilized to increase sensitivity and dansyl chloride is by far the most utilized derivatization reagent for steroids. However, in some cases the sample can be injected after just addition of solvent and centrifugation.[11,93]

Sample preparation methods can also be combined (LLE, SPE and derivatization) to achieve cleaner samples and hence lower detection limits, even as low as 1pg/mL[26]. However, there are studies that achieved such low detection limits with less sample preparation[40]. It is questionable if it is advisable to have complicated sample preparation techniques for methods aimed for everyday use. An optimal sample preparation method that most studies aim to achieve is fast, robust, and simple. These requirements are easily fulfilled with UHPLC based methods. Many of them are true options for immunoassay or GC-MS/MS methods utilized in doping or healthcare related analysis.

The columns utilized in the articles reviewed are mostly C18 columns having the lengths of 50, 100, or 150 mm. The stationary phase particle size is sub 3 µm (1.7, 1.8, 2.6, 2.7, or 3 µm). The most popular column is the BEH (ethylene bridged hybrid) 100 x 2.1 mm with a 1.7 µm particle size by Waters. The typical mobile phase utilized is acetonitrile and water with 0.1 % of formic acid. The injection volumes are from 2 µL[6] up to even as large volumes as 35 µL[26], typically the injections are 10 or 20 µL. With larges injection volumes of sample, lower LODs can be achieved. Typically, the LODs in these studies are in the size of ng/mL[11,22,36,40,68,70,72], but quite a few of them reached even pg/mL levels[28,40,60,62,68]. Hinchliffe *et al.* managed to get as low LODs as 26 pmol/L.[28]

Surprisingly low flowrates were utilized in these studies. These flowrates were as low as 0.14 mL/min[10] and the highest flowrate was 0.6 mL/min.[20] The reason for the low flowrates are probably restrictions by the MS instruments. The low flowrates make the analyses very long for an UHPLC analysis. Many of the steroid studies have closer to or even over 30 min

runtimes.[6,68,71,76,91] On the other hand, there are many applications with short runtimes of only 5 minutes[28,70,80,82]. The column temperatures are typically 30 to 45 °C, but even as high as 65 °C has been utilized.[72]

In applications, the QqQ instrument is widely utilized for the quantification of target compounds and QTOF and TOF are widely utilized for the identification of compounds. Often both QTOF and QqQ are utilized to get the full power of the MS instruments. For example, Su *et al*. studied women with painful periods.[39] First they processed the data with MassLynx V4.1 and MarkerLynx software, after which they proceeded to do a multivariate analysis with a software called EZinfo. From this they proceeded to check the results with unsupervised PCA analysis, PLS and OPLS and determined the potential biomarkers with these. Lastly, they quantified the biomarkers with UHPLC-QqQ.[39] As can be seen from this, metabolomic studies need a lot of data handling, and not even all the steps are mentioned here. The data handling procedure described here is what most of the UHPLC-QTOF metabolic studies utilize. In steroid applications, it is highly interesting to do untargeted analysis to find a target compound to further analyze.

II. EXPERIMENTAL SECTION

Validation of food additives using ultra high pressure liquid chromatography

1. Background

The Finnish Customs laboratory monitors a variety of products of non-animal origin to ensure that they meet EU standards. There are EU regulations for the labeling of products and upper limits for the allowed concentration of a certain additive. The allowed levels are given in the regulation N:o 1333/2008 appendix II.

Typical matrixes for the Customs laboratory are different beverages, candy, jams, and snacks. In these are screened preservatives, sweeteners and caffeine, among others. It is extremely important that the labelling of the product is correct to ensure consumer safety. The aim of this project was to increase the efficiency of the analysis of soft beverages by combining three different methods into one.

It is very attractive to combine several analyses into one, this saves time in sample preparation, analysis time, and documentation. Especially since some products must be screened for several compounds. An example of this could be an energy drink, which can contain caffeine, preservatives, and sweeteners. The energy drink would earlier have been analyzed with three separate methods.

The Finnish customs acquired a brand new UHPLC, for which the new method was developed. In the new method, the compounds acesulfame potassium (As-K), saccharin (Sac), aspartame (Asp), caffeine (Caf), sorbic acid (SA), and benzoic acid (BA) were analyzed, with only minimum amount of sample preparation and an analysis time of less than two minutes.

Agilent has several application notes for analysis of foodstuff. At least two of these applications had all the compounds of interest separated.[94,95] They were selected for the basis of this method. The major difference between the application notes was that the first one utilized ammonium acetate buffer and the other phosphate buffer. These buffers were compared and the parameters were tuned to achieve excellent separation. Originally the acetate buffer was preferred due to the possibility to change the detector to a mass spectrometer. Different flowrates, column temperatures, injection volumes, and gradient compositions were evaluated and optimized.

The method was lastly validated partially. The within laboratory repeatability, robustness, and measurement uncertainty were calculated. The result is a fully functioning simple method for the analysis of additives in beverages.

2. Instrumentation

The apparatus was bought from Agilent Technologies. The instrument was an Agilent infinity II 1290 UHPLC system, which was combined with a fluorescence and DAD detector. It has two dual piston pumps with integrated degasser, mixing unit, rotary injector with loop (10 µL), column oven, and the detectors. The two dual piston pumps can pump high pressures and cause only minimal noise.

Only the DAD detector was utilized in the experiments, as all the selected compounds absorb light in the ultraviolet region. A column with a C18 stationary phase was utilized, which is considered an all-around column. The model of the column was Zorbax Eclipse Plus C18 Rapid Resolution HD 2.1x50 mm, with a particle size of 1.8 µm. The column was provided with the instrument, but proved fit for purpose.

The instrument is equipped with the software Chemstation. Chemstation was utilized for all the data handling.

3. Chemicals

Chemicals utilized in this project consist of the compounds, solvents, rinsing solution for the needle and water. The compounds of interest were all solids and stored per manufacturers' instructions.

Aspartame and sorbic acid were stored in a refrigerator in a desiccator. The other solids were stored at room temperature protected from humidity. Most of the solvents utilized were LC-MS purity and if not the solution was filtered through a 0.2 µL filter. The buffers were prepared from solid ammonium acetate or concentrated phosphoric acid. The pH was adjusted with acetic acid (concentrated), for the ammonium acetate buffer and ammonium (about 25 %) for the phosphate buffer. A 50% ACN-water solution was prepared for needle wash with LC-MS purity ACN and Millipore water. All the water utilized was ion changed and filtered with Millipore systems combined with a 0.2 µm filter.

All the chemicals used for the standards are presented in table 3 with their manufacturers.

TABLE 3: LIST OF UTILIZED CHEMICALS WITH MANUFACTURERS.

Chemical	**Producer**
Acesulfame K	Fluka
Aspartame, L-Aspartyl-L-phenylalanine methyl ester (98%)	Acros organics
Benzoic acid	Merck
Saccharin	Sigma-Aldrich
Sorbic acid	Sigma-Aldrich
Caffeine	Sigma-Aldrich
Vanillin	Sigma-Aldrich
Ethyl-vanillin	Sigma-Aldrich

4. Preparation of solutions

4.1. Standard preparation

The comparison standards were prepared by diluting 100 mg of the compound into a 100-mL measuring flask to the final concentration of 1000 µg/mL. Sorbic acid does not dissolve in water and hence it was always prepared with a minimum of 25 mL methanol with the addition of water to accurate volume. For determination of the retention time for the compounds and for the creation of a spectral library a 500 µg/mL solution for each compound separately was prepared. In other cases, a mixture was prepared of all compounds was made.

Vanillin standards were prepared separately for analyses of possible disturbance. They were made into 1000 µg/mL standard solution with 50 % methanol-water as solvent. The typical stock solution prepared was 1000 µg/mL mixture in 50 % methanol-water.

4.2. Eluent preparation

A fresh eluent was prepared weekly. The 20 mM phosphate solution was prepared by adding 1 mL of 85 % H_3PO_4 into approximately 800 mL of LC-MS purity water, followed by pH

adjustment to 4.0 by addition of 25 % ammonium solution. About 1.1 mL of ammonium solution was needed. Lastly the solution was diluted quantitatively to 1 L and filtered.

The 20 mM ammonium acetate buffer was prepared by dissolving about 1.5 g of ammonium acetate into 800 mL of LC-MS quality water, the pH was adjusted with about 0.85 mL of concentrated acetic acid to a pH of approximately 4.8. Also, a 10 mM ammonium acetate buffer was made with 0.77 g of ammonium acetate and approximately 0.425 mL of the acetic acid.

4.3. Samples and sample preparation

Some samples were bought from a local super market to try out the method. Typical samples were chosen: cola drinks, energy drinks, jams, and juice concentrate. These were prepared like any other sample. From the drinks, sweeteners, preservatives and caffeine – almost all the compounds of interest – could be found.

The samples were prepared by sonicating them for about 15 min to remove carbon dioxide and then diluting them 1:5 or 1:10 in a 30% methanol-water mixture or only in water, lastly filtering them. The filter types and sonicator are listed in table 4.

TABLE 4: FILTERS AND THE SONICATOR UTILIZED IN THE PROJECT.

Filter (for eluent)	Thermo Scientific, membrane filter, non-sterile, nylon, 0.2 µm, 47mm.
Filter (for others)	Life Sciences, GHP Acrodisc 13 mm Syringe Filter, 0.2 µm GHP membrane
Sonicator	GWB, B220, 45 kHz

The samples used in this study are Vanilla Coca-Cola, Coca-Cola, Redbull and Redbull sugarfree, FunLight Orange, and apple-pear concentrate along with the jams pirkka strawberry and smashed apples. The juice concentrates were first prepared according to instructions on the package. These are typical samples analyzed by the customs laboratory, with typical concentrations of additives. The more specific contents are listed in table 5 for the beverages. In the beverages, there is not much that should cause issues in the analysis after removal of possible gas bubbles. The biggest issues can be caused by sugar and possibly low amounts of the additives.

For the customs laboratory, it is important to know whether the compound is below a certain maximum, which has been set by the European Union, or in the case of caffeine if there are correct markings if the product has high amount of caffeine. Most of the chosen samples have caffeine and none of the samples have all the compounds of interest.

The allowed levels for the different additives depends on what type of product it is and for who it is aimed. In beverages, a warning label is required if the product contains more than 150 mg/L Caf.[96] In flavored drinks the limits for Asp is 600 mg/L, As-K 350 mg/L, Sac 80 mg/L, sorbate/ SA 300 mg/L.[97] From this can be seen that the allowed levels are quite high and thus the method does not have to be very sensitive.

TABLE 5: CONTENTS OF THE UTILIZED SAMPLES PER THE CONTENTS LISTED IN THE PACKAGE SPRING 2016.

Product	Contents
Coca-Cola	water, sugar, carbon dioxide, E150d, E338, natural aromas (incl. caffeine), plant extract.
Vanilla Coca-Cola	water, sugar, carbon dioxide, E150d, E338, natural aromas (incl. caffeine), plant extract.
Redbull	Water, saccharide, glucose, E330, carbon dioxide, taurine (0.4%), E500, E504, caffeine (0.03%), vitamins (niacin, pantone acid, B6, B12), aromas, E150a, E101
Redbull sugarfree	Water, E330, carbon dioxide, taurine (0.4%), E500, E504, sweeteners E950, E951, caffeine (0.03%), vitamins (niacin, pantone acid, B6, B12), aromas, E415, E150a, E101
Fun Light, orange	Water, apple acid, sodium citrate, arabicum, xantangum, E444, aspartame, acesulfame-K, beta-carotene, ascorbic acid, potassiumsorbate.
Pirkka Sokeriton omenan ja päärynän makuinen juomatiiviste	Water, pear juice from concentrate, apple juice from concentrate, citric acid, aromas, aspartame, acesulfame-K, sodium benzoate, potassium sorbate, ascorbic acid, E150d.

The supermarket provided a valuable starting point to see if there are some immediate issues with the method. The peaks seemed to be well resolved for all the samples and the

concentrations for the contents possible. After this, the Food Analysis Performance Assessment Scheme (Fapas) samples were analyzed. Fapas provides samples for laboratories for proficiency testing. The same samples are sold as reference materials. The reference materials come with assigned values and uncertainty determined from the proficiency test results.

The Fapas samples were utilized for estimation of bias and trueness. The contents of the samples are listed in table 6. Fapas samples should give quite a good approximate of how much the sample contains a specific compound before the expiration date. Additionally, some even older Fapas samples were analyzed, the results were however unusable. In the old samples, it could be clearly seen that aspartame had degraded. The levels of the other compounds did not seem to change significantly within one year. The samples were either of cola or tonic water type.

To get a realistic estimation of the noise, Freeway pineapple soda was utilized. This takes into account possible variation caused by the sugar contents. It has none of the compounds of interest, and no peaks could be seen.

TABLE 6: CONTENTS OF FAPAS SAMPLES.

FAPAS sample number	As-K (assigned value/ confidence range)	Sac (assigned value/ confidence range)	Caf (assigned value/ confidence range)	Asp (assigned value/ confidence range)	BH (assigned value/ confidence range)	SH (assigned value/ confidence range)	Applicability date
T03124QC, Cola		22.3/ 17.8-26.8 mg/L			127/ 107-146 mg/L	140/ 118-161 mg/L	29 Aug 2016
T03114QC, Tonic	174/ 148-199 mg/mL			356/ 309-403 mg/mL	115/ 97-133 mg/mL		21 Feb 2015
T03115QC, Tonic	85.1/ 71.2-99.1 mg/L	35.2/ 28.6-41.7 mg/mL	93.9/ 78.8-109.1 mg/mL		140/ 118-161 mg/mL		14 Jun 2015
T03122QC, Cola	53.2/ 43.9-62.6 mg/L	53.7/ 44.2-63.1 mg/L	97.6/ 81.9-113.3 mg/L		115.4/97.3-133.5 mg/mL		5 Jun 2016

5. Method optimization

5.1.Selection of buffer

Agilent has a few application notes with analyses of sweeteners and conserving agents. There was however only two type of buffers, acetate and phosphate. The acetate buffer is based on a mixture of ammonium acetate salt, with a buffering range (3.76-5.76).[24]The phosphate buffer on phosphoric acid, which buffers from 1.14 to 3.14 cannot be utilized with an MS instrument.

A gradient 10% - 30% ACN in 1.40 min was utilized with acetate buffer and a flowrate of 1 mL/min and column temperature 30 °C. All the compounds were well separated. However, the fact that acetate buffer absorbs at 210 nm, causes a baseline drift as the gradient changes. In an isocratic run the problems with this would not be as dominant. In figure 5 the issue can be seen; the base line drifts a lot with a change of the gradient and all the peaks tail. Additionally, there is a lot of background noise in the beginning of the analysis caused by the injection. For a 50 µg/mL solution the baseline drift is not very significant. This is, however not the case for a 5 µg/mL solution.

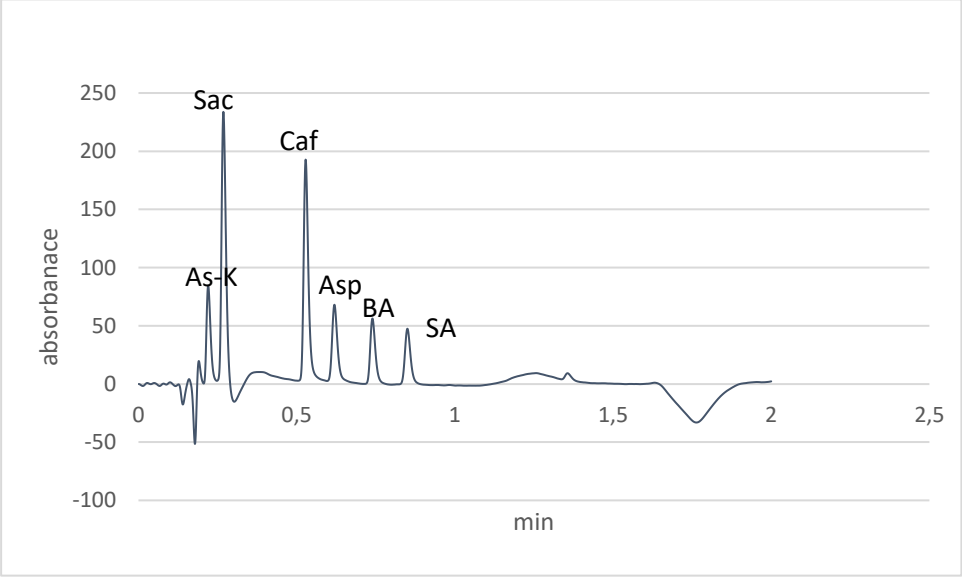

FIGURE 5: UHPLC SEPARATION OF A STANDARD SAMPLE OF 50 µG/ML CONCENTRATION. THE SAMPLE COMPONENTS WERE ACESULFAME-K (AS-K), SACCHARINE (SAC), CAFFEINE (CAF)ASPARTAME (ASP), BENZOIC ACID (BA) AND SORBIC ACID (SA), MOBILE PHASE: ACETATE BUFFER (PH 4.8) AND ACN, UV-DETECTION AT 210 NM, INJECTION VOLUME 2 µL, TEMPERATURE 30 °C.

The baseline drift could be reduced by addition of acetate to the acetonitrile solution. The issues with injection could be reduced somewhat by preparing standards into buffer solution.

As there is only a little retention for As-K, it elutes almost at the same time as the rest of the solution injected. This made it hard to get reproducible results. This combined with the extra work with having acetate in every solution made the phosphate buffer as the eluent of choice, even though the optimal pH was not within the buffering range.

5.2. Selection of pH for the phosphate buffer

The pH optimization was made for the phosphate solution. The method is quite pH dependent, which is problematic as the optimal separation is not within the buffering range of the phosphate solution (1.14 to 3.14; $pK_a \approx 2.1$).[24] In Agilent application note a pH of 3.65 was utilized. This did not give good enough separation in our analysis (figure 7). To further enhance the separation of sorbic acid and benzoic acid, a pH of 4 was chosen (figure 8).[95] There is no major changes in the retention times within the pH range 4.00 - 4.10. With a pH of 4.92 aspartame eluted last and was not completely separated from sorbic acid (figure 9). As the pH is lowered to 3.19 there is no separation of sorbic acid and benzoic acid (figure 10). A pH change from 3 to 5 only caused the retention times of benzoic acid and sorbic acid to change.

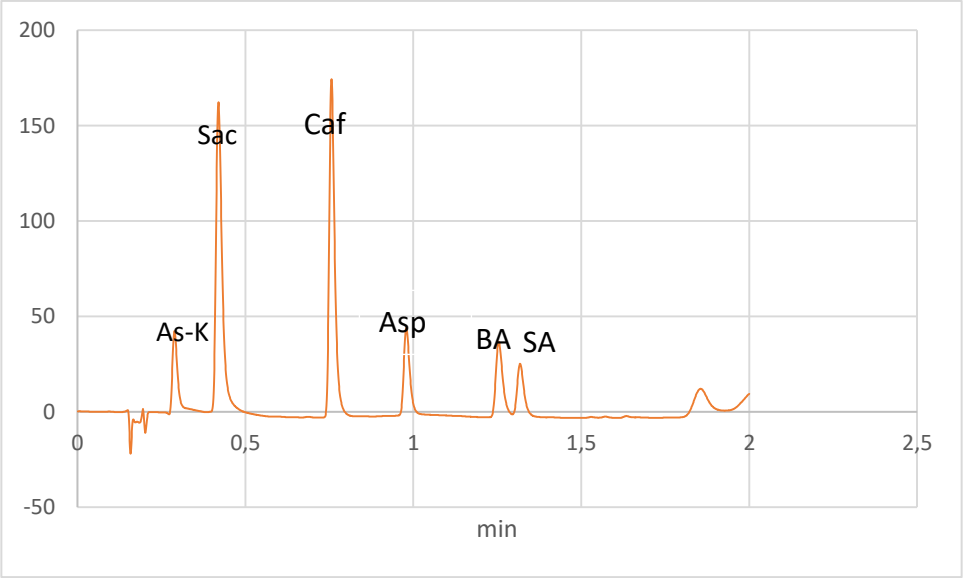

FIGURE 6: UHPLC SEPARATION OF A STANDARD SAMPLE OF 25 µG/ML CONCENTRATION. THE SAMPLE COMPONENTS WERE ACESULFAME-K (AS-K), SACCHARINE (SAC), CAFFEINE (CAF)ASPARTAME (ASP), BENZOIC ACID (BA) AND SORBIC ACID (SA), MOBILE PHASE: PHOSPHATE BUFFER (PH 3.65) AND ACN, UV-DETECTION AT 210 NM, INJECTION VOLUME 2 µL, TEMPERATURE 35 °C.

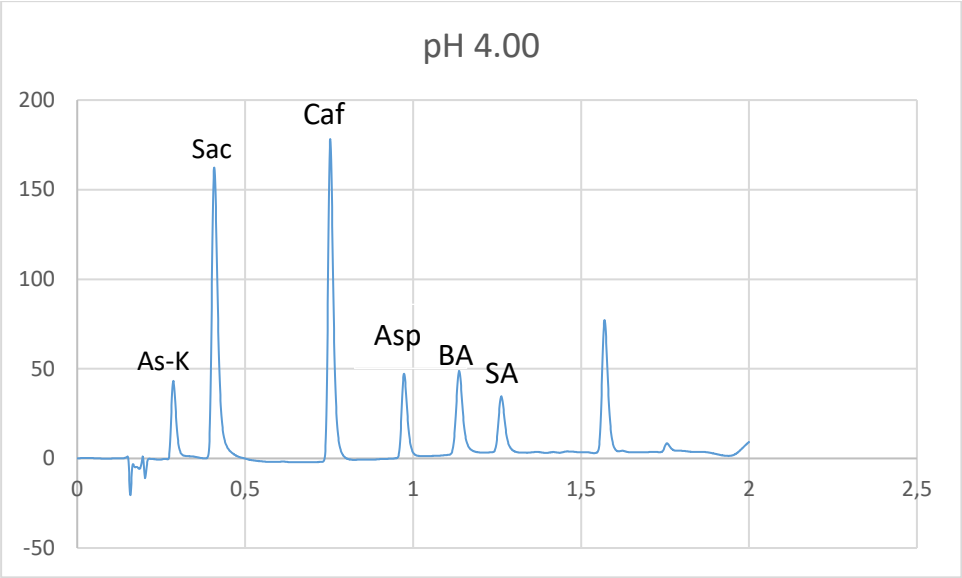

FIGURE 7: UHPLC SEPARATION OF A STANDARD SAMPLE OF 25 µG/ML CONCENTRATION. THE SAMPLE COMPONENTS WERE ACESULFAME-K (AS-K), SACCHARINE (SAC), CAFFEINE (CAF)ASPARTAME (ASP), BENZOIC ACID (BA) AND SORBIC ACID (SA), MOBILE PHASE: PHOSPHATE BUFFER (PH 4.00) AND ACN, UV-DETECTION AT 210 NM, INJECTION VOLUME 2 µL, TEMPERATURE 35 °C.

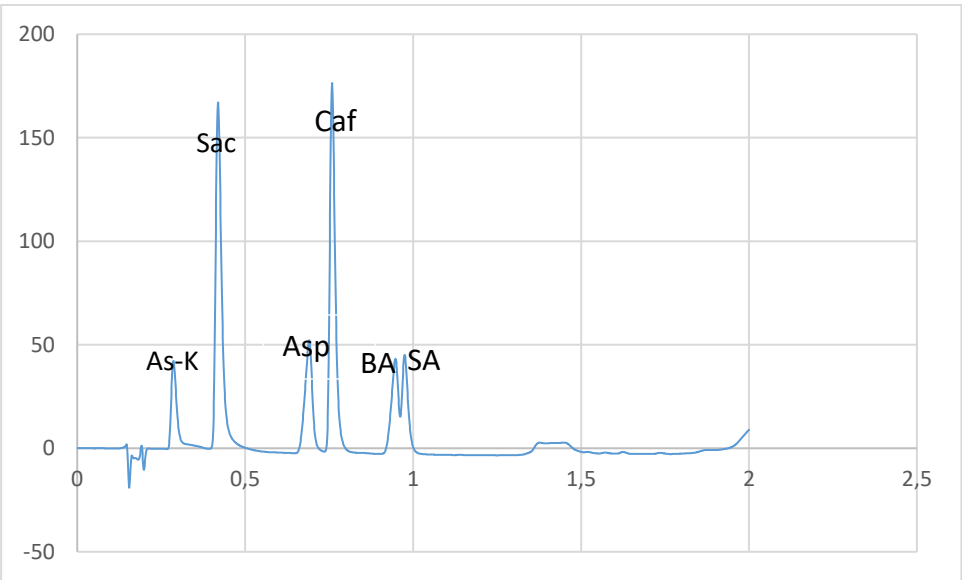

FIGURE 8: UHPLC SEPARATION OF A STANDARD SAMPLE OF 25 µG/ML CONCENTRATION. THE SAMPLE COMPONENTS WERE ACESULFAME-K (AS-K), SACCHARINE (SAC), CAFFEINE (CAF)ASPARTAME (ASP), BENZOIC ACID (BA) AND SORBIC ACID (SA), MOBILE PHASE: PHOSPHATE BUFFER (PH 4.92) AND ACN, UV-DETECTION AT 210 NM, INJECTION VOLUME 2 µL, TEMPERATURE 35 °C

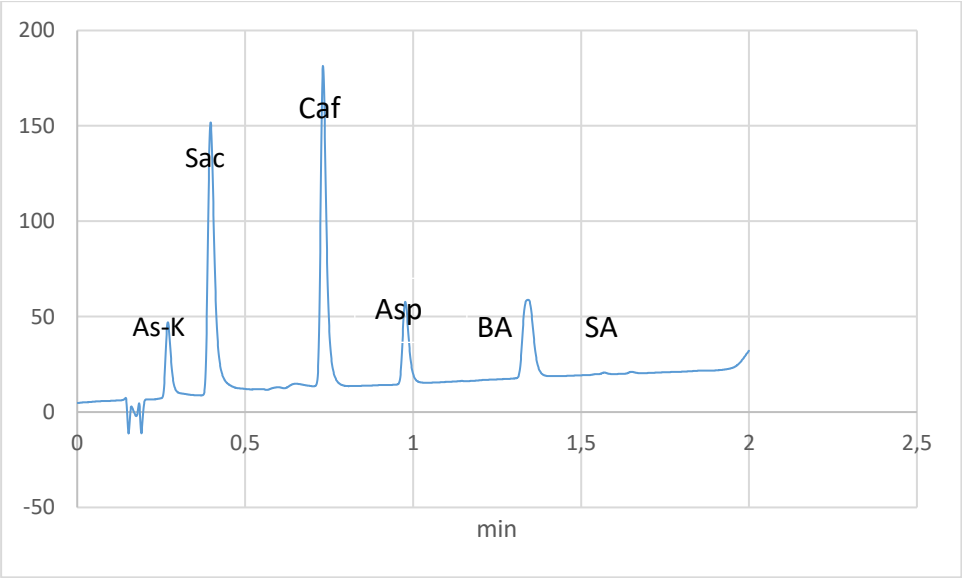

FIGURE 9: UHPLC SEPARATION OF A STANDARD SAMPLE OF 25 µG/ML CONCENTRATION. THE SAMPLE COMPONENTS WERE ACESULFAME-K (AS-K), SACCHARINE (SAC), CAFFEINE (CAF)ASPARTAME (ASP), BENZOIC ACID (BA) AND SORBIC ACID (SA), MOBILE PHASE: PHOSPHATE BUFFER (PH 3.19) AND ACN, UV-DETECTION AT 210 NM, INJECTION VOLUME 2 µL, TEMPERATURE 35 °C

5.3.Gradient

In gradient runs the composition of the mobile phase changes. Usually the amount of the organic modifier increases within the run. This can be done linearly or in steps. The reason for using a gradient is to reduce the analysis time of the slower eluting compounds, and hence to get narrower peaks. On the other hand, also the beginning of the gradient can be adjusted. Very polar compounds are going to be more retained if there is less organic modifier in the eluent.

Better separation of the compounds was done by adjusting the gradient using phosphate buffer. The biggest drawback of the original gradient in the application note was that there was hardly any retention of the first compound. This is not surprising as As-K is ionic with highly polar functional groups.

As the gradient was selected to start with a higher percentage of buffer the retention was somewhat increased. The levels from 11 % of ACN was reduced to 6 %. Also, the percentage was changed to 30 % ACN from 56 % at the end of the gradient. The effect this had on the retention times can be seen in table 7. The most important changes were for the first eluting

compounds. The differences might seem negligible, but in high resolution separation and short runtimes like these, the difference is big.

TABLE 7: CHANGE IN RETENTION TIMES AS THE AMOUNT OF ORGANIC MATTER WAS CHANGED FROM 11 % (RETENTION TIME 1) TO 6 % (RETENTION TIME 2).

Compound	Retention time 1	Retention time 2
As-K	0.256	0.288
Sac	0.333	0.418
Caf	0.636	0.758
Asp	0.866	0.975
BA	1.054	1.121
SA	1.18	1.256

The retention times vary somewhat from run to run, typically with one number on the second decimal. For example, the retention times for Sac varied from 0.41 to 0.42 min. This is not a very big variation, but causes some issues for the peak recognition as the peaks were narrow (especially As-K, Sac, and Caf) and thus the software did not always recognize the compound as the target compound. The software is programed so that it allows a certain amount of variation (percentage) in the retention time, which depends on the peak width.

5.4.Selection of wavelengths for analysis

A UV/Vis library with the spectra for all the compounds of interest was created. From the spectra, the optimal wavelength for analysis could be seen. The idea of the library is that the reference spectrum can be compared to the peak from the sample to reduce the risk for cross referencing. Unfortunately, aspartame and saccharine have very similar spectra. Overall the UV/Vis spectrum is not as compound specific as the infrared spectrum. The reason for this is that very broad peaks are caused by the different possibilities for electron transition from the different rotational and vibrational levels.[2]

The absorbance maximum of Caf, Sac, and Asp are at 200 nm. Normally 210 nm is selected for the detection of these three compounds. This wavelength is not very good for aspartame as its absorbance increases sharply below 220 nm. Caf has a second absorbance maximum at 275 nm, which could be utilized as backup wavelength. As-K and BA have an absorption maximum

227 nm. SA has very strong absorption at 260 nm. To summarize: three wavelengths where chosen for quantification, 210 nm, 227 nm, and 260 nm. Of these, 210 nm was considered the general wavelength, because most compounds that absorb, absorb in that area. This means that the wavelengths are not very selective, which increases the risk of misidentification.

5.5.Injection volume

Injection volume is a parameter which can easily lower the LODs and enhance reproducibility. When the injection volume is doubled also the peak size of the compound is doubled, as there is double the amount of compound present. The injection volume should not be too low or high. If too much is injected the detector can be overloaded. On the other hand, there needs to be sufficient amount of the compound present to be detected. At low concentrations, the peaks are very low and the areas small. Even at an injection of 2 µL aspartame the UV-absorption is only 10 mAu high for a 5 µg/mL solution.

At first a 1 µL injection was utilized. By keeping low injection volumes the risk of blocking the column is reduced. This is especially important for tricky matrixes. The volume of 1 µL was also utilized in one of the application notes provided by Agilent Technologies.[95] The peaks were however too small. With doubling the injection volume to 2 µL better and more reproducible results were achieved. Further 3 µL injections were tested in hope of even more reproducible results, but it turned out that with the injection of 3 µL of the sample Redbull Sugrafree there was no longer baseline separation of As-K and an unknown compound.

5.6.Temperature

To get stable retention times it is important to have constant temperature. In the summer the room temperature can increase up to 30°C. No temperature below that could be considered. The temperatures 30, 35, 40, and 45°C were investigated. As the temperature rises, the retention times get shorter. The changes are presented in table 8. The pressure reduced from approximately 640 bar to 520 bar in the beginning of the analysis. The reason for this is that liquids get less viscous as the temperature rises.[2] The temperature of 40 °C was chosen, because of the lower pressure and the diminished risk of the room temperature being over that.

TABLE 8: CHANGE IN RETENTION TIMES COMPARED TO TEMPERATURE.

Temperature	30 °C	35 °C	40 °C	45 °C
As-K	0.306	0.297	0.286	0.276
Sak	0.460	0.439	0.410	0.388
Kof	0.798	0.778	0.757	0.737
Asp	1.019	0.998	0.976	0.953
BH	1.217	1.174	1.134	1.089
SH	1.328	1.295	1.261	1.226
Pressure (bar)	640	590	550	520

5.7. Flow rate

As the method was based on the Agilent application note, first a flow rate of 1.8mL/min was tested.[95] The pressure was however quite close to the maximum and due to an expected increase of the pressure over time, the flowrate was reduced. The pressure dropped about 100 bars with a reduction of 0.2 mL/min in the flow rate, see table 9. Both the pressure and the flowrate affect the retention times and peak shapes. A flowrate of 1 mL/min was found to be optimal with short analyses time, good peak shapes, and low pressure. There is also no need for large flowrates as the analysis is so short even with 1 mL/min flowrate.

TABLE 9: EFFECT OF FLOWRATE UPON RETENTION TIMES AND PRESSURE.

mL/min	0.8	1	1.2	1.4
As-K	0.357	0.286	0.238	0.204
Sak	0.511	0.410	0.345	0.291
Kof	0.883	0.757	0.667	0.597
Asp	1.115	0.976	0.874	0.795
BH	1.318	1.134	0.993	0.886
SH	1.449	1.261	1.120	1.011
Pressure (bar)	445	550	650	740.0

6. Method validation

Validation of an analysis is the most important part of the development of a new method; without validation, there is no proof that the method works. Validation confirms the fitness for purpose of the analytical method.[98] For validation of this method for analysis of food additives, the trueness, measurement uncertainty, reproducibility, robustness, LOD and LOQ, and the standard solution stability were evaluated.

6.1.Trueness

Trueness consists of accuracy and precision.[2] Accuracy is the value of how close the average value comes to the "real" value and precision is the factor how close the results are too each other.[2] Optimally the results are both accurate and precise. Trueness was measured by comparing Fapas samples and in house measured samples and by statistical evaluation.

Several of the analyzed Fapas sample showed also peaks for other compounds, often sorbic acid, even though they were not included as reference compounds. Bias was used for comparison of achieved values in comparison to assigned values for the FAPAS samples and in house samples.

6.2.Within laboratory reproducibility

Within laboratory reproducibility (R_w) was measured with two different Fapas samples 3122 and 3124. The evaluation of reproducibility is not fully completed. First, the analysis was only carried out during 4 days and for aspartame there is no evaluation. The reproducibility testing was additionally done by the same person in the same laboratory with the same instrument at different days. The method still needs to be tested at least by a different analyst, for a longer term, with different stock solutions, with recalibrations, and for all other factors that change.[99]

The reproducibility was calculated by calculating the standard deviation (SD) from day to day analysis. The results are presented in table 10. These results indicate reasonable to good reproducibility; however, the data is unreliable with so few parallel analyses. The reproducibility for aspartame was not calculated as the sample was analyzed only a few times.

TABLE 10: WITHIN LABORATORY REPRODUCIBILITY

	Average result	SD Value	(RSD%) Relative value
As-K	51.69	1.36	2.64 %
Sac	52.03	1.69	3.25 %
Caf	98.77	5.94	6.01 %
BA	127.8	1.89	1.48 %
SA	132.4	2.23	1.68 %

6.3. Measurement uncertainty

Measurement uncertainty is one of the most crucial parameters to be determined in the validation process. It is highly important to acknowledge the error caused to the method by all the different variables in the analysis. The measurement uncertainty can be evaluated with many different methods. One method is the NORDtest principle.[99] The aim of the NORDtest is to support the evaluation of measurement uncertainty in routine analysis. It provides a practical and easily understandable way to calculate the uncertainty in environmental testing laboratories among others.[99]

The uncertainty consists of many factors in an analysis. It can be hard to identify all factors contributing to the error of the achieved results. There are two types of errors; random errors and systematic errors.[100] Random errors are harder to identify as they are, as the name suggests, random. These can be for example small mistakes done by the analyst. Systematic errors are caused by the system itself. An example here could be an error caused by filtration of the sample. Some of the sample might be adsorbed in the filter. The amount that does this is constant from sample to sample. The systematic error can be evaluated by calculating the bias.[99] This is done by comparison of acquired results and given "true" values for a sample. The equation to calculate the error caused by the bias is presented in equation 7.

$$bias = \bar{x} - RMS_{value} \quad (7)$$

where $\bar{x}$ is the average value of received results and RMS_{value} the "true value" given by the reference material provider. This can be turned into relative bias by dividing the bias with the certified value and multiplying by a 100. If there are several reference materials the average of RMS_{Bias} is utilized. The equation is presented in equation 8.

$$RMS_{bias} = \sqrt{\frac{\sum(bias)^2}{n}} \quad (8)$$

The reference material should be analyzed on a minimum of five different days.[99] The reference material itself, of course, has an error. This must be considered when calculating the error caused by the bias. The final error caused by the bias (u_{bias}) is the bias and the error of the reference material (equation 9), in the case when several reference materials are utilized, and further the standard deviation of the one bias if only one reference material is utilized (equation 10).

$$u_{bias} = \sqrt{RMS_{bias}{}^2 + u(C_{ref})\text{^}2} \quad (9)$$

$$u_{bias} = \sqrt{(bias)^2 + \left(\frac{s_{bias}}{\sqrt{n}}\right)^2 + u(C_{ref})\text{^}2} \quad (10)$$

As earlier mentioned, the measurement uncertainty (u) consists of random error and systematic error. In practice this means that the bias is utilized for the evaluation of systematic error and the day to day variation within the laboratory for the random error, which is represented by the reproducibility within the laboratory (R_w).

Expanded measurement uncertainty (U) is calculated by considering all possible errors. In this analysis, the Fapas samples played a large role in the estimation. The statistical calculations were based on the NORDtest principle.[99] This means that the measured results were first compared with the Fapas assigned values using bias. After that the errors were compared to the standard deviation for the runs.

In chemistry, the results are assumed to be normally distributed.[2] The FAPAS assigned values accompanied with a confidence interval, Z=2, which means that the interval is within a confidence level of 95 %. To calculate the $u(C_{ref})$ the value of the given value (μ) is subtracted from the upper limit (x) and divided by 1.96 (equation 11).[99]

$$u(C_{ref}) = \frac{x-\mu}{1.96}, \quad (11)$$

When several CRMs are utilized the $u(C_{ref})$ is the average of the values.

The u(C_{ref}) can be converted into relative uncertainty by dividing $u(C_{ref})$ with the give value and multiplying with 100.

The measurement uncertainty was calculated from the FAPAS samples 3122 and 3124. The sample 3122 contains As-K (53.2/ 43.9-62.6 mg/L), Sac (53.2/ 43.9-62.6 mg/L), Caf (97.6/ 81.9-113.3 mg/L), and BA (115.4/97.3-133.5 mg/mL). The sample 3124 has Sac (22.3/ 17.8-26.8 mg/L), BA (127/ 107-146 mg/L) and SA (140/ 118-161 mg/L). The samples were analyzed four days: 21.6, 5.7.16, 7.7.16 and 12.7. As can be seen a reference material for Asp is missing, this was analyzed instead with a inhouse prepared material made into a Funlight concentrate. Funlight was measured over a long period to be 136.78 µg/mL (130.99-142.58) by the Customs laboratory. Funlight was only analyzed twice with the new method.

The bias indicated for aspartame is very low. The reason for this is the small uncertainty of the Funlight measurements. The uncertainty is not however very trustworthy as the reference material has only been analyzed with one method. The other biases seem to be around the expected values and are quite similar to each other.

The calculation of the measurement uncertainty is presented for BA.

$$bias\%(3124) = \overline{(x} - RMS_{value})/RMS_{value} \times 100\ \% = (128 - 127)/127 \times 100\% = 1.14\ \%$$

$$bias\%(3122) = \frac{116.7-115.4}{115.4} \times 100\% = 0.66\ \%$$

$$RMS_{bias} = \sqrt{\frac{\sum(bias)^2}{n}} = ((0.66\%^2 + 1.14\%^2)/2)^{0.5} = 0.90\ \%$$

$$\mathrm{u}(C_{ref}) = \frac{x-\mu}{1.96} = \frac{146-127}{1.96} = 9.69, \frac{9.69}{127} \times 100\ \% = 7.63\ \%$$

$$and\ \frac{133.5-115.4}{1.96} = 9.23, \frac{9.23}{115.4} \times 100\ \% = 8.00\ \%$$

$$\left(\frac{7.63\ \%^2+8.00\ \%^2}{2}\right)^{0.5} = 7.82\ \%$$

$$u_{bias} = \sqrt{RMS_{bias}{}^2 + u(C_{ref})\text{^}2} = \sqrt{0.90\ \%^2 + 7.82^2} = 7.87\ \%$$

The estimation of the bias with only one reference material differs after the calculation of the bias. Here is an example for the calculation of the $\mathrm{u}(C_{ref})$ for As-K.

$$u_{bias} = \sqrt{(-2.93\%)^2 + \left(\frac{1.36}{\sqrt{4}}\right)^2 + 9.01\%\text{^}2} = 9.55\ \%$$

The rest of the u_{bias} are listed in table 11.

TABLE 11: THE VALUES FOR THE CALCULATED u_{bias}

Compound	u(bias)
As-K	9.55 %
Sac	12.17 %
Asp	2.87 %
Caf	8.81 %
BA	12.2 %
SA	9.62 %

After estimating the systematic error with the bias, an estimation of the random error needs to be made. The random error can be estimated with R_w. Optimally this would be taken from a control chart or similar over a long period. In this case the best value we have for the estimation is the four analyses done for the FAPAS samples.

The uncertainty (u_c) and the expanded uncertainty (U) was not calculated for aspartame due to lack of data of reproducibility. The equation for u_c (equation 12) and U (equation 13) is presented below. U gives a high confidence interval of 95 %.[99] A model calculation is presented for BA.

$$u_{bias} = \sqrt{{u_{bias}}^2 + R_w^2} \qquad (12)$$

$$U = 2 \times u_{bias} \qquad (13)$$

$$U(BA) = 2 \times \sqrt{12.2\ \%^2 + 1.48\ \%^2} = 24.76\ \%$$

TABLE 12: CALCULATED EXPANDED UNCERTAINTY FOR THE COMPOUNDS.

Compound	U
As-K	19.10 %
Sac	25.20 %
Caf	18.29 %
BA	24.76 %
SA	19.53 %

6.4. Limit of detection and limit of quantification

Limit of detection (LOD) is the lowest concentration, where the peak can be distinguished from the noise and where the signal is larger than the measurement uncertainty related to it.[101]

The limit of detection was calculated by using a zero matrix with an addition of 0.5 µg/mL, in this case Freeway lemonade with pineapple taste diluted to 1:5. Six parallel analyses were carried out and the noise was calculated close to the peaks (see table). The noise is given by the software by calculation of three times of the standard deviation of the noise plus the mean of the noise. The noise varies quite a lot from analysis to analysis, the largest variation is at the beginning of the run, which affects highly As-K, which has a noise at worst above 2.5 mAu.

The LOD was the calculated according to equation 14.

$$LOD = 3 \times N \times \frac{Amount\ of\ analyte_{std}}{Height_{std}} \quad (14)$$

An example for calculation of the LOD is given for BA, the rest are tabled in table 10. The factor of three is utilized to achieve a 99 % confidence limit.[2] The heights of the peaks in these calculations are from a 5 µg/mL standard solution and noise is measured from a 1/5 diluted freeway pineapple soda.

The LOQ was calculated similarly but instead of using a factor of three a factor of ten was utilized (equation 15).

$$LOQ = 10 \times N \times \frac{Amount\ of\ analyte_{std}}{Height_{std}} \quad (15)$$

An example for the calculation of the LOD is given for BA, the rest of the results are given in table 13, the LOQ is as previously mentioned calculated similarly and hence it is not separately presented here.

$$LOD = 3 \times 0.0962 \times \frac{5\ ppm}{17.408} = 0.0753\ ppm$$

TABLE 13: THE LODS AND LOQS FOR AS-K, SAC, CAF, ASP, BA AND SA.

	AVERAGE NOISE	LOD	LOQ
AS-K	2.0111	1.7329	5.7763
SAC	0.1466	0.0613	0.2043
CAF	0.0574	0.0224	0.0747
ASP	0.0601	0.0895	0.2984
BA	0.0962	0.0753	0.2509
SA	0.0611	0.0162	0.0541

6.5.Linearity

The linearity or working range for the analyzed compounds is somewhat different for each compound. The ones that look linear in figure 10 are quite linear with correlation factors above 0.999. The peak shapes are affected and retention times are different, however. Only the peak of sorbic acid clearly overloaded the detector at a concentration of 1000 µg/mL. This is not surprising as the most accurate values given by a spectrometer is between 180-820 mAu.[2] Modern instruments can give excellent values in a larger scale, even up to 2000 mAu.[2] Within the normal working range, aimed for this method, (5-100 µg/mL) all the compounds show excellent linearity (see figure 11).

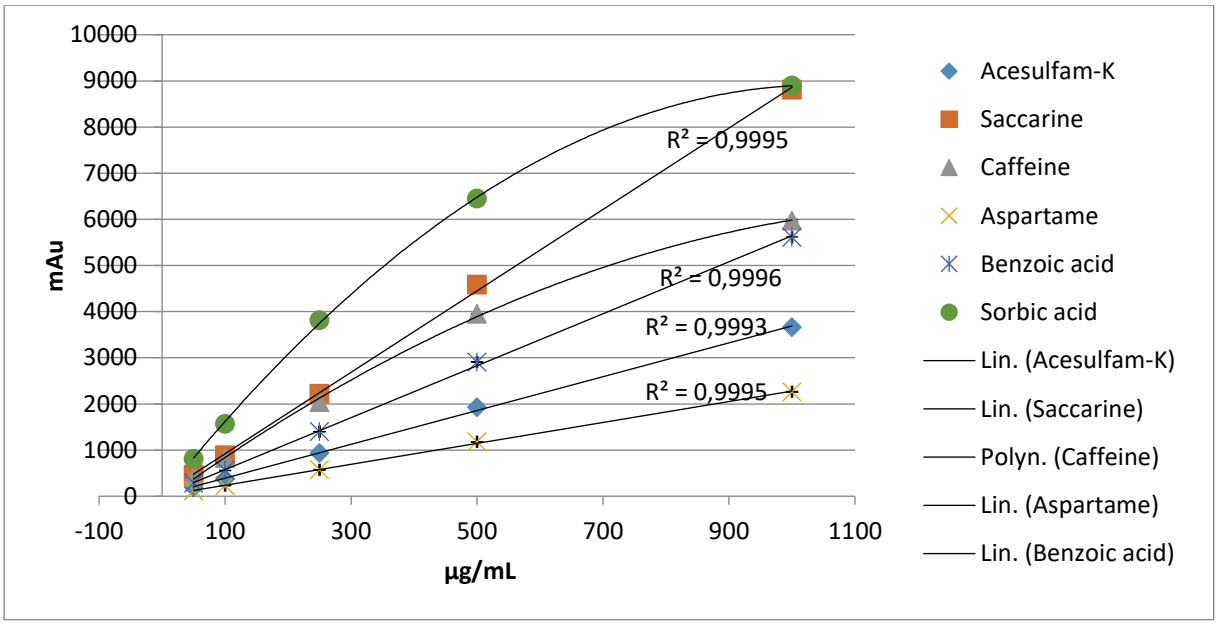

FIGURE 10: THE AREAS FOR 50, 100, 250, 500 AND 1000 µG/ML SAMPLES WITH ERRORS.

6.6. Robustness

Robustness assesses factors in the process that can cause variation, like changes in temperature, fluctuations of mobile phase composition, or pH.[101] As has been shown in the method development section, the method seems to be quite robust.

There are no major changes in the analysis if the pH changes from 4.00-4.10. It is very important that this analysis is not very pH sensitive. It needs to withstand small pH variation, as the buffer utilized is not in its buffering range. As for the temperature changes the major changes have been addressed by having high enough column temperature. Small changes in the mobile phase composition should not matter much, but for example the effect of concentration changes in the buffer was not assessed for the phosphate buffer. For the acetate buffer, there was no change between 10 mM and 20 mM buffers.

The DAD detector is very robust and the nature of electron transitional changes are of the type that small changes in the wavelengths do not cause major changes in the detection process. The biggest issue might be caused by fluorescent compounds, which, however, were not present in this analysis.[2]

The samples Vanilla Coca-Cola, Coca-Cola, Redbull and Redbull sugarfree, FunLight Orange and apple-pear concentrate along with the jams Pirkka strawberry and mashed apple, were analyzed to see if anything unexpected would happen. The method seems to work for all of

these. However, the jams needed more dilution and several filtration steps in order to pass the 0.2 µm filter.

Vanillin and ethyl-vanillin are very common additives in food matrixes and there was some concern for possible issues with vanillin peaks possibly overlapping one of the compounds. A standard mixture with added vanillin and ethyl-vanillin of 25 µg/mL was analyzed. The retention times of the different types of vanillas were the same and did not overlap with any of the compounds of interest. There might of course be other additives that overlap and this should be kept in mind while analyzing new products.

6.7. Durability

Stock solutions are highly practical to utilize as a base for comparison of standards. The problem is that quite often the compounds start to disintegrate in aqueous solution.[16] This can be reduced by adjusting the pH, addition of organic solvents, or with storage in cold and dark.[16] For the customs two different solutions at two different storage temperatures were tested over a period of two months.

The durability was tested by preparing two stock solutions. One was 500 µg/mL containing all of the compounds in 30 % MeOH and 70 % H_3PO_4-solution, and the other one was identical, but the H_3PO_4 solution replaced with water. These solutions were divided into small test tubs with caps and stored in either the fridge (temp 4 °C) or freezer (temp -20 °C). One tube of each type was then analyzed every one or two weeks, after the dilution to 50 µg/mL and 5 µg/mL. The day of preparation was 13.5.2016 after which the solutions were analyzed on the 20.5.2016, 1.7.2016, and 13.7.2016.

The results are a bit inconclusive. For more accurate results, more data points would have been needed. However, it seems that the variation is caused by variation in the preparation of the solution. Especially the results from the solution made from the buffer stored in the freezer 20.5.2016 can be discarded, as it is clearly differs from the other results. Over in all, the areas of the peaks are quite the same over the period measured. The areas are shown for the 50 µg/mL solution of As-K and Sac, which have the biggest variation. The results for the other samples are similar (figures 12 and 13).

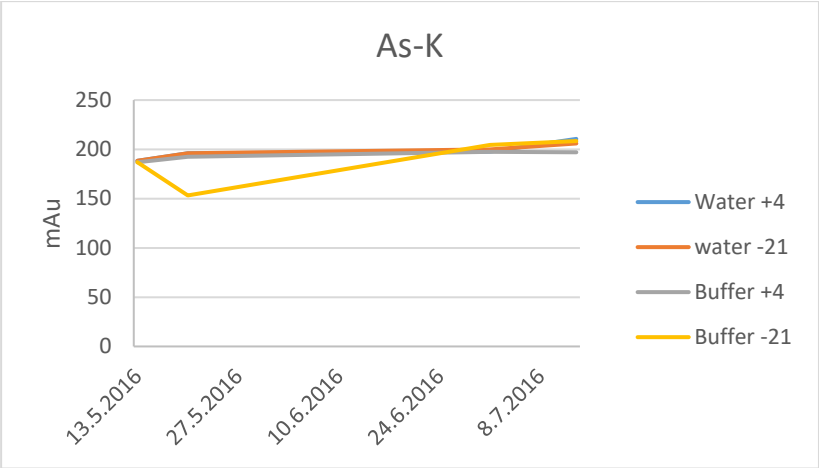

FIGURE 11: STABILITY TEST FOR AS-K OVER THE PERIOD OF TWO MONTHS.

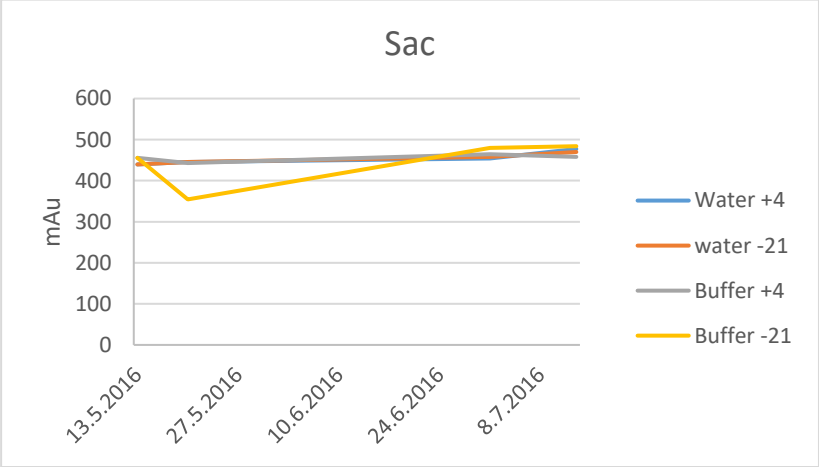

FIGURE 12: STABILITY TEST FOR SAC OVER THE PERIOD OF TWO MONTHS.

From the compounds analyzed, aspartame disintegrated the most. This could be clearly seen in the older Fapas sample analyzed, where the observed amount could be less than half of the given value. Over the period of two months there was no difference in the areas of any of the compounds, whichever way the standard solutions were stored. Furthermore, no division in peaks or unknown peaks could be seen in the chromatograms.

7. Results and discussion

The method developed was validated partly, with the aim to fulfil the needs of the Finnish Customs Laboratory. The optimized method was as follows: an injection volume of 2 µL; gradient elution from 6 % ACN to 30 % ACN in 1.6 minutes, then 1.6-1.7 minutes again 6 %; phosphate buffer at pH of 4.0; a total runtime of 1.70; a flowrate of 1.0 mL/min; detection at

210 nm, 227 nm, and 260 nm. The LODs were between 0.02 µg/mL and 1.73 µg/mL and the LOQ between 0.054 µg/mL and 5.78 µg/mL. This values should be sufficient for the Customs needs in the sense of checking if a product is over a certain limit. The LOQ for AS-K, which is over 5 µg/mL, is a bit problematic as the lowest calibration solution is 5 µg/mL. This is something to take into consideration if low concentrations are quantified. Expanded measurement uncertainties are around 20%.

The method developed is good for analyzing beverages. It is however questionable if it will be robust enough for more complex matrixes. As an example can be mentioned matrixes that contain a lot of proteins and fat, like protein bars. These might have stronger matrix effects. They might also cause clogging, resulting in an increase of the column pressure, without proper sample preparation. Dilution and filtration of samples like this is not going to be sufficient, and some LE procedure combined with protein precipitation, would probably be needed. This could be compared to sample preparation methods for the serum samples prior to MS studies, which were surprisingly simple and have similar components to foodstuff. The biggest difference is the sample amount. In foodstuff, large samples are better and there is no need to miniaturize the sample volume. A large sample is more representative for foodstuff that might have variation from package to package.

Other matrixes than beverages contain a lot of compounds that have not been addressed in this thesis. This might cause some misidentification of the compounds. It is a true downside with UV/Vis detectors that they cannot be utilized for identification of compounds. The original idea to develop a method with an acetate buffer would have allowed the possibility to switch the detector to an MS, which would give more knowledge of the detected compound, as could be seen in the steroidal analyses. For routine analysis, with good idea what the sample contains, the MS instrument might be too elaborate and expensive. The MS instruments are highly efficient, but need a lot more knowhow to operate, need cleaner samples, and are higher in maintenance. Lastly, the analysis is also slower, due to the low flowrates.

Compared to steroidal analysis the developed method for analysis of food additives is very simple. The contents of food stuff are well known and the quantification of the compounds thus simple. In steroidal analysis, the MS and in most of the cases a MS/MS is needed, due to complex matrixes that contain plenty of different compounds of similar structures.

8. Concluding remarks

UHPLC is a versatile instrument, which can be utilized for the analysis of simple beverages to complicated blood samples. It is flexible in the sense that it can be connected to a MS instrument to provide even more information of the sample than could be achieved with only a UV/Vis detector.

The instrument itself provides repeatable results with high resolution. The sample preparation can be simple and the analysis time short, depending on the application. In non-targeted analysis, the analysis tends to be long, but in targeted analysis times less than ten minutes are common. This makes the instrument well fitted for routine analysis of parallel samples.

There are almost no limits for the compounds that can be analyzed with UHPLC. Only ionic compounds can cause issues in RPLC. The composition of the mobile phases and the type of stationary phases can be tuned to fit a variety of samples and compounds. The only limitation is that the samples need to be free from all kinds of particles that might induce clogging of the column.

The main advantage of UHPLC lies in the small particles utilized as stationary phase. It provides short time for the column to equilibrate and high plate numbers for relatively short columns. This reduces the analysis time and the solvent consumption. Even though UHPLC is a powerful tool, the instrument is easy and straightforward to use.

The validation process for UHPLC is simple, but takes time. The method developed for the Customs heroes the speed of the UHPLC in routine analysis. The samples need only minimal pretreatment. This leads to complete analysis in a very short time.

In steroid analysis, the versatility and softness of the instrument is the major advantage. There is no need for extreme heat or labor intense sample preparation, such as derivatization, in order to analyze the compounds.

In the future, the UHPLC instruments will replace all the traditional HPLC instruments. The higher efficiency and the fast analysis is what is needed today. The UHPLC-MS will become more affordable and they will land in regular hospital laboratories. They will be utilized for finding indicators for cancer and other diseases.

9. References

1. Ettre LS, Sakodynskii KI. M. S. tswett and the discovery of chromatography II: Completion of the development of chromatography (1903-1910). *Chromatographia*. 1993;35(5):329-338. https://doi.org/10.1007/BF02277520. doi: 10.1007/BF02277520.

2. Robinson JW, Skelly Frame EM, Frame II GM. *Undergraduate instrumental analysis.* 7th ed. Taylor & Francis group; 2014.

3. Fekete S, Guillarme D. Possibilities of new generation columns packed with 1.3 µm core–shell particles in gradient elution mode. *J. Chromatogr. A.* 2013;1320:86-95. doi: http://doi.org.libproxy.helsinki.fi/10.1016/j.chroma.2013.10.061.

4. What is the difference between UHPLC and UPLC? chromatography today. https://www.chromatographytoday.com/news/hplc-uhplc/31/breaking-news/what-is-the-difference-between-uhplc-and-uplc/30754. Accessed 10/3/2017, 2017.

5. Fountain KJ, Neue UD, Grumbach ES, Diehl DM. Effects of extra-column band spreading, liquid chromatography system operating pressure, and column temperature on the performance of sub-2-µm porous particles. *J. Chromatogr. A.*. 2009;1216(32):5979-5988. doi: https://doi-org.libproxy.helsinki.fi/10.1016/j.chroma.2009.06.044.

6. Xu X, Gao B, Guan Q, et al. Metabolomic profile for the early detection of coronary artery disease by using UPLC-QTOF/MS. *J Pharm Biomed Anal*. 2016;129:34-42. doi: 10.1016/j.jpba.2016.06.040.

7. Gosetti F, Mazzucco E, Gennaro MC, Marengo E. Ultra high performance liquid chromatography tandem mass spectrometry determination and profiling of prohibited steroids in human biological matrices. A review. *J. Chromatogr. A*. 2013;927:22-36. doi: http://dx.doi.org/10.1016/j.jchromb.2012.12.003.

8. Krone N, Hughes BA, Lavery GG, Stewart PM, Arlt W, Shackleton CHL. Gas chromatography/mass spectrometry (GC/MS) remains a pre-eminent discovery tool in clinical steroid investigations even in the era of fast liquid chromatography tandem mass spectrometry (LC/MS/MS). *The J. Steroid Biochem. Mol. Biol.* 2010;121(3):496-504. doi: http://dx.doi.org/10.1016/j.jsbmb.2010.04.010.

9. Liu L, Wang M, Yang X, et al. Fasting serum lipid and dehydroepiandrosterone sulfate as important metabolites for detecting isolated postchallenge diabetes: Serum metabolomics via ultra-high-performance LC-MS. *Clin. Chem*. 2013;59(9):1338-1348. doi: 10.1373/clinchem.2012.200527.

10. Olazarán J, Gil-De-Gómez L, Rodríguez-Martín A, et al. A blood-based, 7-metabolite signature for the early diagnosis of alzheimer's disease. *J Alzheimer's Dis*. 2015;45(4):1157-1173. doi: 10.3233/JAD-142925.

11. Qian X, Zhan Q, Lv L, et al. Steroid hormone profiles plus α-fetoprotein for diagnosing primary liver cancer by liquid chromatography tandem mass spectrometry. *Clin. Chim. Acta*. 2016;457:92-98. doi: 10.1016/j.cca.2016.03.022.

12. Zang X, Jones CM, Long TQ, et al. Feasibility of detecting prostate cancer by ultraperformance liquid chromatography-mass spectrometry serum metabolomics. *J. Proteome Res*. 2014;13(7):3444-3454. doi: 10.1021/pr500409q.

13. Gaikwad NW. Ultra performance liquid chromatography-tandem mass spectrometry method for profiling of steroid metabolome in human tissue. *Anal. Chem*. 2013;85(10):4951-4960. doi: 10.1021/ac400016e.

14. Díaz-Cruz MS, López de Alda MJ, López R, Barceló D. Determination of estrogens and progestogens by mass spectrometric techniques (GC/MS, LC/MS and LC/MS/MS). *J. Mass Spectrom.* 2003;38(9):917-923. doi: 10.1002/jms.529.

15. Fragkaki AG, Angelis YS, Tsantili-Kakoulidou A, Koupparis M, Georgakopoulos C. Schemes of metabolic patterns of anabolic androgenic steroids for the estimation of metabolites of designer steroids in human urine. *The J. Steroid Biochem. Mol. Biol.* 2009;115(1–2):44-61. doi: http://dx.doi.org.libproxy.helsinki.fi/10.1016/j.jsbmb.2009.02.016.

16. Rautio J. *Farmaseuttisen kemian perusteet.* Farmasian opiskelia yhdistys Fortis; 2013.

17. Gosetti F, Mazzucco E, Gennaro MC, Marengo E. Ultra high performance liquid chromatography tandem mass spectrometry determination and profiling of prohibited steroids in human biological matrices. A review. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci*. 2013;927:22-36. doi: 10.1016/j.jchromb.2012.12.003.

18. Jeanneret F, Tonoli D, Hochstrasser D, et al. Evaluation and identification of dioxin exposure biomarkers in human urine by high-resolution metabolomics, multivariate analysis and in vitro synthesis. *Toxicol. Lett*. 2016;240(1):22-31. doi: 10.1016/j.toxlet.2015.10.004.

19. Dandliker WB, Kelly RJ, Dandliker J, Farquhar J, Levin J. Fluorescence polarization immunoassay. theory and experimental method. *Immunochemistry*. 1973;10(4):219-227. doi: http://dx.doi.org/10.1016/0019-2791(73)90198-5.

20. Schwarz E, Liu A, Randall H, et al. Use of steroid profiling by UPLC-MS/MS as a second tier test in newborn screening for congenital adrenal hyperplasia: The utah experience. *Pediatr. Res*. 2009;66(2):230-235. doi: 10.1203/PDR.0b013e3181aa3777.

21. Janzen N, Sander S, Terhardt M, et al. Rapid steroid hormone quantification for congenital adrenal hyperplasia (CAH) in dried blood spots using UPLC liquid chromatography-tandem mass spectrometry. *Steroids*. 2011;76(13):1437-1442. doi: 10.1016/j.steroids.2011.07.013.

22. Kotronoulas A, Marcos J, Segura J, Ventura R, Joglar J, Pozo OJ. Ultra high performance liquid chromatography tandem mass spectrometric detection of glucuronides resistant to

enzymatic hydrolysis: Implications to doping control analysis. *Anal. Chim. Acta*. 2015;895:35-44. doi: 10.1016/j.aca.2015.08.043.

23. van Deemter JJ, Zuiderweg FJ, Klinkenberg A. Longitudinal diffusion and resistance to mass transfer as causes of nonideality in chromatography. *Chem. Eng. Sci.* 1956;5(6):271-289. doi: http://dx.doi.org/10.1016/0009-2509(56)80003-1.

24. Riekkola M, Hyötyläinen T. *Kolonnikromatografia ja kapillaarielektromigraatiotekniikat.* 2nd ed. Helsinki: Yliopistopaino; 2002.

25. Guillarme D, Veuthey J. State-of-the art of (UHP)LC–MS(–MS) techniques and their practical application. *J. Chromatogr. A*. 2013;1292:1. doi: http://dx.doi.org.libproxy.helsinki.fi/10.1016/j.chroma.2013.03.055.

26. Wheaton JP, Chambers EE, Fountain KJ. Challenges in developing an ultra-sensitive bioanalytical method for ethinylestradiol in human plasma. *Bioanalysis*. 2012;4(7):769-781. doi: 10.4155/bio.12.29.

27. Dolan JW. Column packing - what's at the bottom of it? *LC-GC*. 1998;16(4):350-354.

28. Hinchliffe E, Carter S, Owen LJ, Keevil BG. Quantitation of aldosterone in human plasma by ultra high performance liquid chromatography tandem mass spectrometry. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci*. 2013;913-914:19-23. doi: 10.1016/j.jchromb.2012.11.013.

29. Balcke GU, Handrick V, Bergau N, et al. An UPLC-MS/MS method for highly sensitive high-throughput analysis of phytohormones in plant tissues. *Plant Methods*. 2012;8(1):47. https://doi.org/10.1186/1746-4811-8-47. doi: 10.1186/1746-4811-8-47.

30. Niessen WMA. Progress in liquid chromatography–mass spectrometry instrumentation and its impact on high-throughput screening. *J. Chromatogr. A*. 2003;1000(1):413-436. doi: http://dx.doi.org.libproxy.helsinki.fi/10.1016/S0021-9673(03)00506-5.

31. Wilm M. Principles of electrospray ionization. *Mol. Cell. Proteomics*. 2011

32. Korfmacher WA. Foundation review: Principles and applications of LC-MS in new drug discovery. *Drug Discovery Today*. 2005;10(20):1357-1367. doi: http://dx.doi.org.libproxy.helsinki.fi/10.1016/S1359-6446(05)03620-2.

33. 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. http://dx.doi.org.libproxy.helsinki.fi/10.1080/05704920902717872. doi: 10.1080/05704920902717872.

34. Broecker S, Herre S, WÃ¼st B, Zweigenbaum J, Pragst F. Development and practical application of a library of CID accurate mass spectra of more than 2,500 toxic compounds for systematic toxicological analysis by LC-QTOF-MS with data-dependent acquisition. *Anal.*

Bioanal. Chem. 2011;400(1):101-117. https://doi.org/10.1007/s00216-010-4450-9. doi: 10.1007/s00216-010-4450-9.

35. Lacorte S, Fernandez-Alba AR. Time of flight mass spectrometry applied to the liquid chromatographic analysis of pesticides in water and food. *Mass Spectrom. Rev*. 2006;25(6):866-880. doi: 10.1002/mas.20094.

36. Badoud F, Boccard J, Schweizer C, Pralong F, Saugy M, Baume N. Profiling of steroid metabolites after transdermal and oral administration of testosterone by ultra-high pressure liquid chromatography coupled to quadrupole time-of-flight mass spectrometry. *J Steroid Biochem. Mol. Biol*. 2013;138:222-235. doi: 10.1016/j.jsbmb.2013.05.018.

37. Wang W, Zhao Y, Jing W, et al. Ultrahigh-performance liquid chromatography-ion trap mass spectrometry characterization of the steroidal saponins of dioscorea panthaica prain et burkill and its application for accelerating the isolation and structural elucidation of steroidal saponins. *Steroids*. 2015;95:51-65. doi: 10.1016/j.steroids.2014.12.023.

38. Zhang Y-, Bao M-, Wang G, Qu Q, Zhou H-. Induction of human CYP3A4 by huperzine A, ligustrazine and oridonin through pregnane X receptor-mediated pathways. *Pharmazie*. 2014;69(7):532-536. doi: 10.1691/ph.2014.3950.

39. Su S, Duan J, Wang P, et al. Metabolomic study of biochemical changes in the plasma and urine of primary dysmenorrhea patients using UPLC-MS coupled with a pattern recognition approach. *J. Proteome Res*. 2013;12(2):852-865. doi: 10.1021/pr300935x.

40. Ke Y, Bertin J, Gonthier R, Simard J-, Labrie F. A sensitive, simple and robust LC-MS/MS method for the simultaneous quantification of seven androgen- and estrogen-related steroids in postmenopausal serum. *J Steroid Biochem. Mol. Biol*. 2014;144(PB):523-534. Accessed 22 March 2017. doi: 10.1016/j.jsbmb.2014.08.015.

41. Richter BE, Jones BA, Ezzell JL, Porter NL, Avdalovic N, Pohl C. Accelerated solvent extraction:Â€‰ A technique for sample preparation. *Anal. Chem*. 1996;68(6):1033-1039. http://dx.doi.org/10.1021/ac9508199. doi: 10.1021/ac9508199.

42. Pena-Pereira F, Lavilla I, Bendicho C. Miniaturized preconcentration methods based on liquid–liquid extraction and their application in inorganic ultratrace analysis and speciation: A review. *Spectrochim. Acta, Part B*. 2009;64(1):1-15. doi: http://dx.doi.org.libproxy.helsinki.fi/10.1016/j.sab.2008.10.042.

43. Berrueta LA, Gallo B, Vicente F. A review of solid phase extraction: Basic principles and new developments. *Chromatographia*. 1995;40(7):474-483. http://dx.doi.org/10.1007/BF02269916. doi: 10.1007/BF02269916.

44. Hennion M. Solid-phase extraction: Method development, sorbents, and coupling with liquid chromatography. *J. Chromatogr. A.* 1999;856(1):3-54. doi: http://dx.doi.org.libproxy.helsinki.fi/10.1016/S0021-9673(99)00832-8.

45. Anastassiades M, Lehotay SJ, Štajnbaher D, Schenck FJ. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and "Dispersive solid-phase extraction" for the determination of pesticide residues in produce. *J. AOAC Int*. 2003;VOL. 86(NO. 2).

46. Bruzzoniti MC, Checchini L, De Carlo RM, Orlandini S, Rivoira L, Del Bubba M. QuEChERS sample preparation for the determination of pesticides and other organic residues in environmental matrices: A critical review. *Anal. Bioanal. Chem*. 2014;406(17):4089-4116. http://dx.doi.org/10.1007/s00216-014-7798-4. doi: 10.1007/s00216-014-7798-4.

47. Deshmukh N, Hussain I, Barker J, Petroczi A, Naughton DP. Analysis of anabolic steroids in human hair using LC–MS/MS. *Steroids*. 2010;75(10):710-714. doi: http://dx.doi.org.libproxy.helsinki.fi/10.1016/j.steroids.2010.04.007.

48. Thomas A, Guddat S, Kohler M, et al. Comprehensive plasma-screening for known and unknown substances in doping controls. *Rapid Commun. Mass Spectrom*. 2010;24(8):1124-1132. doi: 10.1002/rcm.4492.

49. Tonoli D, Fürstenberger C, Boccard J, et al. Steroidomic footprinting based on ultra-high performance liquid chromatography coupled with qualitative and quantitative high-resolution mass spectrometry for the evaluation of endocrine disrupting chemicals in H295R cells. *Chem. Res. Toxicol*. 2015;28(5):955-966. doi: 10.1021/tx5005369.

50. Westerhuis JA, Hoefsloot HCJ, Smit S, et al. Assessment of PLSDA cross validation. *Metabolomics*. 2008;4(1):81-89. https://doi.org/10.1007/s11306-007-0099-6. doi: 10.1007/s11306-007-0099-6.

51. Wold S, Esbensen K, Geladi P. Principal component analysis. *Chemom. Intell. Lab. Syst.* 1987;2(1):37-52. doi: http://dx.doi.org.libproxy.helsinki.fi/10.1016/0169-7439(87)80084-9.

52. Wehrens R. *Chemometrics with R.* Springer Berlin Heidelberg; 2011. 10.1007/978-3-642-17841-2_4.

53. WOLD S, SJÖSTRÖM M. SIMCA: A method for analyzing chemical data in terms of similarity and analogy. In: *Chemometrics: Theory and application.* Vol 52. American Chemical Society; 1977:243-282. http://dx.doi.org/10.1021/bk-1977-0052.ch012. doi:10.1021/bk-1977-0052.ch012.

54. Jiang M, Klein M, Zanger UM, et al. Inflammatory regulation of steroid sulfatase: A novel mechanism to control estrogen homeostasis and inflammation in chronic liver disease. *J. Hepatol*. 2016;64(1):44-52. doi: 10.1016/j.jhep.2015.07.022.

55. Qian X, Zhan Q, Lv L, et al. Steroid hormone profiles plus α-fetoprotein for diagnosing primary liver cancer by liquid chromatography tandem mass spectrometry. *Clin. Chim. Acta*. 2016;457:92-98. doi: 10.1016/j.cca.2016.03.022.

56. Xu X, Gao B, Guan Q, et al. Metabolomic profile for the early detection of coronary artery disease by using UPLC-QTOF/MS. *J. Pharm. Biomed. Anal*. 2016;129:34-42. doi: 10.1016/j.jpba.2016.06.040.

57. Dong F, Deng D, Chen H, et al. Serum metabolomics study of polycystic ovary syndrome based on UPLC-QTOF-MS coupled with a pattern recognition approach. *Anal. Bioanal. Chem*. 2015;407(16):4683-4695. doi: 10.1007/s00216-015-8670-x.

58. Kotronoulas A, Marcos J, Segura J, Ventura R, Joglar J, Pozo OJ. Ultra high performance liquid chromatography tandem mass spectrometric detection of glucuronides resistant to enzymatic hydrolysis: Implications to doping control analysis. *Anal. Chim. Acta*. 2015;895:35-44. doi: 10.1016/j.aca.2015.08.043.

59. Liang Q, Xu W, Hong Q, et al. Rapid comparison of metabolites in humans and rats of different sexes using untargeted UPLC-TOFMS and an in-house software platform. *Eur. J. Mass Spectrom. (Chichester, England)*. 2015;21(6):801-821. doi: 10.1255/ejms.1395.

60. Upreti R, Naredo G, Faqehi AMM, et al. Simultaneous pharmacokinetic and pharmacodynamic analysis of 5α-reductase inhibitors and androgens by liquid chromatography tandem mass spectrometry. *Talanta*. 2015;131:728-735. doi: 10.1016/j.talanta.2014.07.087.

61. Wang W, Wang S, Tan S, et al. Detection of urine metabolites in polycystic ovary syndrome by UPLC triple-TOF-MS. *Clin. Chim. Acta.*. 2015;448:39-47. doi: 10.1016/j.cca.2015.06.008.

62. Baecher S, Kroiss M, Fassnacht M, Vogeser M. No endogenous ouabain is detectable in human plasma by ultra-sensitive UPLC-MS/MS. *Clin. Chim. Acta.*. 2014;431:87-92. doi: 10.1016/j.cca.2014.01.038.

63. Boccard J, Badoud F, Jan N, et al. Untargeted profiling of urinary steroid metabolites after testosterone ingestion: Opening new perspectives for antidoping testing. *Bioanalysis*. 2014;6(19):2523-2536. doi: 10.4155/bio.14.200.

64. Jeanneret F, Boccard J, Badoud F, et al. Human urinary biomarkers of dioxin exposure: Analysis by metabolomics and biologically driven data dimensionality reduction. *Toxicol. Lett*. 2014;230(2):234-243. doi: 10.1016/j.toxlet.2013.10.031.

65. Zhang Y, Zhang A, Yan G, et al. High-throughput metabolomic approach revealed the acupuncture exerting intervention effects by perturbed signatures and pathways. *Mol. BioSyst*. 2014;10(1):65-73. doi: 10.1039/c3mb70352e.

66. Zhou J, Yu S, Wang Y, et al. Serum metabolite profiling of B-cell non-hodgkin's lymphoma using UPLC-QTOFMS and GC-TOFMS. *Metabolomics*. 2014;10(4):677-687. doi: 10.1007/s11306-013-0596-8.

67. Bell LN, Kilkus JM, Booth JN, Bromley LE, Imperial JG, Penev PD. Effects of sleep restriction on the human plasma metabolome. *Physiol. Behav*. 2013;122:25-31. doi: 10.1016/j.physbeh.2013.08.007.

68. Jäntti SE, Hartonen M, Hilvo M, et al. Steroid and steroid glucuronide profiles in urine during pregnancy determined by liquid chromatography-electrospray ionization-tandem mass spectrometry. *Anal. Chim. Acta*. 2013;802:56-66. doi: 10.1016/j.aca.2013.09.034.

69. Kim B, Moon J-, Choi MH, et al. Global metabolomics and targeted steroid profiling reveal that rifampin, a strong human PXR activator, alters endogenous urinary steroid markers. *J. Proteome Res*. 2013;12(3):1359-1368. doi: 10.1021/pr301021p.

70. Kulle AE, Welzel M, Holterhus P-, Riepe FG. Implementation of a liquid chromatography tandem mass spectrometry assay for eight adrenal C-21 steroids and pediatric reference data. *Horm. Res. Paediatr*. 2013;79(1):22-31. doi: 10.1159/000346406.

71. Liu P, Duan J, Wang P, et al. Biomarkers of primary dysmenorrhea and herbal formula intervention: An exploratory metabonomics study of blood plasma and urine. *Mol. BioSyst*. 2013;9(1):77-87. doi: 10.1039/c2mb25238d.

72. García-Cañaveras JC, Donato MT, Castell JV, Lahoz A. Targeted profiling of circulating and hepatic bile acids in human, mouse, and rat using a UPLC-MRM-MS-validated method. *J. Lipid Res*. 2012;53(10):2231-2241. doi: 10.1194/jlr.D028803.

73. Haag M, Hamann T, Kulle AE, et al. Age and skin site related differences in steroid metabolism in male skin point to a key role of sebocytes in cutaneous hormone metabolism. *Dermato-Endocrinology*. 2012;4(1):63-69. doi: 10.4161/derm.19201.

74. Wang X, Wang X, Xie G, et al. Urinary metabolite variation is associated with pathological progression of the post-hepatitis B cirrhosis patients. *J. Proteome Res*. 2012;11(7):3838-3847. doi: 10.1021/pr300337s.

75. Xiao Y, Chan SW, Hu M, et al. Simultaneous determination of cortisol, cortisone, and 6β-hydroxycortisol in human urine by UPLC with UV detector and application to determine diurnal variations. *Chromatographia*. 2012;75(3-4):169-173. doi: 10.1007/s10337-011-2162-8.

76. Badoud F, Grata E, Boccard J, et al. Quantification of glucuronidated and sulfated steroids in human urine by ultra-high pressure liquid chromatography quadrupole time-of-flight mass spectrometry. *Anal. Bioanal. Chem*. 2011;400(2):503-516. doi: 10.1007/s00216-011-4779-8.

77. Ben Khelil M, Tegethoff M, Meinlschmidt G, Jamey C, Ludes B, Raul J-. Simultaneous measurement of endogenous cortisol, cortisone, dehydroepiandrosterone, and dehydroepiandrosterone sulfate in nails by use of UPLC-MS-MS. *Anal. Bioanal. Chem*. 2011;401(4):1153-1162. doi: 10.1007/s00216-011-5172-3.

78. Boccard J, Badoud F, Grata E, et al. A steroidomic approach for biomarkers discovery in doping control. *Forensic Sci. Int*. 2011;213(1-3):85-94. doi: 10.1016/j.forsciint.2011.07.023.

79. Grata E, Perrenoud L, Saugy M, Baume N. SARM-S4 and metabolites detection in sports drug testing: A case report. *Forensic Sci. Int*. 2011;213(1-3):104-108. doi: 10.1016/j.forsciint.2011.07.014.

80. Kaur-Atwal G, Reynolds JC, Mussell C, et al. Determination of testosterone and epitestosterone glucuronides in urine by ultra performance liquid chromatography-ion mobility-mass spectrometry. *Analyst*. 2011;136(19):3911-3916. doi: 10.1039/c1an15450h.

81. El-Talatini MR, Taylor AH, Konje JC. The relationship between plasma levels of the endocannabinoid, anandamide, sex steroids, and gonadotrophins during the menstrual cycle. *Fertil Steril*. 2010;93(6):1989-1996. doi: 10.1016/j.fertnstert.2008.12.033.

82. Rossi C, Calton L, Hammond G, et al. Serum steroid profiling for congenital adrenal hyperplasia using liquid chromatography-tandem mass spectrometry. *Clin. Chim. Acta*. 2010;411(3-4):222-228. doi: 10.1016/j.cca.2009.11.007.

83. Murray GJ, Danaceau JP. Simultaneous extraction and screening of diuretics, beta-blockers, selected stimulants and steroids in human urine by HPLC-MS/MS and UPLC-MS/MS. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* 2009;877(30):3857-3864. doi: 10.1016/j.jchromb.2009.09.036.

84. Pozo OJ, Van Eenoo P, Deventer K, et al. Detection and structural investigation of metabolites of stanozolol in human urine by liquid chromatography tandem mass spectrometry. *Steroids*. 2009;74(10-11):837-852. doi: 10.1016/j.steroids.2009.05.004.

85. Licea-Perez H, Wang S, Szapacs ME, Yang E. Development of a highly sensitive and selective UPLC/MS/MS method for the simultaneous determination of testosterone and 5α-dihydrotestosterone in human serum to support testosterone replacement therapy for hypogonadism. *Steroids*. 2008;73(6):601-610. doi: 10.1016/j.steroids.2008.01.018.

86. Touber ME, van Engelen MC, Georgakopoulus C, van Rhijn JA, Nielen MWF. Multi-detection of corticosteroids in sports doping and veterinary control using high-resolution liquid chromatography/time-of-flight mass spectrometry. *Anal. Chim. Acta*. 2007;586(1-2 SPEC. ISS.):137-146. doi: 10.1016/j.aca.2006.09.058.

87. Schloms L, Swart AC. Rooibos flavonoids inhibit the activity of key adrenal steroidogenic enzymes, modulating steroid hormone levels in H295R cells. *Molecules*. 2014;19(3):3681-3695. doi: 10.3390/molecules19033681.

88. Rijk JCW, Peijnenburg AACM, Blokland MH, Lommen A, Hoogenboom RLAP, Bovee TFH. Screening for modulatory effects on steroidogenesis using the human H295R adrenocortical cell line: A metabolomics approach. *Chem. Res. Toxicol*. 2012;25(8):1720-1731. doi: 10.1021/tx3001779.

89. Schloms L, Storbeck K-, Swart P, Gelderblom WCA, Swart AC. The influence of aspalathus linearis (rooibos) and dihydrochalcones on adrenal steroidogenesis: Quantification of steroid intermediates and end products in H295R cells. *J. Steroid Biochem. Mol. Biol*. 2012;128(3-5):128-138. doi: 10.1016/j.jsbmb.2011.11.003.

90. Jiang M, Klein M, Zanger UM, et al. Inflammatory regulation of steroid sulfatase: A novel mechanism to control estrogen homeostasis and inflammation in chronic liver disease. *J. Hepatol*. 2016;64(1):44-52. doi: 10.1016/j.jhep.2015.07.022.

91. Dong F, Deng D, Chen H, et al. Serum metabolomics study of polycystic ovary syndrome based on UPLC-QTOF-MS coupled with a pattern recognition approach. *Anal. Bioanal. Chem*. 2015;407(16):4683-4695. doi: 10.1007/s00216-015-8670-x.

92. Becue I, Van Poucke C, Van Peteghem C. The use of library identification and common fragments for the identification of corticosteroids in forensic samples. *Anal. Bioanal. Chem*. 2011;400(9):2837-2846. doi: 10.1007/s00216-011-5029-9.

93. Liang Q, Xu W, Hong Q, et al. Rapid comparison of metabolites in humans and rats of different sexes using untargeted UPLC-TOFMS and an in-house software platform. *Eur. J. Mass Spectrom (Chichester, England)*. 2015;21(6):801-821. doi: 10.1255/ejms.1395.

94. Kailasam S. Ultrafast and sensitive analysis of sweeteners, preservatives and flavorants in nonalcoholic beverages using the agilent 1290 infinity LC system. *Agilent Technologies*. 2010.

95. Mack AE, Long WJ. Fast, low pressure analysis of food and beverage additives using a superficially porous agilent poroshell 120 EC-C18 column. *Agilent Technologies*. 2010.

96. EUROOPAN PARLAMENTIN JA NEUVOSTON ASETUS (EU) N:O 1169/2011.

97. EUROOPAN PARLAMENTIN JA NEUVOSTON ASETUS (EY) N:O 1333/2008:Appendix II.

98. Taverniers I, De Loose M, Van Bockstaele E. Trends in quality in the analytical laboratory. II. analytical method validation and quality assurance. *TrAC, Trends Anal. Chem*. 2004;23(8):535-552. doi: http://dx.doi.org.libproxy.helsinki.fi/10.1016/j.trac.2004.04.001.

99. Magnusson B, Näykki T, Hovind H, Krysell M. *Handbook for calculation of measurement uncertainty in environmental laboratories.* 3.1st ed. Oslo: Nordic Innovation; 2012. www.nordtest.info.

100. Hovind H, Magnusson B, Krysell M, Lund U, Mäkinen I. Internal quality control - handbook fo chemical laboratories (trollboken - troll book). 2012;NT TR 569 Edition 4.

101. Gustavo González A, Ángeles Herrador M. A practical guide to analytical method validation, including measurement uncertainty and accuracy profiles. *TrAC, Trends Anal. Chem.* 2007;26(3):227-238. doi: http://dx.doi.org.libproxy.helsinki.fi/10.1016/j.trac.2007.01.009.