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Pekka Keski-Rahkonen

New LC-MS Assays for Drugs and Endogenous Compounds in Small-Volume Biological Samples

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ABSTRACT

This thesis, which consists of four original publications and a summary, explores the feasibility of combining different unconventional ionization techniques and sample preparation methods to improve the sensitivity of liquid chromatography–mass spectrometry (LC–MS) based bioanalytical assays. It addresses important sensitivity-limiting factors, such as the weak electrospray ionization (ESI) of non-polar analytes, ion-suppressing effect of ion-pairing mobile phase additives, and the loss of analytes during sample pre-processing.

The study demonstrates the suitability of using an atmospheric pressure chemical ionization (APCI) source without a corona discharge for the efficient ionization of selected analytes in the presence of ion-pairing agents. This rarely used technique, termed atmospheric pressure thermospray ionization or no-discharge APCI, is also shown to be suitable for use with a novel microchip heated nebulizer. Moreover, oxime derivatization is presented as a simple and efficient method to improve the proton affinity and thus the ESI ionization of non-polar ketosteroids. Small-scale liquid-liquid extraction and direct injection can be utilized to increase the recovery in the sample preparation process. By combining the above techniques, three different assays were developed and their performance was evaluated by validation. The assays were also used for the analysis of samples from clinical and animal studies.

Taken together, this thesis presents alternative or complementary approaches for bioanalytical LC–MS method development. The assays developed represent significant improvements in sensitivity, dynamic range, and robustness and the novel findings regarding ionization in particular provide important new information about some rarely used analytical techniques.

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TIIVISTELMÄ

Tässä neljästä osajulkaisusta ja niiden yhteenvedosta koostuvassa väitöskirjatyössä tutkittiin erilaisten näytteenkäsittely- ja ionisaatiotekniikoiden soveltuvuutta bioanalyyttisten nestekromatografia–massaspektrometriamenetelmien (LC–MS) suorituskyvyn lisäämiseen. Tutkimuksessa pyrittiin löytämään ratkaisuja tunnettuihin mittausmenetelmän herkkyyttä heikentäviin tekijöihin, kuten poolittomien yhdisteiden heikkoon ionisaatioon sähkösumutuksessa (ESI), ioniparireagenssien aiheuttamaan ionisupressioon sekä analysoitavien yhdisteiden puutteelliseen saantoon näytteen esikäsittelyn aikana.

Tutkimuksen mukaan eräiden lääkeaineiden ja endogeenisten yhdisteiden analysoinnissa voidaan hyödyntää ilmanpaine-kemiallinen ionisaatio (APCI) tekniikkaa täysin ilman sen toimintaan olennaisesti liittyvää koronapurkausta. Tuloksena on ESI:n kaltainen ionisaatio, joka toisin kuin ESI-tekniikka yleensä, mahdollistaa ioniparireagenssien käytön ajoliuoksessa ilman merkittävää ionisupressiota. Tutkimuksen mukaan tekniikka soveltuu käytettäväksi normaalin APCI-ionilähteen lisäksi myös kuumasumutus-mikrosirulla, mikä mahdollistaa mikrofluidististen analyysitekniikoiden, kuten kapillaari-nestekromatografian, hyödyntämisen. Lisäksi tutkimuksessa kyettiin parantamaan nestefaasissa ionisoitumattomien steroidien ESI-ionisaatiota kemiallisella derivatisoinnilla sekä näytteenkäsittelyn saantoa pienen mittakaavan neste-neste uuton ja näytteen suoran injektion avulla. Edellä mainittuja tekniikoita yhdistämällä kehitettiin kolme uutta mittausmenetelmää, joiden käytännön suorituskyky arvioitiin validoinnilla sekä biologisten tutkimusnäytteiden analysoinnilla.

Väitöskirjatyön tulokset tarjoavat uusia, vaihtoehtoisia lähestymistapoja bioanalyyttisten LC–MS menetelmien kehitykseen. Kehitetyillä analyysimenetelmillä on huomattavia herkkyyteen, pitoisuusalueeseen sekä luotettavuuteen liittyviä etuja. Erityisesti ionisaatiotekniikoihin liittyvät tulokset sisältävät merkittävää uutta tietoa harvoin käytetyistä analyysitekniikoista.

Luokitus: QU 25, QV 25, QV 26, QV 744, QY 25, QY 90
Yleinen Suomalainen asiasanasto: analyyttinen kemia; analyysimenetelmät; kvantitatiivinen analyysi; kliininen kemia; nestekromatografia; massaspektrometria; tamsulosiini; labetaloli; asetyylikoliini; steroidit; validointi

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December 14, 2011. Sydney, Australia

Pekka Keski-Rahkonen

List of the original publications

This dissertation is based on the following original publications, hereafter referred to by their Roman numerals (**I-IV**):

I Keski-Rahkonen P, Pärssinen O, Leppänen E, Mauriala T, Lehtonen M, Auriola S. Determination of tamsulosin in human aqueous humor and serum by liquid chromatography–electrospray ionization tandem mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis 43: 606–612, 2007.*

II Keski-Rahkonen P, Lehtonen M, Ihalainen J, Sarajärvi T, Auriola S. Quantitative determination of acetylcholine in microdialysis samples using liquid chromatography/atmospheric pressure spray ionization mass spectrometry. *Rapid Communications in Mass Spectrometry 21: 2933–2943, 2007.*

III Keski-Rahkonen P, Haapala M, Saarela V, Franssila S, Kotiaho T, Kostiainen R, Auriola S. Atmospheric pressure thermospray ionization using a heated microchip nebulizer. *Rapid Communications in Mass Spectrometry 23: 3313–3322, 2009.*

IV Keski-Rahkonen P, Huhtinen K, Poutanen M, Auriola, S. Fast and sensitive liquid chromatography-mass spectrometry assay for seven androgenic and progestagenic steroids in human serum. *Journal of Steroid Biochemistry and Molecular Biology 127: 396–404, 2011.*

The publications were adapted with the permission of the copyright owners. Some unpublished results are also presented.

Contents

Abbreviations

ACh	Acetylcholine
AH	Aqueous humor
APCI	Atmospheric pressure chemical ionization
API	Atmospheric pressure ionization
APPI	Atmospheric pressure photoionization
APTSI	Atmospheric pressure thermospray ionization
CID	Collision induced dissociation
CSF	Cerebrospinal fluid
ESI	Electrospray ionization
FDA	Food and Drug Administration
FT–ICR	Fourier transform ion cyclotron resonance
GC	Gas chromatography
HFBA	Heptafluorobutyric acid
HILIC	Hydrophilic interaction liquid chromatography
HPLC	High performance liquid chromatography
ISO	International Organization for Standardization
IT	Ion trap
LC	Liquid chromatography
LLE	Liquid-liquid extraction
LLOQ	Lower limit of quantification
LPME	Liquid phase microextraction
MEPS	Microextraction in packed syringe
MRM	Multiple reaction monitoring
MS	Mass spectrometer/spectrometry
MS/MS	Tandem mass spectrometry
MSPD	Matrix solid phase dispersion
MTBE	Methyl *tert*-butyl ether
m/*z*	Mass-per-charge
PPT	Protein precipitation

QC	Quality control
QQQ	Triple quadrupole
RAM	Restricted access media
RP	Reversed phase
RSD	Relative standard deviation
SBSE	Stir-bar sorptive extraction
S/N	Signal-to-noise
SPE	Solid phase extraction
SPME	Solid phase microextraction
SRM	Selected reaction monitoring
TEA	Triethyl amine
TFA	Trifuoroacetic acid
TSI	Thermospray ionization
TOF	Time-of-flight
UV	Ultraviolet

1 Introduction

Quantitative analysis of small organic compounds in biological samples is an integral aspect of research in many fields, such as pharmaceutical development, clinical diagnosis, forensic and environmental toxicology, as well as in studying biological organisms. The analytes consist of different exogenous and endogenous compounds together with their metabolites, while the samples are usually different bodily fluids or tissues. Due to the complexity of the biological sample material and the often low sample volumes and analyte concentrations, highly selective and sensitive analytical methods are needed. Currently, the combination of chromatography and mass spectrometry is one of the most commonly used techniques. Although gas chromatography–mass spectrometry (GC–MS) has existed for decades (Ryhage, 1964), the amount of published work based on liquid chromatography–mass spectrometry (LC–MS) has increased rapidly, particularly during the last decade (Figure 1).

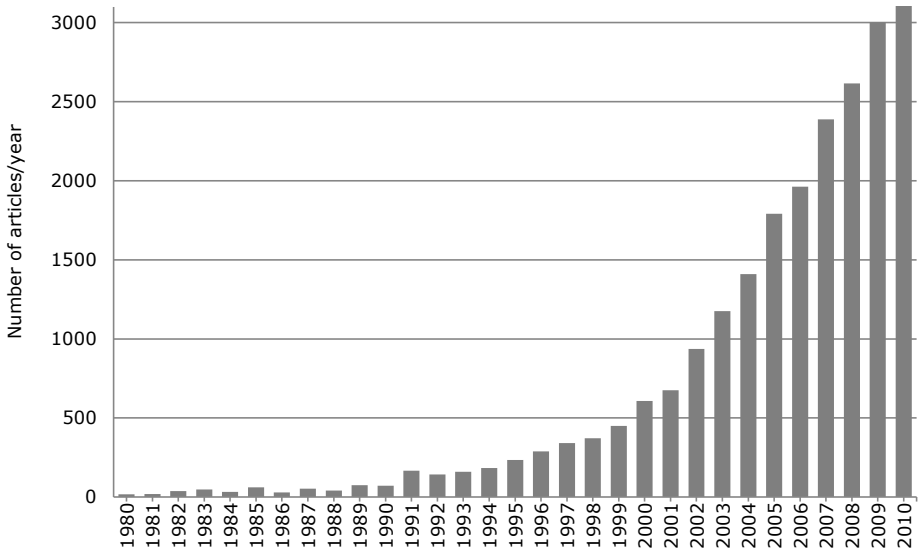

Figure 1. *The number of articles related to liquid chromatography–mass spectrometry. ISI Web of Knowledge, Thomson Reuters. Search string: Topic=("liquid chromatography–mass spectrometry") OR Topic=("lc-ms").*

The popularity of LC–MS is often attributed to its suitability for the analysis of polar compounds without derivatization, soft ionization conditions, fast chromatography, and the straightforward sample preparation techniques that can be used (Niessen, 1999). However, despite the advantages of LC–MS, insufficient sensitivity, selectivity, or some other performance-related aspect often limits the use of these assays. Sensitivity and selectivity not only depend greatly on the mass spectrometer in use, but also on several other factors including sample preparation, type and conditions of the separation stage, and choice of ionization method.

Since the amount of analytes in biological samples is usually low, improving the sensitivity has long been one of the main objectives in developing new bioanalytical assays, being also a strong driving force in the introduction of new MS instruments. However, in addition to adequate sensitivity, a method has to be reliable and reproducible for its intended purpose. Moreover, depending on the purpose of the method, several other factors may need to be considered. As a summary, an ideal bioanalytical assay would be:

- Sensitive enough for its intended purpose
- Accurate and precise across the required concentration range
- Robust in use
- Fast
- Easy to operate
- Safe for its operators
- Environmentally friendly
- Economical

In practice, it may be difficult to achieve all of these requirements in a single method. The work described in the present thesis concentrates on increasing the sensitivity of bioanalytical methods, but also addresses the above-mentioned factors that are related to the assay usability. The sensitivity issue is approached by exploiting different sample preparation techniques to maximize the analyte recovery (**I**, **II**, **IV**), together with new ionization techniques (**II**, **III**) and a derivatization method (**IV**) to improve the analyte recovery and response at the ionization stage. These two stages, sample preparation and ionization, were selected for their significance in the loss of analyte molecules during sample analysis.

Chapter 2 reviews the LC–MS instrumentation and bioanalytical methodology with an emphasis on sample preparation, ionization and quantification aspects.

Chapter 3 presents the specific challenges arising from low analyte amounts, dissecting an assay into stages where loss of analytes occurs and offering methodological solutions for the analyte preservation. Aims of the study are given in Chapter 4 and the experimental details in Chapter 5. The results are divided into three sections in Chapter 6, the first section discussing the sample preparation stage, the second describing the results achieved by modifying the ionization stage, and the third summarizing the developed assays. The overall results of the study are summarized and concluded in Chapter 7.

2 LC–MS in Quantitative Bioanalysis

2.1 CHROMATOGRAPHIC SEPARATION

High performance liquid chromatography (HPLC) has become a major analytical technique, particularly in the field of pharmaceutical and biomedical analysis (Unger et al., 2010; Görög, 2007). Despite the selectivity of the MS detection system, liquid separation is also a fundamental part of LC–MS assays for biological samples. The main reason for this is the complexity of the biological sample material. The presence of isobaric compounds is obvious, as is the possibility of similar product ions from the isobaric compounds. Without differences in the mass-to-charge (m/z) values either at MS or MSn levels, no mass spectrometric selectivity can be obtained. In addition, ionization enhancement or suppression due to coeluting sample components is also possible (Matuszewski et al., 1998; Matuszewski et al., 2003). If the compounds of interest are susceptible to this phenomenon, it can have a detrimental impact on assay sensitivity or reliability. Moreover, depending on the efforts made during sample pre-processing, the sample material can contaminate the ion source over time, although this can be avoided if selected chromatographic fractions can be diverted away from the mass spectrometer inlet.

Chromatographic techniques

In its modern form, the chromatographic separation of small organic compounds is most often performed with reversed phase (RP) columns (Majors, 2009). Since many analytes of biological activity are relatively polar, other forms of liquid chromatography can also be used, such as hydrophilic interaction chromatography (HILIC) (Alpert, 1990; Alpert, 2011), ion exchange chromatography, or techniques that utilize mixed column functionalities, such as reversed phase with embedded ion exchange capabilities (Nogueira at al., 2005; Ma et al., 2008). The dimensions of the chromatographic column used in LC–MS methods have been mostly dictated by different practical aspects such as the resolution needs and the mode of ionization in use. Electrospray ionization (ESI) is a concentration-sensitive process that benefits from the use of low eluent flow rates, which are best combined with sub-3 mm (i.d.) columns, while atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) are considered as mass-flow sensitive techniques that allow much higher flow rates and thus the use of wider column diameters

(Kebarle and Ho, 1997; Voyksner, 1997). However, since high assay sensitivity is usually desirable, column diameter can be also used to control the sample dilution within the column. For instance, decreasing the diameter may be used to increase the MS response obtained from a certain amount of analytes being injected, or to preserve the response when the injection volume is decreased. Small-diameter columns have several other attractive features, which were noticed very early in the development of HPLC; research on the miniaturization of the column dimensions was started already in the late 1960's, with the first international meeting on microcolumn separation methods held in 1982 (Takeuchi, 1990). However, due to difficulties in the operation of the capillary LC systems and the limited detection techniques, columns with internal diameters between 2 and 5 mm have remained more popular in routine use. Recently there has been increasing interest in microfabricated devices and other microanalytical systems that can manipulate extremely small sample volumes. With these techniques, capillary- and microfluidic LC is usually employed to maintain sensitivity (Yin and Killeen, 2007; West et al., 2008).

Current status

The development of LC column technology has been largely focused on RP columns, with silica as a major base material for the stationary phase, despite its limited pH stability (Unger et al., 2008). Monolith columns have been available for more than a decade, but despite the advantages of monoliths over particles, they have become overtaken by the recent advances in the particle technology (Majors, 2011). Decreasing the particle size, while maintaining the quality of the packing, has been an area of particular interest. The reason for the use of particles with sub-2 μm mean diameter in place of the previously utilized 3–5 μm can be seen from the following theoretical resolution equation (Snyder et al., 2010):

$$R_S = (1/4)\,(\alpha - 1)\,N^{1/2}\,\{k/(1 + k)\} \qquad (1)$$

where the retention factor k, selectivity α, and the number of theoretical plates N (column efficiency) contribute to the chromatographic separation. Since N is inversely proportional to particle diameter (Snyder et al., 2010), by decreasing the particle size by a factor of three, from 5 μm to 1.7 μm, N is also changed by three and the R_S by the square root of three, or 1.7. As N is also inversely proportional to the square of the peak width (Snyder et al., 2010), the resulting narrower peaks improve

the separation, resulting also in higher peaks, as the peak area remains the same. Thus, in theory, the separation efficiency of a column that has certain internal dimensions can be improved by decreasing the particle size of the packing. Alternatively, the column can be shortened to achieve faster separations, without affecting the separation efficiency. The small particles can also offer greater flexibility in selecting the optimal mobile phase flow rate, which can be predicted using van Deemter calculations (van Deemter et al., 1956). The advantages of the described columns are obvious and their use has been rapidly increasing (Guillarme et al., 2010). However, one downside of the sub-2 μm particles is that the column backpressure (Δp) increases at a greater rate than N when the particle size (d_P) is decreased ($\Delta p \propto 1/d_P^2$) (Meyer, 2010). The resulting increase in the operational pressures has had an profound effect on the entire HPLC instrument development and to enable the efficient use of the new small-particle columns, systems capable of operating at and beyond 1000 bar (14000 psi) have been introduced (Nováková and Vlcková, 2009).

Despite the new generation of HPLC instruments, the full theoretical advances from the use of small particles are still not always realized (Fekete et al., 2010; Petersson et al., 2011) and the very high pressures and the increased susceptibility to plugging of the small-pore column frits have contributed to another field of column development: by using slightly bigger particles (2–3 μm) with a solid core and porous surface, several advantages, such as lower backpressures, can be realized without losing the improvements in separation efficiency (Abrahim et al., 2010; Oláh et al., 2010). Since 2011, these superficially porous particles have also been commercially available in 1.7 μm size and experimentally synthesized in even smaller diameters (Blue and Jorgenson, 2011). The porous surface layer of these particles can be as little as 0.1 μm, which provide a significantly lower diffusion path and higher rate of mass transfer in comparison to the fully porous particles of the same diameter.

Liquid chromatography–Mass spectrometry

Despite the recent improvements in the field, achieving the full potential of HPLC is limited in LC–MS due to specific needs associated with the two techniques, as noted by the pioneers of LC–MS already in 1975: "*The chief problem at this time is that of achieving a useful balance between retaining the advantages of LC separation methods and accepting the requirement for vaporization of the solute*" (Carroll et al., 1975). Certain mobile phase additives are usually required in the LC stage to achieve or improve

the separation, while at the ion source, the analyte molecules must be transferred from the condensed phase into the gas phase. The required phase-transfer necessitates the use of fully volatile eluents, precluding some of the most useful buffers, such as phosphate, that are commonly used to control analyte ionization in HPLC. It can be difficult to find substitutive volatile additives with adequate buffer capacity working in an appropriate pH range. In addition, many ion-pairing additives are problematic for MS ionization, particularly with ESI. It is well known that halogenated carboxyl acids, such as trifluoroacetic acid (TFA) and heptafluoroacetic acid (HFBA), usually produce severe ion suppression with ESI (Kuhlmann et al., 1995). Some ion pairing agents, such as triethylamine (TEA), are also known to strongly contaminate the MS instrument through surface adsorption, regardless of the ionization mode (Trufelli et al., 2011). In bioanalytical assays, high sensitivity is often required, and thus ion-pairing agents are usually avoided or used at low concentrations.

Sometimes the most efficient ionization is achieved with an eluent that is incompatible with the chromatography being employed. This is common with APPI that typically requires the use of a dopant solvent, which mediates the ionization process (Kauppila et al., 2002). Since the dopant cannot be added to the mobile phase without affecting the separation, it is infused post-column using a separate pump, which adds complexity to the instrumentation.

In some cases, derivatization is an efficient way to improve analyte ionization (Gao et al., 2005; Iwasaki et al., 2011). Particularly with ESI, this can be used to improve the gas-phase proton affinity of non-polar compounds (Higashi and Shimada, 2006; Liu et al., 2000). However, derivatization reactions can produce a variety of isomeric products from a single precursor that may be separated chromatographically, dividing the analyte response into separate chromatographic peaks, or otherwise complicating the separation (Kalhorn et al., 2007).

2.2 ANALYTE IONIZATION

As mentioned in the previous section, the early difficulties of combining LC and MS arose from the two instruments' different requirements for the sample (Arpino, 1982). However, after several different approaches in combining LC and MS (Niessen, 2006), the development of APCI (Horning et al., 1974a; Horning et al., 1974b; Carroll et al., 1975) and ESI (or ionspray) (Dole et al., 1968; Yamashita and Fenn, 1984a, Yamashita and Fenn, 1984b; Bruins et al., 1987; Fenn et al., 1989) has enabled the simple and efficient coupling of the instruments. In addition to the above methods,

many other ionization techniques and their modifications have been described later, but with the exception of APPI (Robb et al., 2000; Syage et al., 2000), they have remained marginal players and are not commercially available from the major MS instrument producers. In this section, the three most commonly employed techniques will be shortly presented.

Electrospray ionization

The basis of the ESI is the spraying of the mobile phase from the tip of a needle with the help of potential difference between the needle and the mass spectrometer inlet. The applied electric field leads to the formation of a Taylor cone at the needle tip (Taylor, 1964). The charged liquid escapes through the cone apex as small droplets, with charge imbalance generated by the electrical current applied to the needle. When the droplets shrink by evaporation, the charge density within the droplets increases. The increasing charge repulsion at the droplet surface finally exceeds the Rayleigh limit, leading to the formation of new droplets by Coulombic fission (Gomez and Tang, 1994). The process continues until all solvent molecules have been evaporated, or the droplet radius is sufficiently small to allow the charged analyte molecules to escape the droplet by field desorption. Modern ion sources employ heat and gas flow to assist the ESI process (Kebarle and Verkerk, 2009).

Atmospheric pressure chemical ionization

APCI is a chemical ionization process. The mobile phase is vaporized in the ion source with the help of heat and gas flow. A sharp needle with a high voltage is positioned in the forming gas cloud. A corona discharge at the needle tip generates high-energy ions from air, water, and solvents. Since the mobile phase is usually a mixture of water and an organic solvent, such as methanol or acetonitrile, the secondary chemical reagents are protonated or deprotonated ions of these solvents (e.g. H_3O^+ or OH^-) that can react with the analytes, leading to analyte ionization (Carroll et al., 1981; Dzidic et al., 1974).

Atmospheric pressure photoionization

APPI is another form of chemical ionization that is based on similar ion source architecture as APCI. The main difference is that an ultraviolet (UV) lamp is used in place of a corona discharge needle. Most often, post-column infusion of suitable dopant solvent is employed. When the ionization energy of the dopant is sufficiently low, it can start the ionization process by absorbing photons generated by the UV

lamp. In the process, the formed dopant radical cation either loses a proton to the analyte with a higher proton affinity, or accepts an electron from a species with lower electron affinity, in which case the latter will be seen as a radical cation (Robb and Blades, 2006; Kauppila et al., 2002; Kauppila et al., 2004a; Robb et al., 2008; Robb and Blades, 2005).

The suitability of these ionization techniques for the analysis of different compounds is often classified by their applicability to analytes with different polarities and molecular weights (Figure 2).

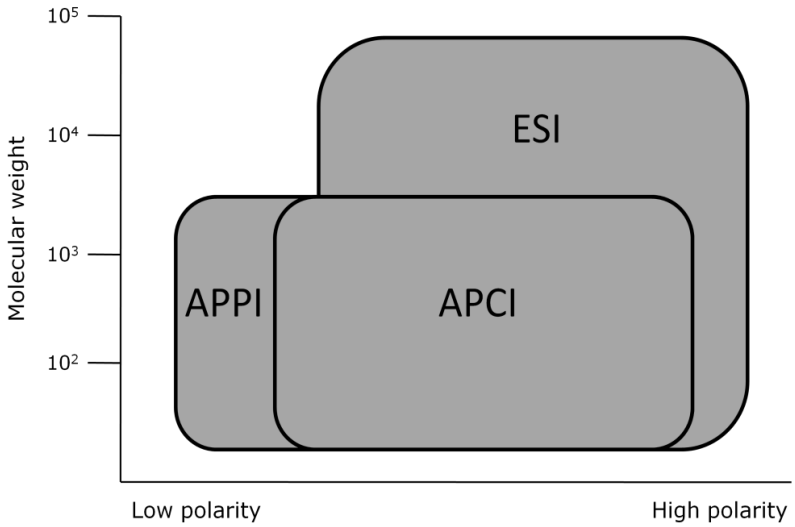

Figure 2. *Approximation for the suitability of ESI, APCI, and APPI ionization techniques for analytes with different polarity and molecule weight*

ESI is thought to be a softer ionization technique than APCI and APPI, being suitable for the analysis of macromolecules such as proteins (Mann et al., 1989). It also results in an efficient ionization of compounds with structures that can be ionized in solution, such as many drugs. However, ESI is associated with low mobile phase flow rates and stronger dependency on the eluent composition than APCI and APPI (Kostiainen and Kauppila, 2009), and is also more susceptible to matrix effects (ME) (Enke et al., 1997; Souverain et al., 2004a). For the ionization of non-polar compounds, APCI and APPI are more feasible and APPI in particular has been found to be superior for the analysis of non-polar compounds in complex biological matrices, such as endogenous steroids present at low concentrations (Harwood and

Handelsman, 2009). In an attempt to combine the advantages of the mentioned ionization techniques and to cover broader range of analytes, such as in non-targeted metabolomic studies (Nordström et al., 2008), combinatory or multimode techniques (e.g. ESI and APCI or APPI in the same ion source) have also been developed (Gallagher et al., 2003; Short and Syage, 2008). Some of these multiple ionization techniques are commercially available from different MS manufacturers.

Current use of different ionization techniques

ESI continues to be the most widely used ionization technique. A search made in the Web of Science (ISI Web of Knowledge, Thomson Reuters) reveals the role of ESI in the field of LC–MS (Figure 3). The number of publications describing ESI–MS has continuously increased over the past 20 years, while APCI has remained as a complementary technique. APPI appears for the first time in the year 2000 (Robb et al., 2000; Syage et al., 2000) with 79 reports in 2010. Older techniques, such as thermospray ionization (TSI) (Blakley et al., 1980; Blakley and Vestal, 1983) have become obsolete at the turn of the millennia.

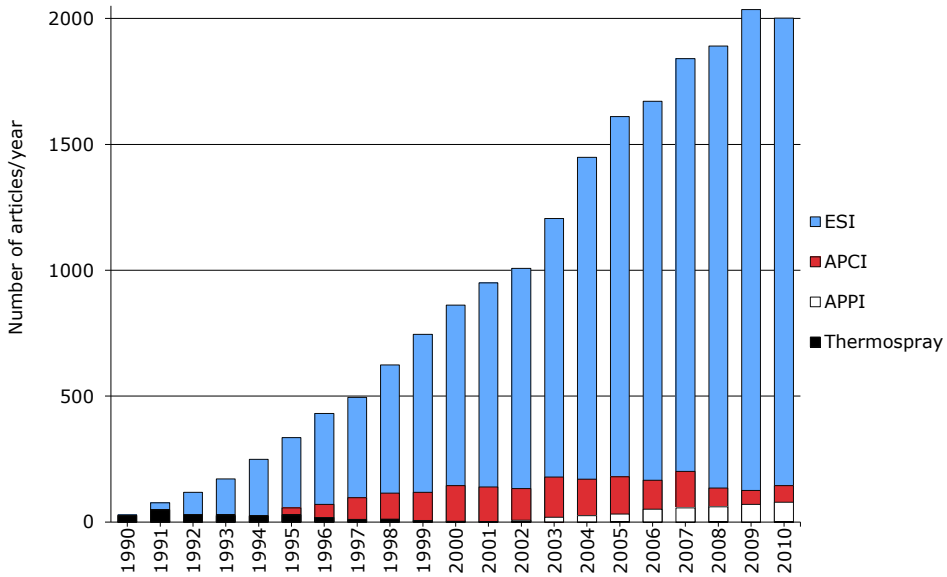

Figure 3. *The different ionization techniques referred to in the published articles during the last 20 years. ISI Web of Knowledge, Thomson Reuters. Representative search string: Topic=("electrospray ionization") OR Topic=("electrospray ionisation") AND Topic=("mass spectrometry").*

In addition to ESI, APCI, and APPI, several other atmospheric pressure ionization techniques or methods have been described in recent years. Many of these are based on the modifications or the unconventional use of existing ion sources, including sonic spray ionization (Hirabayashi et al., 1994), no-discharge APCI (Cristoni et al., 2002), zero needle voltage ESI (Sørensen et al., 2008), photon independent ionization (Hommerson et al., 2007), atmospheric pressure laser ionization (Constapel et al., 2005), and cold-spray ionization (Sakamoto et al., 2000). The majority of the mentioned techniques rely on the use of ESI, APCI, or APPI source, which however is operated only as a pneumatic nebulizer or thermal vaporizer, without applying any electrical potential, discharge, or photo irradiation for the generation of ions. Although these ionization techniques have some advantages over the more established counterparts, they have not generated widely implemented commercial solutions.

Some inherent drawbacks of ESI, mostly its susceptibility to ion suppression (Enke et al., 1997; King et al., 2000) and dependency on the flow rate have been reduced by the recent advances in ion source design. This has mainly been achieved by addition of heating capabilities and through the improvements in the use of drying and nebulizing gases (Ikonomou and Kebarle, 1994; Kebarle and Verkerk, 2009).

The decrease in the dimensions of the chromatographic columns has created a need for smaller ion sources to reduce sample dilution after the separation stage. A number of techniques are described that combine ESI with microfluidic separations, often spraying directly from a capillary column (Koster and Verpoorte, 2007). ESI in itself has also some advantages when operated at very low flow rates and with small diameter electrospray emitters (Schmidt et al., 2003; Marginean et al., 2008), so the efforts in downscaling the dimensions of this ionization technique are not always dictated by the dimensions of the LC. The combination of an entire separation system and an ESI ion source within a single microchip has been available from Agilent Technologies since 2005. In addition, several different micro- and nano-ESI appliances also exist that can be used to improve the interfacing of LC and MS.

Challenges related to ionization

In bioanalytical method development, the choice of ionization technique is usually based on the nature of the analyte. For compounds that can be charged in solution, ESI is a straightforward choice, leaving APCI or APPI usually as a second alternative, often used for less polar analytes. However, in addition to the ionization capabilities

of the analyte, the sample matrix and the chromatographic conditions have an effect on the suitability of the ionization mode. As noted earlier, ESI is sensitive to the mobile phase constituents, flow rate, and the presence of matrix based compounds that co-elute with the analyte (Kostiainen and Kauppila, 2009). The pH of the mobile phase, for instance, has a strong effect on the ionization of cationic and anionic analytes. Furthermore, if the chromatographic separation necessitates the use of an ion pairing agent, such as TFA, to generate retention for polar cationic compounds, some degree of ion suppression is usually inevitable. This can also result from the co-elution of matrix compounds, often of an unknown nature. The actual mechanisms of ion suppression are debated, but in the case of ESI, it appears to be related to the processes taking place at the droplet surface (Enke et al., 1997). Since APCI and APPI do not involve any competition between analytes to enter the gas phase from the surface of the shrinking droplets, they can, in theory, produce a better analyte response in the presence of ion suppressing agents (Marchi et al., 2007; Souverain et al., 2004a). However, some compounds, quaternary ammonium ions being a good example, cannot be detected with APCI (Sakairi and Kato, 1998) or APPI (Robb and Blades, 2009; Syage et al., 2004), leaving ESI as the only choice, despite its limitations.

Derivatization has been particularly useful for the formation of $[M]^+$, $[M+H]^+$, or $[M-H]^-$ ions of compounds that are poorly ionized in their native form (Higashi and Shimada, 2006; Singh et al., 2000). However, it is usually avoided in the development of quantitative LC–MS assays, as it makes the sample preparation more laborious and is a potential source of measurement errors.

Lastly, as mentioned above, the use of capillary and nanoscale columns has created a demand for miniaturized ion sources. However, the majority of the compatible designs have been based solely on ESI, which has constrained the development of microscale LC–MS assays (Wood et al., 2003; Koster and Verpoorte, 2007). Only recently, an experimental heated microchip nebulizer suitable for APCI and APPI ionization was described (Östman et al., 2004; Kauppila et al., 2004b), which triggered great interest in the development of microchip based ion sources that could be used for ionization techniques other than ESI (Sikanen et al., 2010).

2.3 MASS SPECTROMETRIC DETECTION

Within the mass spectrometer, analyte ions generated in the ion source are directed into a system that consists mainly of mass analyzers and a detector. The most widely used mass analyzers are quadrupole mass filters, different types of ion traps (IT) and

orbitraps, magnetic sectors, Fourier transform ion cyclotron resonance spectrometers (FT–ICR or FTMS), and time-of-flight (TOF) analyzers. Different hybrid instruments also exist, including quadrupole–quadrupole (or triple quadruple; QQQ), quadrupole–time-of-flight (Q–TOF), ion trap–time-of-flight (IT–TOF), time-of-flight–time-of-flight (TOF–TOF), and different combinations of trap analyzers, such as quadrupole–ion trap (Q–Trap). When used for quantitative analysis in combination with LC, the type of MS is usually based on quadrupole, TOF, or IT analyzers. Depending on the intention of the analytical work, the mass analyzers' different characteristics regarding sensitivity, scan speed, dynamic range, resolving power, mass accuracy, and mass range can be exploited. The highest possible mass accuracy, resolving power and the constant acquisition of spectra are seldom essential for quantitative bioanalysis, but sensitivity, dynamic range, and speed are important. These properties are usually considered best obtained with quadrupoles, certain IT analyzers, and hybrid instruments based on their combinations. However, whereas triple quadrupole technology is considered fairly mature (Bennett, 2011), recent instrument developments have improved the quantitative capabilities of other analyzers, particularly of the TOF-type, that now have a dynamic range of around four orders of magnitude, in addition to their inherently high speed and good resolving power (Williamson et al., 2008; Bristow et al,. 2008; Pelander et al., 2011; Fung et al., 2011). Moreover, as the quantitative performance of orbitrap based instruments approaches or even matches that of QQQ (Zhang et al., 2009; Kaufmann et al., 2011; Romero-González et al., 2011), the use of high resolution MS instruments is likely to become more wide-spread in quantitative bioanalysis (Ramanathan et al., 2011).

Tandem mass spectrometry (MS/MS) is usually employed for increased analyte selectivity in quantitative bioanalysis. With QQQ instruments, this is achieved by operating the first quadrupole as an m/z selective filter, fragmenting the ions through collision-induced dissociations (CID) in the second quadrupole that acts as a collision cell, and transferring the product ions on to a third quadrupole that is used similarly to the first one (selected reaction monitoring or multiple reaction monitoring, SRM or MRM, respectively). Although the collision cells in the modern QQQ instruments may not be of quadrupole design, the instruments are still commonly known as "triple quadrupole". MS/MS can also be performed with IT analyzers, but whereas QQQ instruments achieve MS/MS in space, an IT mass spectrometer performs MS/MS in time. This is achieved by injecting ions into a trap for a certain time, destabilizing the trap for all except the selected m/z ions, applying

molecule-fragmenting conditions and passing the formed ions out in a scanning function. These multiple steps usually limit the achievable scan speeds, making QQQ instruments more favorable for LC–MS use if fast data acquisition of numerous MS/MS transitions with maximum sensitivity is needed. However, especially the more recent linear quadrupole IT instruments (Douglas et al., 2005) can perform well in many quantitative applications requiring high sensitivity (Schwartz et al., 2002) and a CID process that is distinct from that of QQQ instruments, may also result in improved sensitivity (Shipkova et al., 2008).

Notes on MS/MS detection

Despite the compound selectivity available with the MS/MS technique, some detection-related challenges in bioanalytical LC–MS still remain. As noted earlier, isobaric compounds must have either chromatographic resolution or different product ions, in order to be separated. However, as a result of a cross-talk effect, selectivity issues can be experienced even if the compounds have different precursor ions. In QQQ instruments, this occurs when the dwell times of individual MS/MS transitions are short enough to enable the fragment ions from a previous transition to be monitored. The effect has particular importance with assays employing fast chromatography and numerous MS/MS transitions, as adequate cycle times can usually be obtained only by decreasing the dwell times of individual transitions (Tong et al., 1999). Developments in collision cell technology have reduced the possibility of cross-talk (Loboda et al., 2000), but it must be taken into account if very short dwell times are being used (Gergov et al., 2003).

Another issue affecting the use of MS/MS is related to the analysis of compounds with low proton affinities. Especially with ESI, the formation of different adducts, such as $[M+Na]^+$ and $[M+K]^+$, is sometimes favored over protonation of the analyte molecules. These adducts may be used for single ion monitoring (SIM) and their formation can be even promoted by using different mobile phase additives. However, these adducts are generally not considered suitable as precursors for MS/MS, since structurally relevant fragment ions may not be produced in the CID process of the alkali-metal adducts of non-ionizable compounds due to the dissociation of the complexes to bare alkali-metal ions (Maleknia and Brodbelt, 1992). Some adducts however, such as lithium adduct of vitamin D, are reported to be sufficiently stable for quantitative MS/MS (Casetta et al., 2010; Yuan et al., 2011).

Finally, with MS/MS, the fragmentation characteristics of the analyte and the number of different product ions resulting from the dissociation of the precursor ion

are reflected on the limits of detection and quantification of an assay – distribution of the charge to a great number of different product ions leads to difficulties in selecting a single high-intensity product ion. However, the overall sensitivity of the LC–MS/MS assay depends also to a great extent on the MS instrument in use.

2.4 SAMPLE PREPARATION TECHNIQUES

A search made in Web of Science for different sample matrices using the words "liquid chromatography-mass spectrometry" as a publication topic reveals the significance of blood sample analysis (usually in form of plasma and serum) in bioanalytical research (Figure 4).

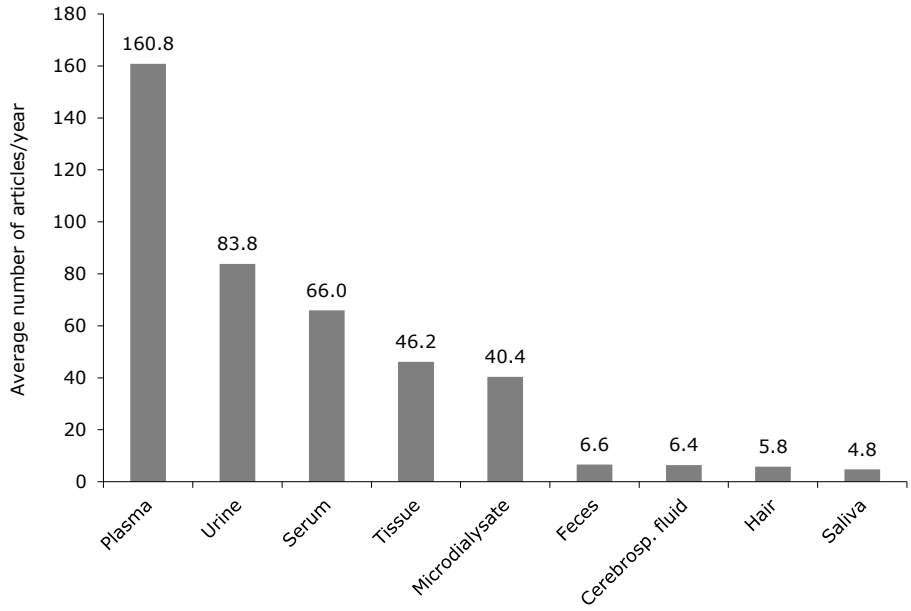

Figure 4. *Occurrence of some commonly analyzed biological sample matrices in the articles associated with LC–MS during the years 2006–2010. ISI Web of Knowledge, Thomson Reuters. Representative search string: Topic=("liquid chromatography–mass spectrometry") AND Topic=("plasma").*

However, even if sample preparation is a potential source of measurement errors (Skonberg et al., 2010), direct injection of plasma, serum, or some other commonly analyzed sample matrix into the separation column is rarely feasible. Even if most sample types are aqueous in nature, they usually contain material that will not be dissolved in the mobile phase and thus can obstruct the chromatographic column.

The sample may also contain ion-pairing substances that change the retention behavior of the column by accumulating in the stationary phase. Depending on the ionization technique employed, MS detection is also sensitive to ion suppression, for example due to phospholipids, nonvolatile material, different extractables from plastics, and other sample-related, often unknown, factors (Souverain et al., 2004a). In addition to ion suppression, any non-volatile material in the sample solution will accumulate on the ion source surfaces. Moreover, despite the combined selectivity of chromatography and MS, in some cases, the sample may need additional fractionation before the LC–MS analysis.

Current use of different sample preparation techniques

There are several different sample preparation techniques (Kole et al., 2011). For the LC–MS analysis of drugs and endogenous compounds, the main objective is removal of the abundant proteins. The protein content in biological matrices is high; in human serum, for instance, the normal albumin concentration is around 3–5 grams per 100 ml (Rustad et al., 2004). However, even after the protein removal, additional clean-up may be needed, depending on the sample and the LC–MS instrumentation in use. To gain some insight into the current use of different methods, 21 recent issues of the *Journal of Chromatography B* and the *Journal of Pharmaceutical and Biomedical Analysis* were reviewed. In these issues, 141 LC–MS assays for human or animal samples were presented (Table 1).

Table 1. *Current use of different sample preparation techniques. Number of times described in 21 recent issues of J Chromatogr B (Volume 878, issues 15–25) and J Pharm Biomed Anal (Volumes 51–52).*

Sample matrix	PPT	LLE	SPE	Direct injection	Other
Plasma	43	32	18	0	4
Urine	4	3	6	1	0
Tissue	6	2	3	0	0
Feces	3	2	0	0	0
Serum	1	2	2	0	0
Whole blood	2	1	1	0	0
CSF	0	0	1	1	1
Microdialysate	0	0	0	1	1
Total	**59**	**42**	**31**	**3**	**6**

PPT: protein precipitation
LLE: liquid-liquid extraction
SPE: solid phase extraction

Of all the described methods, the vast majority were employing protein precipitation (PPT) with miscible organic solvents, liquid-liquid extraction (LLE) with immiscible organic solvents, or solid phase extraction (SPE) with various stationary phases. Only three methods described direct injection of samples without any pre-treatments. The number of sample preparation techniques other than PPT, LLE, and SPE was less than 5 percent. These techniques included solid phase microextraction (SPME) (Arthur and Pawliszyn, 1990), stir-bar sorptive extraction (SBSE) (Baltussen et al., 1999) matrix solid phase dispersion (MSPD) (Long et al., 1990) and the use of an on-line column based on restricted access media (RAM) (Hagestam and Pinkerton, 1985; Šatínský and Solich, 2007).

Although not present in the reviewed articles, several additional sample preparation techniques such as microextraction in packed syringe (MEPS) (Abdel-Rehim, 2004) and liquid phase microextraction (LPME) (Jinno et al., 1996) are available. A significant new addition to the repertoire of commercially available sample preparation products is an SPE-type cartridge that combines PPT with specific removal of the ion-suppressing phospholipids (Pucci et al., 2009). However, despite these new techniques, LLE, PPT, and SPE have retained their popularity, most probably due to their simplicity. In particular, PPT and LLE are straightforward procedures that can be performed in basic test tubes or centrifuge tubes (Figure 5). They are also relatively easy to scale down, and performing PPT or LLE well plates has gained popularity. Even though usually relying on the use of commercial supplies, SPE can offer more variability in analyte selectivity due to the great number of different packings currently available.

Challenges related to sample preparation

Despite their popularity, LLE, PPT, and SPE all retain some negative aspects. Samples that are prepared with PPT are typically associated with stronger ME than those made using LLE and SPE (Souverain et al., 2004a). This is significant, as PPT and the ion-suppression prone ESI are the two most widely used techniques. With LLE, high recoveries cannot be realized for hydrophilic compounds, as the extraction process is based on the use of solvents that are immiscible with the aqueous sample media. SPE is an efficient technique, but the method development can be time consuming, especially if the physicochemical properties of the analytes are diverse (Marchi et al., 2009). Moreover, all these techniques are usually performed off-line, which limits the sample throughput. Instruments for automated sample preparation are available and there is increasing interest in on-line

techniques (Mitchell et al., 2010). However, as evidenced by Table 1, this interest has not yet challenged the popularity of the traditional techniques, as only four of the 141 presented methods employ on-line sample preparation.

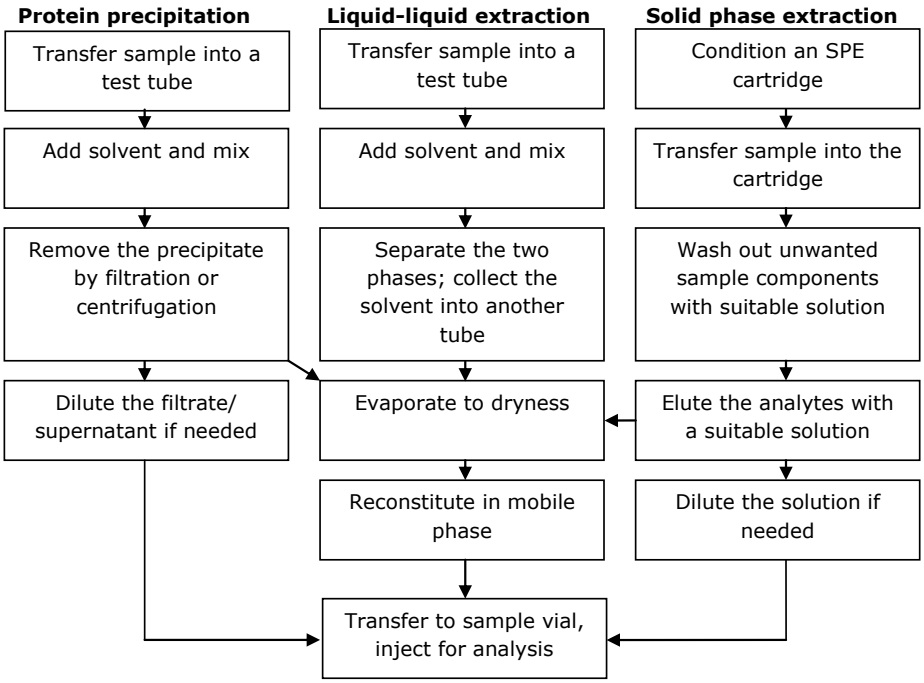

Figure 5. *Principles of the three most commonly used sample preparation techniques*

Lastly, in addition to influencing the throughput, clean-up efficiency, recovery, and precision of the method, the sample preparation technique contributes significantly to the total cost and environmental impact of the analyses. In the ideal case, the biological sample would be injected as such, without a separate sample preparation stage. Certain commercial instruments are available that do achieve this possibility, incorporating on-line SPE or RAM in the analytical instrumentation (Souverain et al., 2004b). For a bioanalytical laboratory, this is an attractive approach, but its implementation requires considerable investments, compared to the traditional methods.

2.5 QUANTIFICATION, VALIDATION, AND RELIABILITY OF RESULTS

A bioanalytical LC–MS assay is built on the same principles as a typical quantitative HPLC method. Chromatographic peak areas are usually taken as the index of analyte response and the concentrations of the unknowns are determined by using the response-concentration equation of the calibration curve. Due to the complexity of biological matrices, the calibration standards are usually prepared by adding the analyte to a blank matrix and processing it similarly to the so-called incurred samples (samples taken from human or animal subject after administration of the analyte). To compensate for the variable loss of analyte in the different stages of sample preparation and analysis, internal standards (IS) are often employed. In this case, peak area ratios of the analyte to the IS are calculated as a function of the concentration of the analyte.

Assay validation

In addition to the technical aspects of a LC–MS assay, it must fulfill its intended purpose, which is usually the sufficiently accurate determination of the analyte concentration in a sample with long-term reproducibility. Exactly what is sufficient is dictated by the intended purpose for which the results will be used (Lee et al., 2006). According to the International Organization for Standardization (ISO), method validation is required to "*confirm the fitness for the purpose of an analytical method*." This is to verify that the "*defined method protocol, applicable to a specified type of test material and to a defined concentration range of the analyte is fit for a particular analytical purpose*" (ISO/IEC 17025:2005). Demonstrating the validity of bioanalytical assays is not a trivial task and it has been the subject of much debate over the past 15 years. As a result, there are now reasonably well-established principles for the validation, with different authorities, having slightly different guidelines for the method validation, governing many analytical laboratories (European Commission, 2002; Torbeck, 2002). However, guidance by the U.S Food and Drug Administration (FDA) on bioanalytical method validation (FDA, 2001) with supplemental material from the 3rd AAPS/FDA Bioanalytical Workshop (Viswanathan et al., 2007), has been of particular value to practitioners of quantitative bioanalytical LC–MS, especially for drug analysis. The FDA guidance covers the following aspects of method validation:

Construction of the calibration/standard curve

- Qualities of the calibration samples
- Amount of concentration levels and blanks
- Criteria for the lowest standard of the calibration curve
- Requirements for the concentration-response curve

Selectivity

- Lack of interference from biological matrix; matrix effect should not compromise method performance

Determination of accuracy, precision, and recovery

- Analysis of multiple spiked samples across the expected range
- Criteria for the variability of the measurement results
- Criteria for the closeness of the measured and calculated concentrations
- Requirements for the analyte recovery during sample preparation

Instructions for studying analyte stability

- Long term storage stability
- Stability during multiple freeze and thaw cycles
- Short-term stability after thawing
- Stability after sample preparation
- Stability of the standard and IS stock solutions

In addition, it sets recommendations for the application of the method for routine analysis. However, the guidance is not a comprehensive collection of all relevant issues that must be taken into account when the validity of the measurement is of concern. It is also intended solely for the drug analysis and cannot be directly applied for the analysis of endogenous compounds.

Quantitative bioanalysis

The prevailing practice of comparing the instrument response between spiked and incurred samples involves the assumption that the analyte is extracted similarly from both samples. However, depending on the sample material, the analyte may be distributed in the samples differently, for example due to the different nature of protein binding. The determination of method accuracy according to FDA may not

fully reveal this, being determined by analyzing spiked matrix against a calibration curve of spiked matrix.

For endogenous analytes, the accurate quantification is more complicated. As an analyte-free sample matrix is usually not available, alternative strategies for calibration have to be used. Two techniques exist: the spiking of surrogate matrix with authentic analyte, or spiking of authentic, analyte-containing matrix with surrogate analyte (van de Merbel, 2008). The first approach assumes that the solubility and extractability from the surrogate matrix is comparable to the sample matrix. The second assumes that the physicochemical properties of the surrogate analyte are identical to the analyte of interest. For this, stable isotope labeled analogues are usually employed (Petucci et al., 2010).

Matrix effects and quantification of low concentrations

Ion suppression can prevent the analysis of compounds for which there is a low response, but in many cases, the sensitivity of the assay can be adequate despite the presence of ME. However, the variability in the quantity of ME between samples and species leads to the variability in analytical results if adequate IS is not used. There is debate whether matrix matching is necessary or if surrogate standards are acceptable when isotope labeled IS is used (Jacobson et al., 2011, Hewavitharana, 2011). Despite its positive effect on accuracy, the use of IS cannot compensate for the loss of detector response due to ion suppression.

Quantification at the sensitivity limits of the method has been addressed in the FDA guideline by setting several requirements for the lowest concentration level of the calibration curve (lower limit of quantification, LLOQ). With these conditions, higher LLOQ concentrations are usually obtained in comparison to the values based on S/N ratios or response linearity (ICH, 2005). However, when constructing a calibration curve for bioanalysis using standards in a surrogate matrix, even the FDA-defined LLOQ can represent an overestimate. This is due to the need to use the surrogate matrix for both standards and the samples that are used for the estimation of LLOQ. If surrogate standards are employed, the absolute response from a standard at LLOQ has to be reasonably strong to leave some headroom for the possible ion suppression in the samples at the same concentration level. Otherwise, false negative results will be reported if ion suppression is strong enough to diminish the analyte response below the detection limit. In addition, the RSD values gathered from the analysis of samples in surrogate matrix at LLOQ can overestimate the method precision for analyzing biological samples at the same concentration

level. For this reason, when using surrogate standards for biological samples at LLOQ levels, lack of ME should be assured, or its actual percentage amount should be determined (Viswanathan et al., 2007), and its effect on the results taken into consideration.

There is also a selectivity-related issue in the LC–MS analysis of samples near to the LLOQ. A common practice to increase the confidence of the analyte identification is to monitor a second, qualifier MS/MS transition in addition to that being used for quantification. A branching ratio of the precursor ion fragmentation to the qualifier and quantifier ions is usually used as an acceptance criterion for the identification (Kushnir et al., 2006). However, if the qualifier ion response is lower than that of the quantifier, the qualifier signal may be absent at the LLOQ.

3 *Towards maximum assay sensitivity*

Despite the low amount or poor ionization of the analytes, injection of the sample has to result in an adequate detector response to enable successful quantification. As reviewed in the previous sections, the detector response is related to the employed ionization technique, the type of mass spectrometer in use, and the dimensions and operational conditions of the chromatographic separation. Furthermore, the sample preparation can have a strong effect on the response, determining the recovery of the analyte and the amount of possible ion suppression, sometimes described as "process efficiency" of the sample preparation (Matuszewski et al., 2003).

In this study, before setting the aims and objectives for the method development, all of the above-mentioned aspects that contribute to the LC–MS/MS assay sensitivity were considered. As a result, the following list of conditions was compiled, which summarizes the theoretical criteria for achieving maximum detector response:

1. All available sample material is used
2. All analyte molecules are recovered during the sample preparation process
3. All of the prepared sample is injected
4. Chromatographic peak volume is infinitesimally small
5. All the analyte molecules are ionized to a single form of ions that enter the gas phase and remain ionized
6. All the analyte ions are taken into the MS – simultaneously
7. If tandem mass spectrometry is being used, the amount of monitored product ions is equal to the number of precursor ions
8. All of the analyte ions are preserved during the transfer, focusing, selection, and fragmentation within the MS
9. Every analyte ion contributes to the mass spectrometric signal; the detector has an unlimited dynamic range
10. The instrument creates noiseless data

In reality, few of the above conditions can be realized. In particular, the loss of analyte ions during the MS stage is largely related to the MS instrument design, and thus out of the control of the analyst. However, some stages before the MS analysis

are critical for preserving the analyte and preventing the sample dilution. Sample preparation should be performed using techniques that cause a minimum amount of analyte loss. If the sample size is significantly larger than the injection volume used for the method, sample preparation may be used to concentrate the analyte in the injection solvent. Further concentration can usually be made in the column head using gradient elution, before the chromatographic separation. During the separation, minimal peak volumes should be generated by employing suitable chromatography and downscaling of the internal volumes of the column and the connections. The most efficient ionization technique and ion source conditions should be used. Analyte structure may be manipulated to improve its ionization properties. If possible, the dimensions and positioning of the ion source should be optimized to minimize sample dilution and analyte loss during the ionization process, which is particularly important if very low flow rates are used in conjunction with capillary columns.

4 *Aims of the study*

The aim of this study was to develop new approaches to overcome three major sensitivity-limiting factors of bioanalytical LC–MS assays: low analyte concentrations, small sample volumes, and poor ionization. The work was focused on sample preparation and ionization. Overall, the aim was to combine different techniques and demonstrate their feasibility by developing complete, validated assays that were applied for routine sample analysis. The following cases were selected for the experimental work:

- Quantitative analysis of α_1-adrenoceptor antagonist drug, tamsulosin, in human serum and 30 µl of aqueous humor (AH). The objective was to maximize the analyte recovery during the sample preparation and enable the analysis of AH and serum samples within a single analytical sequence (**I**).

- Quantitative analysis of the neurotransmitter acetylcholine in 15 µl of rat brain microdialysate. The objective was to achieve efficient analyte ionization, despite the presence of an ion-suppressing mobile phase, and maximum analyte recovery during the sample preparation (**II**).

- Miniaturization of the atmospheric pressure thermospray ionization technique (APTSI) that was employed in the second case. The objective was to study the feasibility of the technique for use with microscale separation systems using a novel microchip nebulizer (**III**).

- Simultaneous analysis of seven endogenic steroids in 150 µl of human serum. The objective was to improve the analyte ionization through derivatization, and to achieve maximum analyte recovery during sample preparation (**IV**).

5 Experimental

5.1 INSTRUMENTS AND SUPPLIES

The analytical instruments and other supplies used for the study are listed in Table 2.

Table 2. *A list of instruments and supplies*

HPLC and MS	**Manufacturer**	**Paper**
Thermo LTQ linear ion trap (ESI, APCI)	Thermo Scientific (San Jose, CA, USA)	I, II, III
Agilent 6410 Triple Quadrupole (ESI)	Agilent Technologies (Palo Alto, CA, USA)	IV
Microchip-APCI	Custom, described in the paper	III
ADPC-IMS PicoFrit ion source adapter	New Objective Inc. (Woburn, MA, USA)	III
Surveyor HPLC system	Thermo Scientific	I, II, III
Agilent 1200 Series RRLC	Agilent Technologies	IV
Xcalibur 1.4 SR1 Acquisition software	Thermo Scientific	I, II, III
LCquan 2.0 Quantification software	Thermo Scientific	I, II
MassHunter Acquisition software B.01.04	Agilent Technologies	IV
Quantitative Analysis software B.04.00	Agilent Technologies	IV
HPLC columns	**Manufacturer**	**Paper**
Zorbax SB-C18 (50×2.1 mm; 1.8 µm)	Agilent Technologies	IV
Zorbax SB-Aq (100×2.1 mm; 3.5 µm)	Agilent Technologies	II
Zorbax XDB-C8 Guard Column	Agilent Technologies	I
Zorbax SB-Aq Guard Column	Agilent Technologies	II
Atlantis HILIC Silica (50×2.1 mm; 3 µm)	Waters (Milford, MA, USA)	II
Oasis WCX (20×2.1 mm; 30 µm)	Waters	II
Waters XTerra C8 (50×2.1 mm; 3.5 µm)	Waters	I
polyHYDROXYETHYL A (100×2.1 mm; 5 µm)	PolyLC (Columbia, MD, USA)	II
General supplies	**Manufacturer**	**Paper**
Water purification system, Milli-Q Gradient	Millipore (Milford, MA, USA)	All
DC power supply, Iso-Tech IPS-603	RS Components (Northants, England)	III
Analytical balance, AX205	Mettler-Toledo (Greifensee, Switzerland)	All
Mass flow controller, Aalborg GFC-17	Aalborg Instruments & Controls, Inc., Orangeburg, NY, USA	
Test tube shaker, Multi Reax	Heidolph (Schwabach, Germany)	IV
Laboratory oven, ULE500	Memmert (Schwabach, Germany)	IV
Nitrogen evaporator, N-EVAP 112	Organomation Assoc. (Berlin, MA, USA)	IV

Mass spectrometer tuning and calibration

The mass spectrometer used in papers **I**, **II**, and **III** (Thermo LTQ) was tuned by infusing the analyzed compounds and employing the semi-automatic tuning function of the instrument software. The same tuning was used in papers **II** and **III**. The mass spectrometer used in paper **IV** (Agilent G6410A) was calibrated and tuned by using the instrument built-in automatic tuning function and the associated tuning solution.

5.2 CHEMICALS AND REAGENTS

The chemicals and reagents used in the work are listed in Table 3. The analyzed compounds are presented in Figure 6.

Table 3. *A list of analytical instruments and supplies*

Chemicals	Manufacturer/Supplier	Paper
17α-hydroxypregnenolone	Sigma-Aldrich Chemie (Steinheim, Germany)	IV
17α-hydroxyprogesterone	Sigma-Aldrich Chemie	IV
2,2′-bipyridyl	Sigma-Aldrich Chemie	III
6-ketocholestanol	Sigma-Aldrich Chemie	III
Acetonitrile, HPLC Ultra Gradient	Mallinckrodt Baker (Deventer, Netherlands)	II, III
Acetonitrile, HPLC-grade	Rathburn (Walkerburn, UK)	I
Acetylcholine iodide	Fluka (Buchs, Switzerland)	II, III
Acetyl-β-methylcholine	Sigma-Aldrich Chemie	III
Aldosterone	Sigma-Aldrich Chemie	IV
Ammonium acetate	Sigma-Aldrich Chemie	III
Ammonium formate	Sigma-Aldrich Chemie	III
Ammonium hydroxide solution	Sigma-Aldrich Chemie	III
Androstanedione	Steraloids (Newport, RI, USA)	IV
Androstenedione	Sigma-Aldrich Chemie	IV
Androsterone	Sigma-Aldrich Chemie	IV
Angiotensin I	Sigma-Aldrich Chemie	III
Arginine	Sigma-Aldrich Chemie	III
Bovine serum albumin (BSA)	Sigma-Aldrich Chemie	IV
Calcium chloride	Merck (Darmstadt, Germany)	II
Choline chloride	Fluka (Buchs, Switzerland)	II, III
D3-17α-hydroxypregnenolone	C/D/N Isotopes (Quebec, Canada)	IV
D3-testosterone	Sigma-Aldrich Chemie	IV
D4-pregnenolone	C/D/N Isotopes	IV
D6-dehydroepiandrosterone	Sigma-Aldrich Chemie	IV
D7-androstenedione	Steraloids	IV
D8-17α-hydroxyprogesterone	C/D/N Isotopes	IV
D9-acetylcholine chloride	C/D/N isotopes	II
D9-progesterone	Steraloids	IV
Dehydroepiandrosterone	Sigma-Aldrich Chemie	IV

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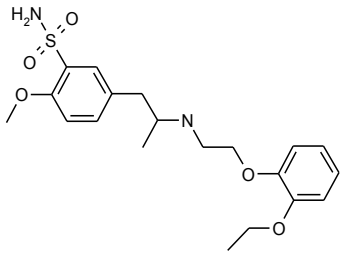
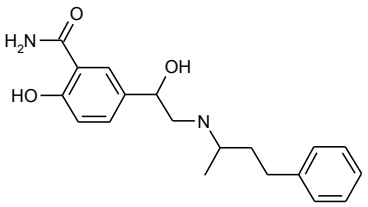
Chemicals	**Manufacturer/Supplier**	**Paper**
Dihydrotestosterone	Steraloids	IV
Estradiol	Sigma-Aldrich Chemie	IV
Estrone	Sigma-Aldrich Chemie	IV
Ethyl acetate	LabScan (Dublin, Ireland)	I
Etiocholanolone	Gift from United Medix Laboratories Ltd.	IV
Formic acid	Sigma-Aldrich Chemie	I, III
Formic acid, LC/MS grade	Sigma-Aldrich Chemie	IV
Glucose	Sigma-Aldrich Chemie	III
Heptafluorobutyric acid (HFBA)	Fluka	II
Hydroxylamine hydrochloride	Sigma-Aldrich Chemie	IV
Iron (II) sulphate	Sigma-Aldrich Chemie	III
Labetalol hydrochloride	Sigma-Aldrich Chemie	I
Lidocaine	Sigma-Aldrich Chemie	III
Magnesium chloride	Riedel-de Haën (Seelze, Germany)	II
Methanol, HPLC Gradient Grade	Mallinckrodt Baker	III
Methanol, LC/MS grade	Sigma-Aldrich Chemie	IV
Methyl *tert*-butyl ether (MTBE)	Sigma-Aldrich Chemie	IV
Neostigmine bromide	Sigma-Aldrich Chemie	II
Nitrogen, pharmacopeial grade	Oy AGA Ab (Espoo, Finland)	III
Ofloxacin	Sigma-Aldrich Chemie	III
Phenylalanine amide	Sigma-Aldrich Chemie	III
Potassium chloride	Merck	II
Pregnenolone	Sigma-Aldrich Chemie	IV
Progesterone	Steraloids	IV
Sodium bicarbonate	Riedel-de Haën	I
Sodium chloride	Mallinckrodt Baker	II
Sterofundin ISO	B.Braun (Melsungen, Germany)	IV
Tamsulosin hydrochloride	Fermion Oy (Espoo, Finland)	I

Paper I

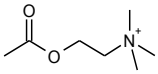


Tamsulosin
(408.2)



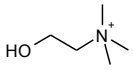
Labetalol (IS)
(328.2)

Paper II

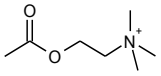


Acetylcholine
(146.1)

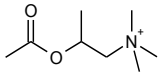
Paper III



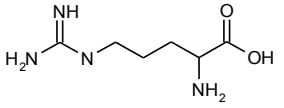
Choline
(104.1)



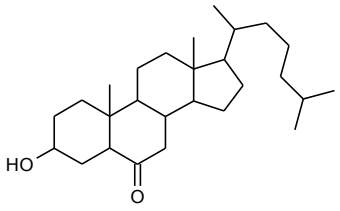
Acetylcholine
(164.1)



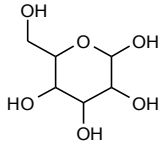
Acetyl-β-methylcholine
(160.1)



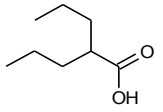
Arginine
(174.1)



6-ketocholestanol
(402.4)



Glucose
(180.1)



Valproic acid
(144.1)

Figure 6. *Structures and monoisotopic masses of compounds used in the study. Isotope labeled internal standards are not included. Numbering of the steroid carbons and the derivatization reaction (**IV**) are presented using DHEA.*

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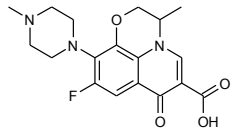
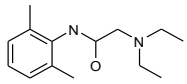
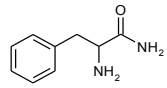
Paper III



Ofloxacin
(361.1)

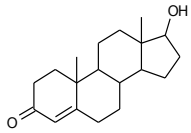


Lidocaine
(234.2)

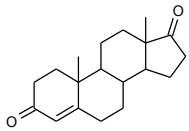


Phenylalanine amide
(164.1)

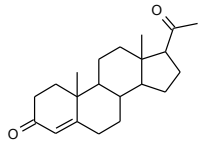
Paper IV



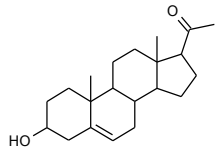
Testosterone
(288.2)



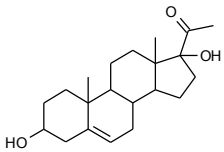
Androstenedione
(286.2)



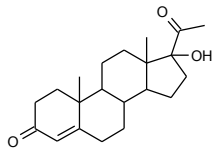
Progesterone
(314.2)



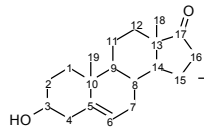
Pregnenolone
(314.2)



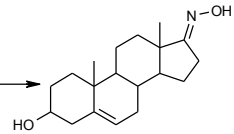
17-OH-pregnenolone
(332.2)



17-OH-progesterone
(330.2)



DHEA
(288.2)



DHEA-oxime
(303.2)

Figure 6. *Continued*

6 *Results and discussion*

This chapter is divided into three sections. In the first section (6.1), results of the sample preparation stage of the developed assays are presented and discussed. The second section (6.2) concentrates on the ionization stage. In the third section (6.3), the developed assays are summarized and the contribution of the chosen approaches to the assay performance and usability are critically assessed. The papers are referred to by their Roman numerals.

6.1 INCREASING THE ANALYTE RECOVERY

Particularly with small-volume samples, a significant percentage of the analytes may be lost during the various steps of the sample preparation process. The term "analyte recovery" is used here to describe the percentage fraction of analytes in the initial sample that were preserved during the sample preparation and injected into the column. In each case of this study, the challenges in realizing maximum analyte recovery were different: ionizable analyte of medium polarity in an aqueous biological matrix (**I**), permanently ionized polar analyte in aqueous dialysis fluid (**II**), and non-ionizable, non-polar analytes in serum (**IV**) (Figure 6). The results demonstrate how analyte polarity can govern the later stages of an assay, including the chromatographic technique, injection volume, and ionization.

6.1.1 Direct sample injection (I, II)

In the analysis of tamsulosin, the objective was to prevent unnecessary analyte loss by keeping the sample preparation steps at minimum. AH is derived from plasma within the ciliary body of the eye, containing primarily the substrates and by-products of the metabolic processes of the vascular cornea and the lens (Goel et al., 2010). The protein content of human AH is minimal, between 0.12 and 0.50 mg/mL (Chowdhury et al., 2010). Since this is around 150–600 times less than in human serum (Rustad et al., 2004), the typical sample preparation methods (PPT, LLE, SPE) were not considered necessary for protein removal. It was also found that the aqueous tamsulosin solution could be injected in high volumes using the developed chromatographic method. Hence, direct injection of AH, subsequent to the IS addition, was employed. A pre-column was included and provided sufficient

protection against possible protein precipitation on the column head. The maximum amount of AH that could be accurately pipetted from all the sample tubes was 30 µl. After adding the IS, the total volume was 50 µl, of which 35 µl could be injected. A larger injection volume would have been desirable, but the lack of precision in the autosampler needle height setting prevented injecting the entire sample. By using special vials with a spring between the vial and the insert, higher sample recoveries would have been attainable. However, since the sample was prepared directly in an insert without any extraction steps, relatively high analyte recovery (70%) was obtained even with the more economical standard vials and inserts.

Direct injection of the sample was also used for the analysis of ACh. However, the assay development was not as straightforward as with tamsulosin, mainly due to the very high polarity of the analyte. With RP columns, ion-pair reagents were needed to generate sufficient retention, but the resulting mobile phase was unfavorable for ESI ionization, both because of the ion-pair additives and the low amount of organic solvent that had to be used (2% acetonitrile). APCI could not be employed, since ACh was not detectable with this technique. Therefore, an attempt was made to use HILIC, which employs a mobile phase with better compatibility with ESI. With HILIC, adequate retention was achieved for ACh, but the retention of the inorganic salts of the microdialysate buffer affected the chromatographic selectivity. Attempts to remove these salts and to concentrate the sample with a short on-line cation exchange column failed, most probably due to the strong salt concentration of the dialysate sample. In addition, the use of HILIC required heavy dilution of the microdialysate with acetonitrile to decrease the elution strength of the injected solution, ruling out direct injection of the samples. Fortunately, ACh was found to produce intense MS response when APCI source was used without the corona needle, even if TFA was included in the mobile phase. The accidental discovery of this special ionization technique, which will be discussed in more detail in the next chapter, allowed the use of reversed phase separation with TFA and the direct injection technique.

For the same reasons encountered with the tamsulosin assay, the microdialysis sample was prepared by pipette-mixing the sample and the IS in a pulled-point vial insert (Figure 7). Due to the high sampling frequency of the microdialysis experiment, dialysate fractions of only around 20 µl were collected. Of these samples, 15 µl could be transferred to the vials and used for the analysis. After adding equal volume of aqueous IS solution to the sample (bringing the total volume to 30 µl), 20 µl was injected. Using this method, analyte recovery of 67% was achieved (20 µl

injection of a 30 µl sample). Larger injection volumes could not be employed without disturbing the chromatography, which was based on isocratic elution with a mobile phase containing only 2% acetonitrile. For this reason, the maximum amount of microdialysate was used and the total sample volume after IS addition was kept as low as practical.

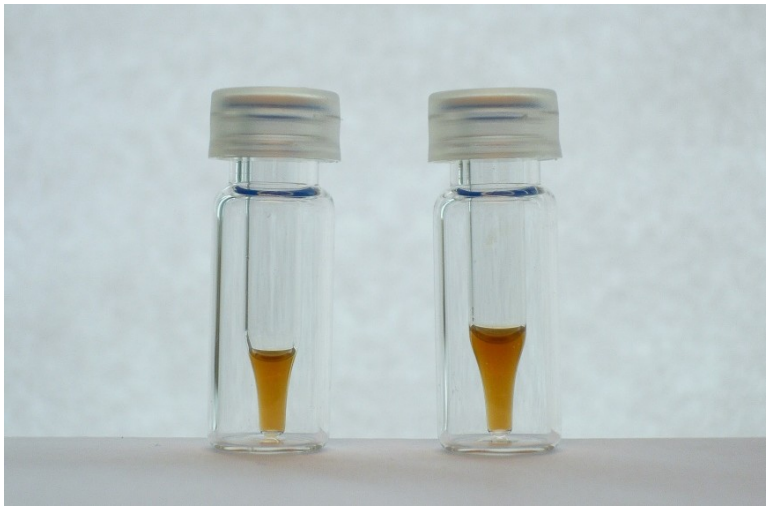


Figure 7. *Sample solution levels at the pulled-point vial inserts used for the direct injection analyses (**I**, **II**). Left: 30 µL (15 µL of microdialysate, 15 µL of internal standard), of which 20 was injected. Right: 50 µL (35 µL of aqueous humor, 15 µL of internal standard), of which 35 µL was injected. In the photograph, colored water was used for illustrative purposes instead of real samples.*

Based on the described studies, direct injection of small-volume samples with IS dilution was shown to be an efficient way of delivering the maximum amount of analytes into the column and should be used when sample pre-processing is not absolutely necessary. Since it does not involve any extraction steps or transfers of the sample, analyte recovery is theoretically the ratio of injection volume to volume of the sample in the vial. In addition, variability in the extraction recovery and the possible measurement errors arising from the multiple pre-processing steps are avoided.

However, direct injection is limited to samples that are relatively free from protein or particulate material that can obstruct the column. It is also necessary to resolve any possible matrix effects during subsequent stages of an assay. In addition, the sample volume has to be within the injection volume limits of the autosampler. Depending on the sample composition, large injection volumes may also overload

the separation column, resulting in poor chromatography. This has to be considered during the selection of the column dimensions and type; the new superficially porous particles for instance have a lower mass loading capacity in comparison with the fully porous ones. Moreover, the amount of sample that is left in the vial after injection depends on the design of the vial and the instrument. In both discussed studies, pulled-point vial inserts were used to maintain a high solution surface level (Figure 6), but since the employed autosampler was not able to withdraw the sample from the very bottom of the vials, some loss of sample solution was inevitable.

6.1.2 Sample concentration and recovery maximization (IV)

Concentrating the samples during the sample preparation process has been mentioned as a means to increase the analyte recovery. This technique was applied in the human serum steroid assay. Direct injection of serum is not feasible and hence a different technique had to be employed. However, the aim was to maintain the sample preparation as simple and robust as possible, while ensuring efficient clean-up. In addition, analyte derivatization was known to be necessary and its suitability for integration with the sample preparation was taken into account.

Protein precipitation with organic solvent was not considered, as the technique leads to large volumes of partly aqueous solution, which cannot be easily concentrated by evaporation. Moreover, PPT has been shown to be more susceptible to ion suppression than some other popular techniques, like SPE and LLE. After experimenting with LLE, it was found to produce high extracting recoveries when diethyl ether or MTBE were used. These volatile organic solvents could also be rapidly evaporated. However, since a typical LLE process involves the transfer of the sample from the evaporation tube into the injection vial or well, the resulting analyte loss can be significant, particularly if the volume of the reconstituted sample is small. To ensure maximum analyte recovery during the LLE, a simplified method was developed: the sample extraction, evaporation, derivatization, and injection were performed using only two autosampler vials. The extraction was performed in a standard 1.5 ml vial using 1 ml of MTBE. After the extraction, the organic phase was transferred into a cone-bottomed vial, into which it was evaporated. The sample was then redissolved in derivatization solution, the vial was capped, heated for derivatization, and injected directly from the same vial (Figure 8).

With this technique, the initial serum volume of 150 µl was transformed into 50 µl of derivatization solution, of which 40 µl could be injected. This translates into

80% recovery of the original serum volume. The extraction recovery of the assay, calculated as a mean of all the analytes from both male and female serum, was 85%.

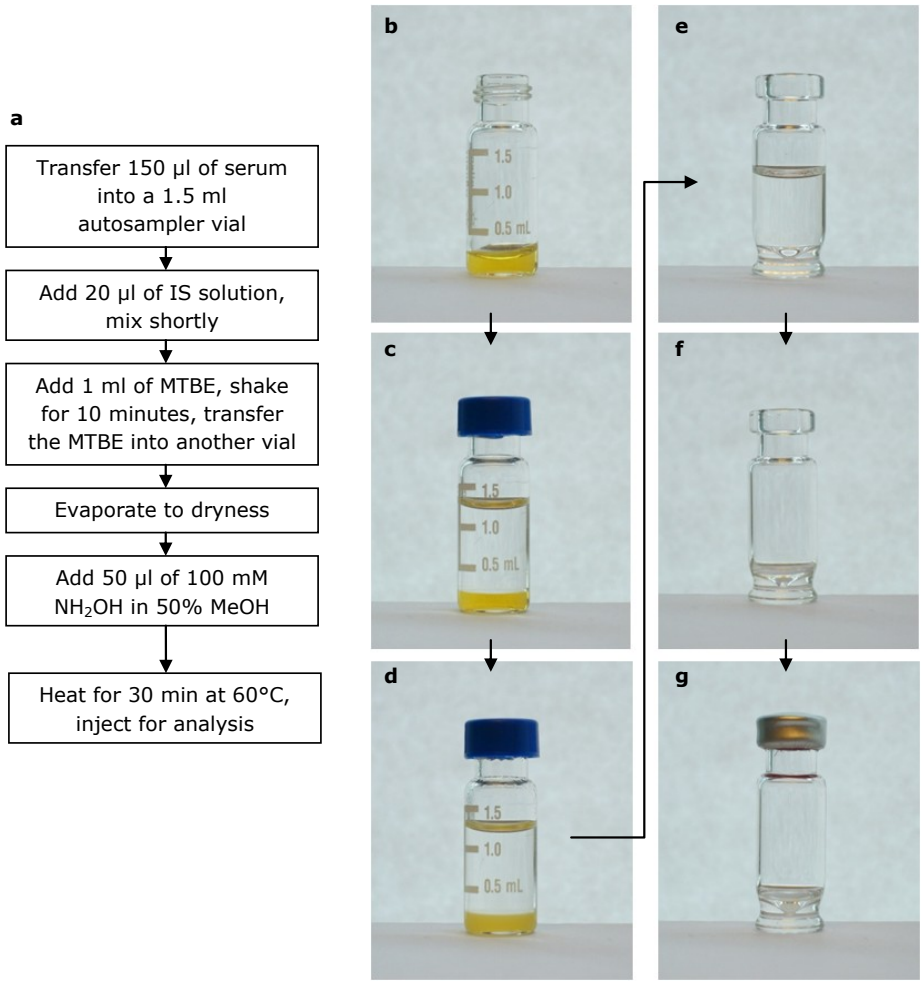


Figure 8. *Combined liquid-liquid extraction and oxime derivatization of serum steroids using two autosampler vials (**IV**). (**a**) The sample preparation process. (**b**) A serum sample spiked with IS solution, (**c**) after adding the MTBE, (**d**) after the extraction and 5-minute equilibration time. (**e**) The MTBE phase transferred to a cone-bottomed vial, (**f**) the vial after solvent evaporation, (**g**) after reconstituting in the derivatization solution and heating.*

Thus, the overall mean analyte recovery (extraction recovery + percentage of the sample injected) was 68%. Once again, injecting the entire contents of the vial would have increased the recovery, but dispersion of the sample on the inside surface of the

vials reduced the volume of the injectable solution to around 40 µl. The extraction recovery would also have been slightly higher if the samples were extracted more than once. However, the resulting volume of the MTBE would have necessitated either two evaporation cycles, or abandoning the concept of evaporation straight into the injection vial. As the assay usability and reliability were strongly emphasized, single extraction was employed. The described sample preparation technique resulted in fully satisfactory sensitivity after the analyte derivatization was supplemented into the extraction process.

6.2 INCREASING THE MASS SPECTROMETRIC RESPONSE

In this study, there were two major ionization-related challenges. In the first case, the mobile phase composition did limit the response, even though the analyte was permanently ionized (**II**). In this case, a special ionization technique was employed to counteract the negative effects of the mobile phase. As this technique had never been described for microscale analysis, an additional study was devoted to the characterization of the technique in microchip scale (**III**). In the second case, the low proton affinity of the analytes resulted in weak ionization and the formation of different adducts, irrespective of the mobile phase composition (**IV**). In this case, modification of the analyte structures was used to improve the proton affinity of the compounds.

6.2.1 Atmospheric pressure thermospray ionization (II, III)

As discussed in chapter 6.1.1, the mass spectrometric response for ACh was extensively affected by the chromatographic conditions that were needed for the highly polar analyte. The mostly organic mobile phase of a HILIC separation technique would have favored the ESI process, but due to the reasons discussed above, this technique could not be employed, necessitating the use of an ion-pair agent (TFA) and an almost completely aqueous mobile phase. Although ionization of the permanently cationic ACh was not of concern, the TFA-containing mobile phase resulted in a suboptimal ESI response. Interestingly, however, a clear ACh peak was seen with APCI, when the corona discharge was accidentally set to zero. When a discharge current was applied, the observed $[M]^+$ ion of ACh disappeared. It was speculated that by using the heated APCI vaporizer without any corona discharge, more efficient desolvation and hence response for the analyte could be realized than with the ESI source. This was indeed the case. Particularly with TFA-containing mobile phase, this technique, which was later termed atmospheric

pressure thermospray ionization (APTSI) produced significantly higher ion counts for acetylcholine than could be achieved with ESI (Figure 9).

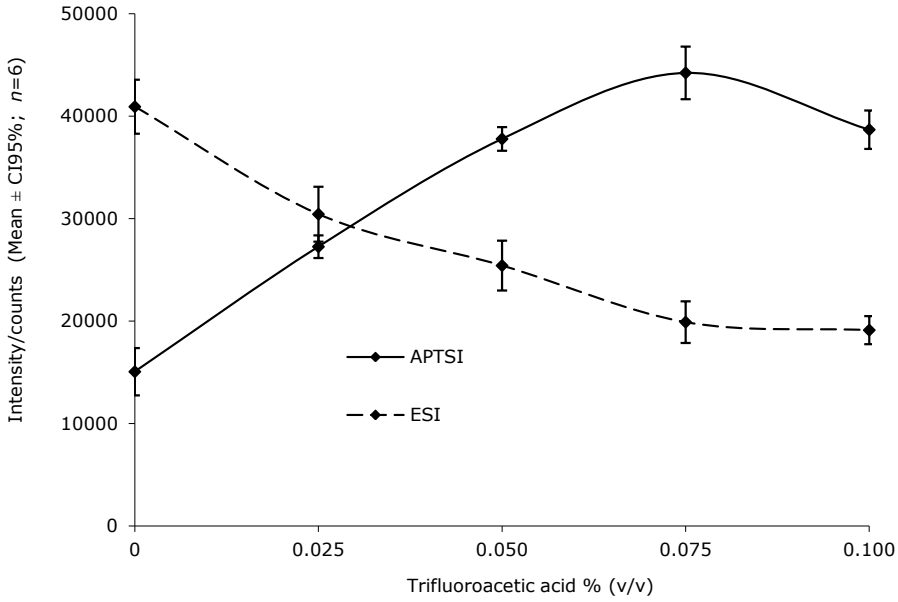


Figure 9. *Comparison of acetylcholine signal intensity using electrospray (ESI) and atmospheric pressure thermospray ionization (APTSI). Mobile phase: 2% acetonitrile, flow rate 0.3 mL/min.*

Thus a novel sensitive assay for ACh was developed by combining APTSI with the previously discussed ion-pair chromatography. The use of APCI source in this unconventional manner has been previously reported in few papers (Turnipseed et al., 2006; Andersen et al., 2009; Favretto et al., 2010). Its favorability for ACh analysis or mobile phases containing TFA has not been previously described.

APTSI in microchip scale

Increasing the ACh assay sensitivity further to enable the analysis of samples without acetylcholinesterase may be difficult, without changing the described instrumentation. For instance, sensitivity improvements that could be achieved by downscaling of the separation system volumes are limited by the size of the employed ion source; as discussed earlier, only ESI sources are commercially available in capillary or microchip scale. However, shortly after the ACh assay development, the opportunity arose to use the experimental heated microchip

nebulizer mentioned in chapter 2.2 (Saarela et al., 2007). When attached to a nano-ESI adapter probe originally designed to enable ESI spraying directly from a capillary column (Figure 10), the microchip could be operated inside a closed ion source housing as a miniature APCI source (Figure 11).

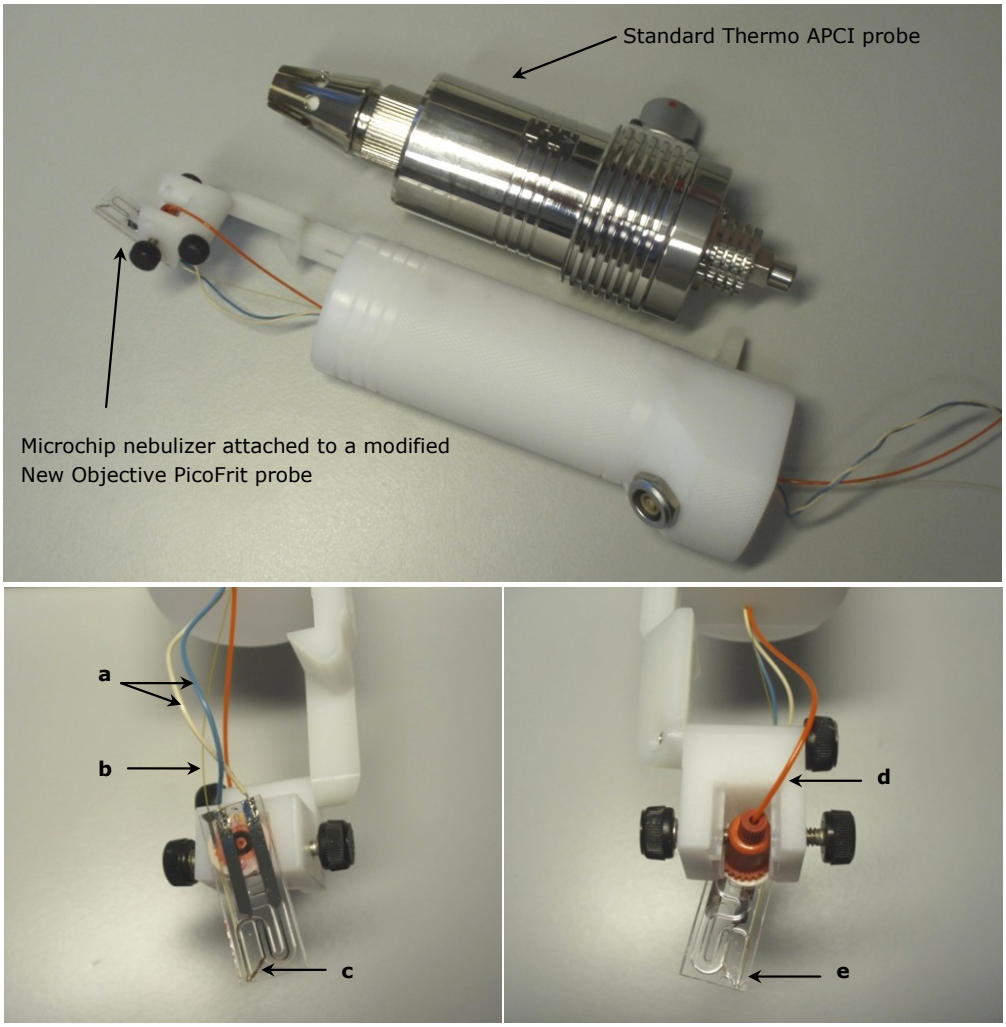


Figure 10. *Photographs of the standard APCI ion source probe and the microchip heated nebulizer used for the miniature APTSI experiments. (**a**) Electrical wires for the resistive heating of the microchip, (**b**) fused silica sample inlet capillary, (**c**) platinum heater wire, (**d**) vaporizer gas tubing, (**e**) vaporizer channel and the nozzle of the microchip.*

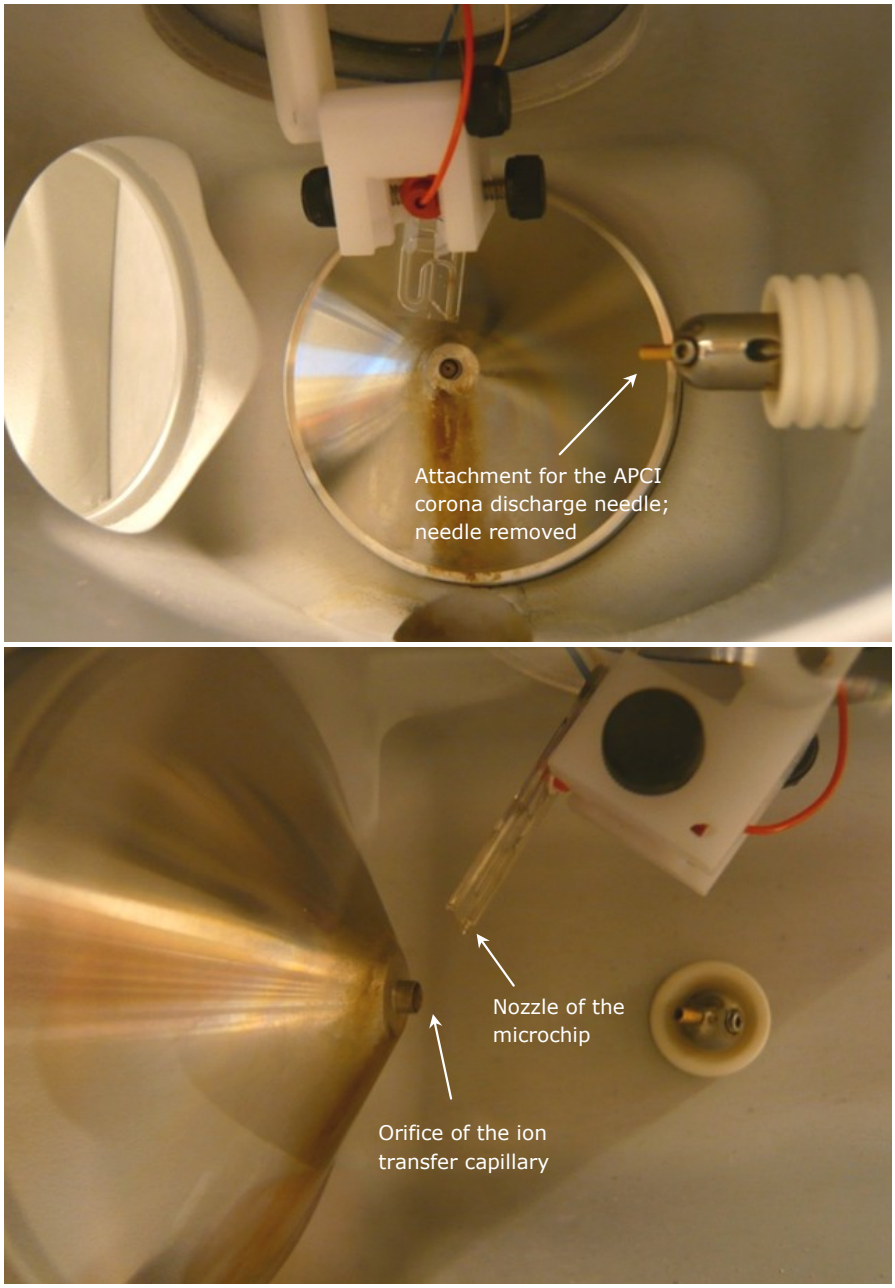


Figure 11. *Positioning of the microchip nebulizer inside a Thermo ion source housing*

As the first prototype of the microchip had been already successfully used for APCI and APPI (Östman et al., 2004; Kauppila et al., 2004b), the feasibility of utilizing the later version for APTSI was studied. Similarly to the previous experiments with a standard APCI source, the $[M]^+$ ion was observed for ACh when the corona discharge was turned off. The most suitable flow rate was 2–20 μl/min in contrast with the 200 μl/min that was used for the ACh assay.

In an attempt to further characterize this novel ionization technique, several additional compounds were included in the study and the comparisons were made with microchip-APCI and standard ESI. As a result, the microchip-APTSI (μAPTSI) was found to produce ESI-like spectra, with weaker overall intensities but remarkably good signal-to-noise ratios that were comparable with the standard-sized ESI. For instance, for the test compound ofloxacin (Figure 6), similar S/N ratios were observed, despite the 10-times greater response of ESI. The test compound spectra were comparable with ESI, showing adduct ions and multiple charging of peptides, as demonstrated with angiotensin II. In addition, similarly to the earlier results with the standard-sized APTSI, the microchip-APTSI was found to tolerate well the ion-suppressing mobile phase constituents, including TFA and ammonium salts of formic and acetic acid. It also favored the use of a mobile phase with a high organic content, like ESI, being suitable for use with HILIC separations.

Although the aim of the study was not to investigate the theoretical mechanisms of the APTSI ionization process, the results suggest that at least in its microchip variation, the ion formation proceeds through ion evaporation or charged residue process. The inability of APTSI to ionize non-polar test compounds, such as 6-ketocholestanole, and the strong response for quaternary ammonium compounds are comparable to ESI and in contrast with APCI. Moreover, the disappearance of the signal when high nebulizer temperatures were applied implies that, after reaching a certain temperature, complete vaporization of the solution occurs and the ionization process driven by the shrinkage of droplets is no longer realized. The subject is discussed in more detail in the paper **III**.

Overall, the results indicate that APTSI ionization can be used as an alternative ionization technique also on a smaller scale, in combination with capillary separation systems. With the studied microchip, the separation column could be integrated into the fused silica inlet capillary, or within the chip structure. This opens interesting new options especially for the development of more sensitive ACh assays, since in theory the smaller dimensions of the microchip source would result

in sensitivity advances. However, direct comparison of the chip and a standard APCI source was not made using the mobile phase composition of the ACh assay.

6.2.2 Derivatization of the analytes for increased proton affinity (IV)

All the analytes in the serum steroid assay were poorly ionized with ESI. Using positive polarity, $[M+H]^+$ ions were observed, but the adduct formation was pronounced, even if the mobile phase consisted of solvents and reagents of MS grade. This is a well known phenomenon for steroid compounds (Ma and Kim, 1997). However, it was not possible to use these adduct ions as precursors as the product ions were left uncharged and were not detected. Loss of water was also evident for compounds with a hydroxyl group in 3-position (pregnenolone, 17-OH-pregnenolone, and DHEA), likely resulting in the formation of conjugated double bond between carbons 3 and 4 (Figure 6).

In some cases, the ESI response of underivatized steroids can be improved by adding ammonia to the mobile phase to suppress the adduct formation (Leinonen et al. 2002; Saito et al., 2010). Although this can increase the amount of $[M+H]^+$ ions, the inherent weak gas phase proton affinities of steroids remain and many factors, including the proton affinity of the organic solvent used for the mobile phase, have a significant effect on the ion intensity (Ma and Kim, 1997).

With derivatization, however, the ionization efficiency of the analytes can be modified, which has particular importance for ESI based methods, where the gas phase proton affinities of all the sample and mobile phase components contribute to the analyte ion intensity (Amad et al. 2000). In the present case, all the analytes had ketone functionality, which was indicative of the suitability for the recently introduced oxime derivatization technique to increase the proton affinity of the analytes (Liu, Sjövall, Griffiths, 2000). Indeed, the reaction was fast and efficient and had a substantial positive effect on the mass spectrometric response of all the analyzed compounds, resulting in 1.7–16 fold increase in the analyte $[M+H]^+$ intensities (Figure 12).

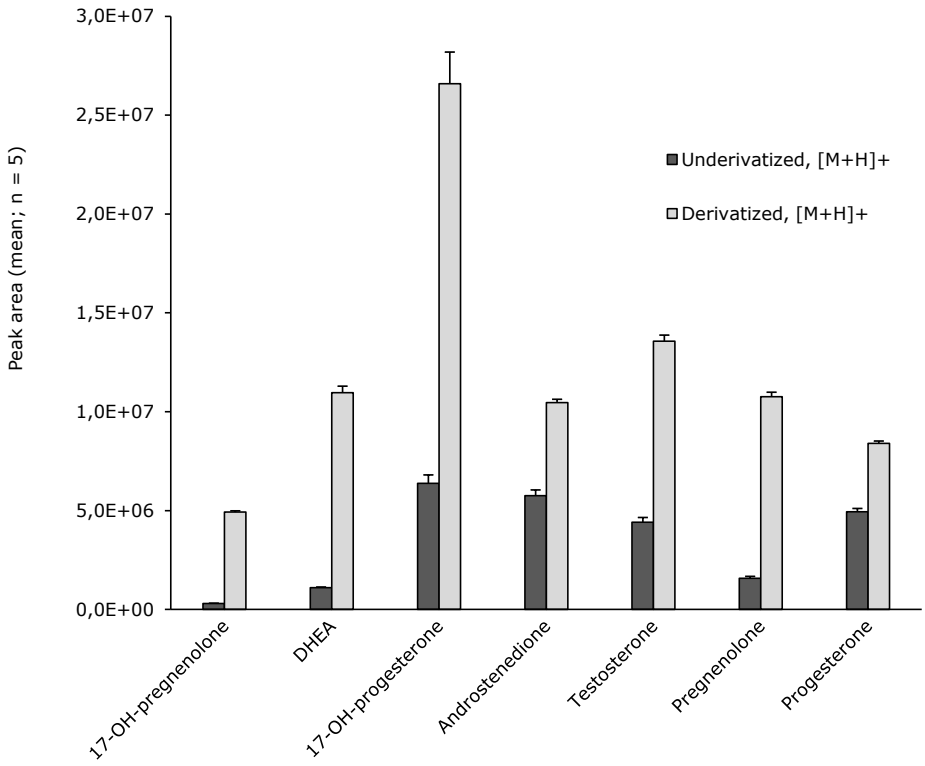


Figure 12. *The effect of derivatization on the $[M+H]^+$ ion intensity of ketosteroids*

After derivatization, the steroids were detected as $[M+H]^+$ ions, without adducts (Figure 13), and with loss of water only evident for 17-OH-pregnenolone. The derivatives were stable when stored in the reaction mixture at ambient temperature, allowing the derivatization to be made up to 18 hours before the sample analysis. Moreover, since the derivatization reaction mixture could be injected without any additional clean-up procedures, it provided a simple and efficient method for improving the analyte ionization, without adding unnecessary complexity to the assay.

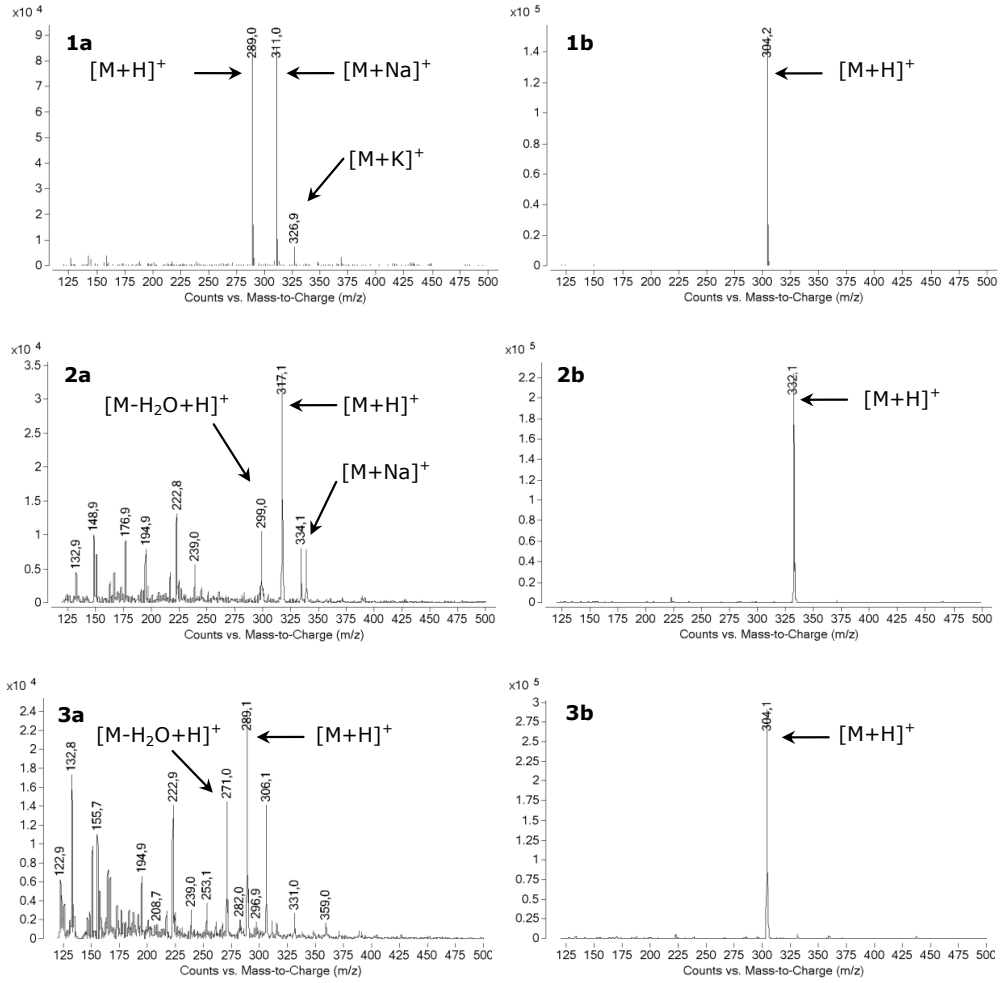


Figure 13. *The effect of oxime derivatization on the spectra of selected ketosteroid analytes. The spectra are marked (**a**) before and (**b**) after the derivatization. **1**: testosterone, **2**: pregnenolone, **3**: DHEA.*

6.3 DEVELOPED ASSAYS

The next paragraphs provide a brief discussion of each developed assay regarding the method development, validation, and routine use. The performance characteristics related to the study objectives are presented in Table 4. More detailed results of the method performance and validation results are described in the individual papers.

Table 4. *Summary of the assays developed in the study*

Assay	Sample preparation	Ionization	LC–MS	Recovery			LLOQ (ng/mL)
				Extract[a]	Inject[b]	Total[c]	
Tamsulosin (**I**)	Direct injection	(+)ESI	RP, Linear IT	–	70 %	70 %	0.08
Acetylcholine (**II**)	Direct injection	(+)APTSI	Ion-pair, Linear IT	–	67 %	67 %	0.02
Steroids (**IV**)	LLE, derivatization	(+)ESI	RP, QQQ	85 %	80 %	68 %	0.01–0.10

[a]Extraction recovery
[b]Percentage of available sample injected on column
[c]Combined extraction and injection recovery

Tamsulosin (I)

Tamsulosin is an exogenous compound, which as per FDA guidance requires matrix based standard samples for quantification and demonstration of the assay selectivity and accuracy. However, due to ethical reasons, such amount of samples cannot be obtained. In the presented case, the availability of blank AH was limited to a single patient that was not receiving tamsulosin. This sample was used for selectivity and accuracy studies, but was not sufficient for the calibration and quality control (QC) purposes as described in the FDA guidance. Therefore, water was used as a surrogate matrix. The AH samples that were used for the stability studies were from the operation of the other eye of the patients enrolled in the study, if both eyes were operated.

At the time of the study, stable isotope labeled tamsulosin was not available. Instead, labetalol was used as an IS, because of its structural similarity with tamsulosin (Figure 6). Uniform ionization environment for both compounds was achieved through chromatographic coelution. However, because labetalol is an antihypertensive drug, some clinical samples may contain this drug. In the study in question, the medication of all the enrolled patients was known and did not include labetalol, justifying its use as an IS. Nonetheless, to ensure the compatibility of the assay with a broader range of clinical samples, the use of isotope labeled tamsulosin would have been desirable. This underlines the importance of careful IS selection for drug analysis if isotope labeled analogues are not available. Many structurally close compounds that are commercially available and could be used as an IS are also drugs or drug metabolites and may potentially be present in the patient samples.

In the tamsulosin assay, no comprehensive study of the ME was performed. However, since the absolute recovery from serum was around 70% (combination of

extraction recovery and the possible ME, calculated by comparing pure standard solution with extracted sample), this points to the absence of strong ion suppression. In the AH samples, ME was not studied due to the shortage of blank sample material. However, assaying the spiked blank AH against water based standards resulted in adequate accuracy, indicating that if present, the ion suppression was at an acceptable level.

Using dedicated standard samples and separate quantification, both serum and AH samples could be analyzed within the same run. During the tamsulosin medication, the concentrations in the serum samples were expectedly high (mean concentration 12.7 ng/ml or 31.1 nM), whereas the AH levels were low (mean concentration 0.4 ng/ml or 0.98 nM). Since the LLOQs for serum and AH were 0.1 ng/ml (0.25 nM) and 0.08 ng/ml (0.20 nM), respectively, the concentrations of tamsulosin were well within the sensitivity limits of the assay. After a pause of 7–28 days in the tamsulosin medication, most of the AH samples still had concentrations above LLOQ (Pärssinen et al., 2006). However, to further study the kinetics and the clearance of tamsulosin from the eye, more sensitive assay would be needed. It is noteworthy that the sensitivity of the ion trap MS used for this study was not comparable with the current QQQ instruments. However, this was the first published assay for the determination of tamsulosin from the human AH, revealing the ophthalmic penetration of the drug and its prolonged presence in the anterior chamber of the eye, which had been recently found to complicate eye operations.

Acetylcholine (II)

At the time of its publication, the ACh assay was the first to use a stable isotope labeled IS. Unlike the AH assay for tamsulosin, the availability of microdialysis samples enabled the careful validation of the method. In this study, the absence of sensitivity-affecting ME could be proved (Figure 14).

Probably due to the special ionization technique, the concentration range of the assay was wider than in any of the previously published ESI based methods, ranging from 0.15 nM (0.02 ng/ml) to 73 nM (10.7 ng/ml). The assay was used for the determination of hippocampal levels of ACh in the rats treated to mimic Alzheimer's disease (Ihalainen et al, 2010; Ihalainen et al, 2011). All the study samples were within the standard curves, requiring however, the full use of a wide concentration range as the concentrations in the study samples were between 0.3 and 70 nM (0.04–10.2 ng/ml).

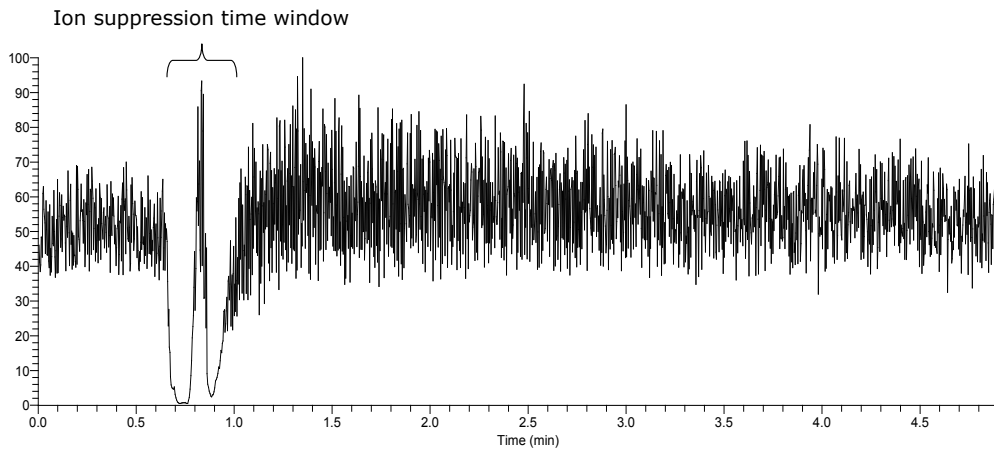


Figure 14. *A post-column infusion chromatogram after injecting a rat brain microdialysate sample. APTSI ionization, MRM transition of ACh monitored. Retention time for ACh: 1.35 min.*

Although the sensitivity of the developed assay enabled the collection of small-volume samples, which enabled fine temporal resolution of the microdialysis experiment, an acetylcholinesterase inhibitor was needed in the perfusion fluid to prevent the enzymatic breakdown of ACh. This technique is commonly used to increase the ACh levels in the microdialysis samples, but it is also controversial, as the inhibitors may leak into the studied tissues and contribute to the biological processes. For the measurement of ACh in the perfusates that do not contain inhibitors, assays with significantly greater sensitivity are needed.

Steroids (IV)

In addition to the improvements in sensitivity in the ketosteroid analysis, the oxime derivatization also improved the chromatographic selectivity, increasing the number of analytes that could be included in the assay. Under identical chromatographic conditions, the peaks of testosterone, 17-hydroxyprogesterone, and dehydroepiandrosterone were overlapping, but became baseline-separated after derivatization (Figure 15). This was important, as MS/MS selectivity could not be obtained for these compounds due to their isobaric precursor ions or similar fragmentation patterns.

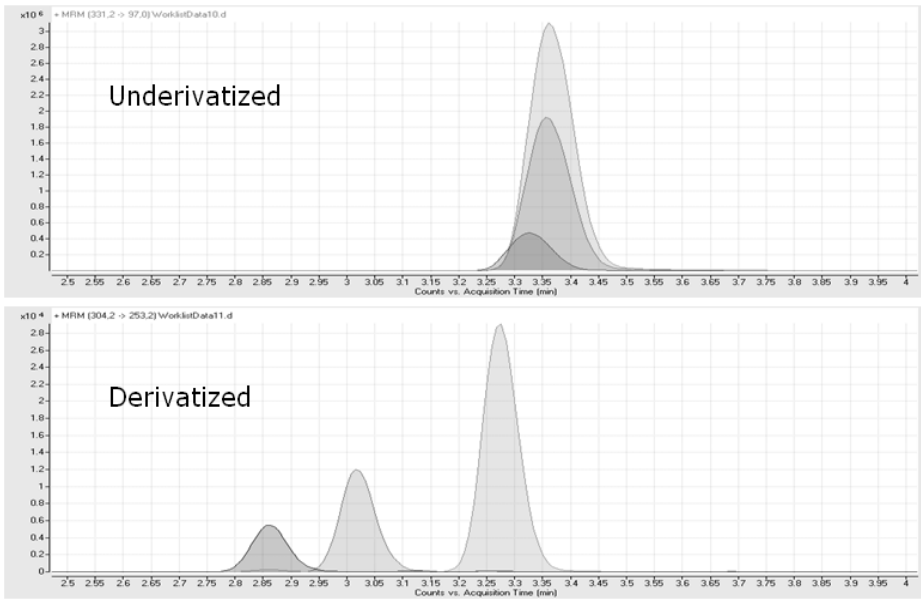


Figure 15. *Separation of testosterone, 17-hydroxyprogesterone, and dehydroepiandrosterone after their derivatization to oximes. Identical chromatographic method in both runs. Details of the separation system and conditions are described in paper* ***IV****.*

Depending on the analyte, the LLOQs were between 0.03 and 0.34 nM (0.01–0.10 ng/ml), enabling the analysis of all of the included steroids from adult female and male serum. However, some important analytes had to be excluded due to their insufficient response. Dihydrotestosterone could be measured from males, but not from females. This compound, as well as androstanedione that could not be detected from the serum of either sex, showed pronounced separation of the oxime isomers, which resulted in decrease of peak height (Figure 16). Estrone was detectable in the serum of both sexes, but its low levels resulted in poor precision. Androsterone could not be chromatographically separated from its biologically active isomer etiocholanolone. It also produced fragmentation that was identical with etiocholanolone and hence these two coumpound could not be included in the assay. Despite the shortcomings in the analysis of these compounds, the steroid assay was the first to describe a simultaneous analysis of all of the main ketosteroid metabolites of progestagen and androgen groups in both female and male serum.

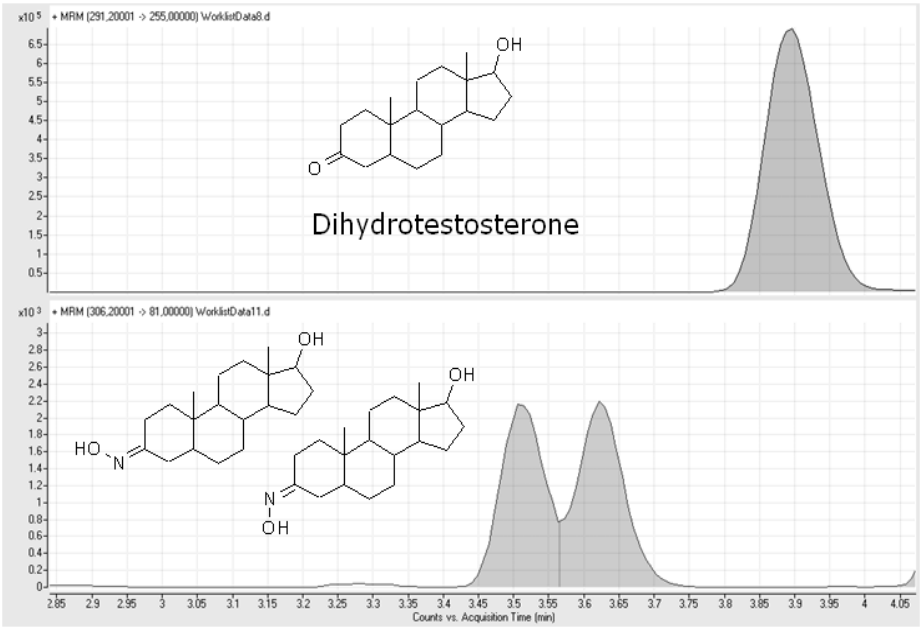


Figure 16. *Formation of chromatographically separable isomers during oxime derivatization. Identical chromatographic method in both runs. Details of the separation system and conditions are described in paper* ***IV****.*

The ketosteroid assay was used for the serum steroid profiling of endometriosis patients (n=155) and healthy controls (n=64). All the included analytes could be reliably assayed with a typical throughput of 90–100 samples per day. The high sensitivity of the assay was essential for the successful analysis of progesterone, 17-hydroxyprogesterone, and 17-hydroxypregnenolone.

7 *Summary and conclusions*

In this thesis, different techniques to increase the sensitivity of bioanalytical LC–MS assays were studied. The experimental work was focused on improving the sample preparation and the analyte ionization, with two different approaches taken for both stages of an assay. For the aqueous, protein-deficient samples with a volume below 50 μL, direct injection was employed. For the larger serum samples that could not be injected as such, small-scale LLE technique was developed. Ionization of permanently charged analyte ACh was improved by using a novel APTSI technique. For poorly ionized ketosteroids, oxime derivatization was used in conjunction with ESI. The techniques were implemented in three complete assays, with validation and sample analysis data to support the evaluation of their performance. In addition, the feasibility of using the APTSI technique at the microchip scale was evaluated in a preliminary manner prior to further studies.

The assays demonstrate the feasibility of direct injection for aqueous samples with a low protein content, such as AH or brain microdialysate. Diluting the sample with IS increases the analyte recovery by diluting the solution that remains in the vial after the injection. However, the injection volumes are limited by the analyte polarity and column capacity. Increasing the injection volumes may not be a feasible option when very small columns or particles with low mass loading capacities are employed. Direct injection of aqueous samples also relies on RP or ion-pair chromatography, since injection of aqueous samples into a HILIC column is not feasible. For the serum samples that required sample pre-processing, LLE was used. In the case of steroid analysis that required derivatization in addition to the extraction, minimal sample loss was achieved by performing the entire process using only two autosampler vials. This method is also feasible with large injection volumes, though having the same limitations related to the capacity of the separation column as the methods based on direct injection.

APTSI was found to be a remarkably suitable ionization technique for ACh analysis, enabling the use of TFA in the mobile phase without ion-suppression. Experiments with a heated microchip nebulizer highlighted the feasibility of using APTSI with the microchip technique, enabling its use in combination with microscale separation systems. For the first time, APTSI was compared against APCI and ESI, and was found to result in ESI-like ionization of various compounds that

can be charged in solution. The low noise and the tolerance for ion-suppressing mobile phase additions seem to be the major advantages of this ionization technique over ESI. For the ketosteroid analytes with weak proton affinity, oxime derivatization was used. This resulted in improved ionization efficiency, permitting the use of ESI. The derivatization reaction could be efficiently incorporated into the LLE process, maintaining the high analyte recovery of the sample preparation method.

Future perspectives

The advantages of APTSI should be fully probed. The studies with APTSI and different mobile phase additives suggest that APTSI may be less sensitive to matrix based ion-suppression than ESI. In this case, APTSI could represent a feasible alternative to ESI, when APCI and APPI cannot be used. In addition, a direct comparison of standard-sized APTSI with ESI, APCI, and APPI would allow its establishment among the other ionization techniques.

Due to the decreased dimensions, a microchip APTSI in combination with a capillary column should offer significantly improved sensitivity for ACh analysis. Performance characterization of the described setup in an analytical setup is needed to allow comparisons against standard-sized systems. If the increase in sensitivity is sufficient to allow the quantification of the baseline ACh levels in rat brain without requiring the inclusion of acetylcholinesterase inhibitors in the perfusion fluid, it would be the preferred technique. In addition, the smaller injection volumes possible with the capillary systems would allow smaller sample volumes and greater sampling frequency, increasing the temporal resolution of the microdialysis experiments. However, it must be noted that the performance of the experimental microchip used in this study was not optimal when the amount of organic solvent in the mobile phase was below 10%. This indicates that the design of the microchips, such as the efficiency of the heater and the type and dimensions of the vaporizer may have strong effect on their performance.

In the present study, the IS dilution and mixing were performed manually in the vials using a micropipette. To increase the method throughput, this step could be performed using the autosampler functions. This would enable the collection of the samples directly into the vials or well plates and their subsequent analysis without any need to manually process the samples.

The described oxime derivatization method can only be used for compounds containing a ketone functionality. Consequently, ionization of some steroids of

significant biological importance, such as estradiol, cannot be improved with this technique. Other derivatization techniques exist, but they are not compatible with the steroids described in this study. However, there is a great need for the simultaneous measurement of estradiol and ketosteroids. To achieve this goal, an approach different from the one described in this study would be needed. The use of APPI is a promising alternative to older techniques in the analysis of underivatized steroids, and together with suitable derivatization, further increase in the sensitivity may be obtained, with the ability to analyze a greater range of steroids.

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This thesis presents alternative approaches for bioanalytical method development using liquid chromatography–mass spectrometry. It explores the feasibility of using atmospheric pressure chemical ionization without corona discharge, the use of this technique on a microchip scale, derivatization of non-polar analytes for improved electrospray ionization, and different pre-processing methods for sub-150 µl biological samples.

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