

Mass spectrometric analysis of steroid hormones and other apolar molecules

PhD thesis

Dr. Péter Avar

Supervisors: Dr. László Márk and Dr. Zsolt Pirger

Doctoral School of Interdisciplinary Medical Sciences

Program Director: Prof. Dr. Balázs Sümegi



University of Pécs, Medical School

Institute of Biochemistry and Medical Chemistry

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Content

Abbreviations	3
1. Introduction.....	5
1.1 General introduction	5
1.2 Mass spectrometry.....	6
1.3 Steroids	8
1.4 Carotenoids.....	13
2. Aims.....	14
2.1 Steroid measurements.....	14
2.2 Structural elucidation of carotenoids.....	14
3. Instrumentation, Materials and Methods	15
3.1 Instruments	15
3.2 Mass spectral calibration and blanks.....	17
3.3 Materials and standards	19
3.4 Sample preparation	19
3.4.1 Environmental studies.....	20
3.4.2 Mouse brain and blood E2 study	23
3.4.3 Preparation of dansyl-derivates.....	24
3.5 HPLC-MS/MS methods	25
3.5.1 Analysis of DRO, LNG and PRG as environmental contaminants	25
3.5.2 Analysis of E2 and EE2 as environmental contaminants	26
3.5.3 Analysis of endogenous E2 in blood and in brain tissue	27
4. Results and discussion	28
4.1 Steroid measurements.....	28
4.1.1 Environmental analysis of steroids	28
4.1.2 Mouse brain and blood E2 study	45

4.2 Structural elucidation of carotenoids.....	49
5. Summary.....	52
6. Novel findings.....	53
7. Acknowledgements.....	54
8. List of Publications.....	55
8.1 Thesis related publications.....	55
8.2 Other publications.....	57
9. References.....	59

Abbreviations

APCI – atmospheric pressure chemical ionization

DC – direct current

DRO – drospirenone

DRO-d4 – drospirenone-d4

DSCI – dansyl chloride

E1 – oestrone

E2 – oestradiol

E2-d3 – oestradiol-d3

E2-DS – 3-dansyl-oestradiol

E3 – oestriol

EE2 – ethinyl-oestradiol

EE2-DS – 3-dansyl-ethinyl oestradiol

EI – electron impact

ESI – electrospray ionization

GC-MS – gas chromatography - mass spectrometry

HCD – higher-energy collision induced dissociation

HPLC – high performance liquid chromatography

HRT – hormone replacement therapy

IR – infrared

LC-MS – liquid chromatography – mass spectrometry

LNG – levonorgestrel

LNG-d6 – levonorgestrel-d6

LOD – limit of detection

LOQ – limit of quantification

MALDI – matrix assisted laser desorption ionization

MS/MS – tandem mass spectrometry

NF – not found

OVX – ovariectomized

PBS – phosphate buffered saline

PRG – progesterone

PRG-d9 – progesterone-d9

RF – radio frequency

RIA – radioimmunoassay

RSD – relative standard deviation

SOCs – synthetic oral contraceptives

SPE – solid phase extraction

T – testosterone

TOF – time-of-flight

tSIM – targeted single ion monitoring

TBME – tert-butyl methyl ether

UHPLC – ultra high performance liquid chromatography

UV-VIS – ultraviolet-visible spectroscopy

WWTP – waste water treatment plant

1. Introduction

1.1 General introduction

During my years spent in the mass spectrometry laboratory of the Department of Biochemistry and Medical Chemistry at the Medical School of the University of Pécs I had the opportunity to learn analytical chemistry using modern instruments, mostly mass spectrometers. Within mass spectrometry, metabolic profiling became my area of interest. Metabolomics deals with the study of the metabolic (small molecule) content of the analysed tissue, cell or other sample. Metabolomics covers a large scale of research. It is interdisciplinary, but most important in the fields of biochemistry, genetics, molecular biology and medicine. According to Scopus, the largest database for peer-reviewed literature, more than 12000 articles indicated their topic as metabolomics in the last five years in scientific journals of these four fields. In the laboratory of mass spectrometry at the University of Pécs I had the chance to work with excellent research resources. Using MALDI-TOF, HPLC-UV-VIS and LC-MS I have analysed various molecules amongst them several biochemically important polar components, amino acids, small acidic molecules like succinic acid, nucleotides and others, but my main focus became apolar molecules, lipids. Lipidomics represent a major part in metabolomics and in mass spectrometry, and has earned a central analytical role in lipidomics (Harkewicz and Dennis 2011). As it is in the chemical and biomedical analysis generally, I was confronted with two major tasks: identification and quantitation. Identification of purified samples are typically achieved with nuclear magnetic resonance, IR or UV spectroscopy, in some cases with X-ray diffraction, but mass spectrometric analysis contributes to structural studies in an increasing manner (Vékey, et al. 2008). Sensitivity is a great advantage of mass spectrometry in structural elucidation, thus it needs considerably less amount of sample, than other methods and samples do not have to be 100 percent pure. Quantitation of previously identified compounds is another, more frequently occurring task, which I could practice in the laboratory. Quantitation similarly to structural elucidation is based principally on molecular characterization such as spectral features, chromatographic retention time and comparison with external or internal standards or reference materials. Chromatographic retention time alone is no longer a proof of identification, more specific and reliable information is needed nowadays. Researchers must

always intend to apply the best techniques, which are affordable and available for them, but the exact requirements of reliable or acceptable identification or accurate quantification are not regulated in all the fields of analysis. However mass spectra and accurate mass measurements, which I both did use in my work, are increasingly expected. In my thesis I am presenting my small contribution to the mass spectrometric analysis of two different subgroup of lipids, steroids and carotenoids. The main part was achieved by LC-quadrupole-orbitrap analysis. Only a few, but important steroid type molecules have been quantified in several studies. A minor part displays the analysis of tetraterpenoids (carotenoids), where the aim was the identification and chromatography was not coupled with mass spectrometry. It is common in the two groups of analytes, that they both belong to subclasses of the lipid class isoprenoids, thus these natural compounds are ultimately derived from the mevalonate biosynthetic pathway.

1.2 Mass spectrometry

Mass spectrometry is a modern analytical technique, although its history leans back to the 19th century. There are numerous types of instruments, but it is common, that molecules or atoms must be ionised and get into gaseous phase prior to analysis. These ions are manipulated (e.g.: transferred, filtered to select a smaller range of them, collected, injected to analyser) with the help of electric fields. Applied electric fields can be static or alternating current (AC) fields, in some cases both are applied. Static fields are called direct current (DC), applied AC fields are called radio frequency (RF) fields. Magnetic fields are also used in some instruments. The mass to charge ratio (m/z) of the ions is measured in order to identify and quantify ions of interest in simple or complex mixtures.

The main components of mass spectrometers are ion source, analyser and detector. Samples are introduced into the ion source of the instrument, where a portion of them is converted into molecular or atomic ions. In the mass analyser ions are separated based on their mass to charge ratios, then the analyser transmits them to the detector where they are detected and later converted to a digital output. Additional important components make sample introduction, ion filtering and transferring of ions possible. To avoid unwanted collisions of analytes and air components, ion beams in mass spectrometers are travelling from the place of introduction or after the ion source from the transfer capillary on, in vacuum. Modern instruments therefore employ more than one vacuum pump as well. There are backing pumps

and turbomolecular pumps. Oil-based rotary pumps are in use for decades as backing pumps and they remain the most common way to generate rough vacuums. High vacuum is provided by turbo pumps. A turbo pump is basically a sophisticated fan spinning at very high speeds.

Different ion sources are available: electron impact (EI), chemical ionization (CI), matrix assisted laser desorption ionization (MALDI), atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionization (APPI), electrospray ionization (ESI), laser ablation electrospray ionization (LAESI) etc., but the most common sources are MALDI, EI and ESI (Greaves and Roboz 2014).

Mass spectrometry is often coupled with a separation technique, which can be capillary zone electrophoresis (CZE), gas chromatography (GC) or liquid chromatography (LC). GC is mostly followed by EI type ionization, while in LC-MS ESI is the most widely used ion source.

Most common analysers are time-of-flight (TOF), linear quadrupole (Q), which is usually referred simply as quadrupole, iontrap and orbitrap. TOF analysers measure the flight time of accelerated ions in a field-free drift tube. Flight time is proportionate to the square root of the m/z value. Quadrupoles and iontraps work with the same basic principles. The quadrupole mass filters and linear quadrupole analysers consist of a linear array of four symmetrically arranged rods to which RF and DC voltages are supplied. Ions oscillate in the x,y -plane with frequencies which depend on their m/z values and with excursions which depend on the amplitudes of the applied potentials and their initial positions. If the oscillations of an ion in this plane are stable, the ion will continue to drift down the rod assembly and reach the detector. Detectors (ion current detectors) determine the abundance of the ions emerged from the analyser. Detectors are multipliers, because they multiply/amplify the very low current produced by the ions arriving at the detector surface. Most common detectors are electron multipliers. Orbitrap analyser, achieve orbital trapping of ions around a central electrode, an image current is recorded, than a mass spectrum is gained via Fourier transformation (FT) of the data. (Eichhorn 2012) FT instruments do not apply electron multipliers, detection is based in them on the phenomenon that the passage of ions close to a metal surface induces an electric current in the surface, similarly to the action of the armature in electrical generators. The result is an alternating current with a sinusoidal path the frequency of which is proportional to the m/z of the ion that induced the current.

Modern mass spectrometers are sensitive instruments, they are able to detect trace levels of compounds and measure their concentrations in complex matrices. During the analysis mass spectra are collected. If mass resolution is high enough elemental formulas of the components of a mass spectrum can be calculated, but mass spectra generally contain structural information. Fragmentation of selected ions can yield finer structural information. It is called tandem mass spectrometry (MS/MS). Tandem spectra are characteristic, therefore, MS/MS is frequently used as a proof of selectivity. In quantitative studies, peaks of selected ions or parent ion-daughter ion transitions are integrated. Their intensities are proportional to the amount of their corresponding compounds in the samples, which is the basis of quantitative measurements.

1.3 Steroids

Steroids belong to the lipid group of sterol lipids. All the natural steroids contain a fused four-ring sterane core structure. The basic sterol synthesized in animal cells is cholesterol. In the ovary, different subclasses of sterols can be formed from it. Based on the number of carbon atoms in their structure there are C18 steroids (oestrogens), C19 steroids (androgens) and there is the C21 subclass, which includes progestogens, glucocorticoids and mineralocorticoids (Abduljabbar 2012). Steroids along with the glycerophospholipids and sphingomyelins are important membrane components. Some of them are well-known endogenous hormones or have a role in the molecular signalling. Following the changes of steroid hormones in physiological or pathological states is a main focus of endocrinology. Steroids, however, can also be measured as environmental contaminants. Steroids can be categorised based on their functions as sex hormones, corticoids and anabolic steroids. I investigated only progestogens and oestrogenic compounds, which all belong to the group of sex hormones.

Progestins (also called progestogens, progestagens or gestagens) encompass progesterone (PRG) and structural relatives of it. There are also synthetic steroid drugs such as levonorgestrel (LNG) or drospirenone (DRO) which possess common progestogenic activity. The biological effects of individual progestins however can differ from each other. (Schindler, et al. 2008) These progestins are widely used as synthetic oral contraceptives (SOCs), in hormone replacement therapy (HRT) and in the treatment of several gynecological disorders.

According to literature data, more than 100 million women use SOCs every year.(Huezo 1998) After oral, vaginal or other kind of administration progestins enter the blood stream and take effect. In the meanwhile, a certain amount of progestins will be metabolised. (Besse and Garric 2009; Liu, et al. 2011c) Metabolites and also unmetabolised drugs are eliminated from the human body mainly through the renal system. A remarkable amount is excreted unchanged or in the form of active sulfate or glucuronide metabolites. These metabolites also have pharmacological activity. Household derived contamination enters watercourses fortunately through waste water treatment plants (WWTP) where progestins are partially degraded. This decomposition however is not perfect, because these contaminants can be found in WWTP effluents (Andrasi, et al. 2013; Aris, et al. 2014). Progestins enter the aquatic environment not only through WWTP effluents but also through paper mill plant effluents and animal agricultural runoffs. There is a continuously increasing amount of evidence about the influence of progestins on the hormone systems of aquatic species. Both vertebrate (fish, amphibians) and invertebrate (insects, molluscs) freshwater species are highly sensitive to progestins.(Diniz, et al. 2005; Jobling, et al. 2003; Jobling, et al. 1998; Kahl, et al. 1997; Kroupova, et al. 2014; Liu, et al. 2011c; Svensson, et al. 2014; Watts, et al. 2003). For example, the fecundity or egg production of fathead minnows (*Pimephales promela*) were dramatically decreased due to a 21 day long treatment by synthetic progestins (noretindrone). Plasma concentrations of the endogen 11-ketotestosterone in male and E2 in female fathead minnows after 21 days of exposure were significantly decreased (Paulos, et al. 2010). Toxicity experiments report that both LNG (0.80 ng/L) and DRO (0.66 ng/L) at low concentrations reduce fertility in adult fathead minnows. 3.3 ng/L LNG induces masculinization of female frog (*Xenopus laevis*). The synthetic progestin LNG impresses the reproductive system of pubertal roach (*Rutilus rutilus*) by affecting the pituitary gonadotropin expression and the endogenous sex steroid levels. This disruption was determined to occur in male animals after exposure to an environmentally relevant concentration (31 ng/L). It was reported also that 6.5 ng/L LNG could disrupt the androgen-dependent reproductive cycle of male three-spined sticklebacks (*Gasterosteus aculeatus*). Bioaccumulation of progestogenic chemicals was also observed in the zebra mussel (*Dreissena polymorpha*) and in the rainbow trout (*Oncorhynchus mykiss*) (Liu, et al. 2011c).

Oestrogens are principally known as female human sex hormones, but they can be found generally in vertebrate species and are physiologically present in males also (Jones, et al. 2006). Oestrogens possess not only reproductive functions. They influence various cellular

processes. The most potent natural oestrogen is β -oestradiol (E2). E2 is an endogenous steroid hormone present in both human and animal tissues and body liquids. E2 has a reputation as an effective medicine in menopausal/postmenopausal treatment (HRT), and in aging related diseases in woman. It is not only “the woman’s hormone”, thus tissues other than the female reproductive tract, such as the brain, are important targets of its action (Gillies and McArthur 2010). In mammalian species both oestrogens, mostly E2 but others also, and androgens, such as testosterone (T), are principally synthesized in the gonads and in the adrenal glands. The blood stream contains a relatively low level of circulating E2, except in females of reproductive age. In nonpregnant females of reproductive age a greater amount of oestrogens are produced in a cyclical fashion. E2 is also produced from circulating steroid precursors, such as T, in several tissues, regardless of gender by aromatase enzymes (CYP19) (Jones, et al. 2006). The hypothalamus is most actively expressing aromatases in the brain, but moderate level is synthesized in the amygdala, hippocampus, midbrain and cortical regions in both rodents and humans. Gonadal steroids regulate brain-aromatase activity in the hypothalamus, the above mentioned regions are known to produce aromatases in a steroid and gender independent manner. In the brain, oestrogens can act both rapidly (within seconds to minutes) via membrane actions as paracrine enzymes and through classic nuclear receptors (Acconcia and Kumar 2006; Cheong, et al. 2012; Kwakowsky, et al. 2014).

Other natural oestrogens are oestrone (E1) and oestriol (E3). They are less potent and less prevalent than oestradiol. Their investigation is not included in my thesis. There is a synthetic analogue of E2, ethinyl oestradiol (EE2), which is a component in many frequently used contraceptives and its environmental occurrence was also investigated. Due to their intensive human therapeutic usage, E2 and EE2 are the most abundant steroid molecules with oestrogenic function, which can be found in the environment (Aris, et al. 2014). Domestic effluents and livestock waste excrete a considerable amount of hormones in addition to agriculture runoffs and industrial sources. Similarly to progestins, excreted urine contains a large part of the hormones in the form of sulfate and glucuronide conjugates, but the bacterium *Escherichia coli* in wastewater is able to deconjugate these metabolites due to its β -glucuronidase and sulfatase activity. E2, EE2 and their watersoluble sulfate and glucuronide metabolites enter natural streams, rivers, lakes mainly through cleaned wastewater (Aris, et al. 2014). There are feasible cleaning methods for these compounds, but the generally used cleaning methods in wastewater treatment plants apparently cannot eliminate these compounds totally (Kim, et al. 2015). Consequently, similarly to progestins an unquantified

load of oestrogens is released into the aquatic environment, where it can be absorbed by sediment and persist for long periods or taken up by aquatic biota (Matozzo, et al. 2008). E2 is principally a vertebrate hormone, although it was detected in some invertebrates, such as molluscs. Many aquatic species express oestrogene receptors. Some species are known to have mechanisms, which allow them to maintain their endogenous hormonal levels in the case of exogenous E2 exposure, but a wide range of species are very sensitive to steroid contaminants (Bhandari, et al. 2015; Huang, et al. 2015; Janer, et al. 2005). Bioaccumulation and biotransformation of xenoestrogens, their effects on individual development and alterations in adult behaviours have been reported in numerous publications (Liu, et al. 2011a; Yan, et al. 2012). Increased hepatosomatic index and decreased gonadosomatic index have been described in E2 and EE2 treated fish. Hepatosomatic index is a frequently used indicator in limnology, and it means the ratio of liver weight to total body weight. Gonadosomatic index is a similar indicator, it means the ratio of the weight of gonads to total body weight. Changes of these indices suggest that molecular changes appear in the whole body of treated fish. Changes are described in the liver, kidneys, and in the brain of two species, in silver catfish and in zebrafish (Costa, et al. 2010; Martyniuk, et al. 2007). The immune system and the mortality of leucocytes of fish can also be affected (Bado-Nilles, et al. 2014). The most used indicator of exposure of exogenous oestrogens in aquatic species is plasma vitellogenin. EE2 induces vitellogenin production and affects anxiety and shoaling behavior in adult male zebrafish and induces anxiogenic behavior in guppies (*Poecilia reticulata*) (Hallgren, et al. 2011; Reyhanian, et al. 2011). The exposure to mixtures of E2 and EE2 effects sperm motility, fertilization, embryo- and larval survival even at relevant environmental concentrations in prejerrey fish (*Odontesthes bonariensis*)(Garriz, et al. 2015). Bioaccumulations of E2 and EE2, their effects on individual development, and alterations in behavior have also been reported in numerous publications (Bhandari, et al. 2015; Huang, et al. 2015; Yan, et al. 2012). Considering their diverse role in the regulation of endogenous processes, quantitation of these two chemicals is very useful not only in the clinical assessment but also in environmental investigations. In the European Union, pharmaceuticals authorization procedures include Commission directives, which contain a requirement to develop a strategic approach to the pollution of water by pharmaceutical substances. E2 and EE2 were newly added to the EU watch list of emerging pollutants in 2013 (Directive 2013/39/EU). They were also included in EU Commission Implementing Decision 2015/495 in which a watch list of substances has been established for Union-wide monitoring in the

field of water policy pursuant to earlier Directive 2008/105/EC. Pharmaceuticals on the watch list are not on the EU priority list of environmental pollutants, but they can be added to it in the future. There are several ways to analyse these pollutants. depending on the aim of the study and their opportunities analysts use GC-MS, LC-MS and immunoassays, but chromatography based methods with UV-VIS, fluorescence or electrochemical detection are also available. Recent developments in analytical chemistry enable the mass spectrometric detection of steroids even at the low ng/L level (Andrasi, et al. 2013; Liu, et al. 2011c; Ripolles, et al. 2014; Tomsikova, et al. 2012; Williams, et al. 2003). There is an important difference in the MS ionisation of progestins and oestrogens. Progestins, probably through forming protonated ions, can be ionised easily in positive mode. In the case of E2 and EE2 deprotonation of a hydroxyl-group, thus ionisation in negative mode is observed commonly. Negative mode ionisation generally, and in the case of oestrogens too, enables only higher detection limits compared to positive mode methods. Sensitivity can be though increased in mass spectrometry by chemical transformation of the analyte. Chemical transformations designed to enhance analytical capabilities are called derivatisation. Derivatisation of E2 and EE2 prior to mass spectrometric analysis makes positive mode detection possible, which improves both sensitivity and specificity of their analysis. MS methods measuring E2 or EE2 without derivatisation can be applied in the clinical assessment, where samples contain high concentrations, and are difficult to use in environmental studies. Considering biological (human, rodent or fish blood) samples E2 can be measured from less than 100 µl plasma with or without derivatisation. The analysis of mouse brain tissue extracts however needs derivatisation to meet satisfactory quantification criteria. Full MS and targeted MS/MS methods without derivatisation, which are generally used in environmental monitoring applications, detect 40-100 or even more target compounds. These screening methods however often do not include or cannot detect E2 and EE2 due to poor detection limits. (Chitescu, et al. 2015; Kuster 2008; Liu, et al. 2011b; Loos, et al. 2010) Evaluation of derivatives of oestrogens makes positive mode electrospray ionization (ESI) mass spectrometric detection possible and improves generally the sensitivity of MS methods. (Anari, et al. 2002; Xu and Spink 2008) A novel comparison of derivatisation methods for the determination of E2 using standard solutions was recently published. (Li and Franke 2015) We applied dansyl chloride (DSCl) derivatisation in our method because it was found to be very sensitive and DSCl is approximately 10 fold cheaper than 1-methylimidazole-2-sulfonyl chloride, which produced the lowest limit of detection in the comparison. Our own

chromatographic method with mass spectrometric detection has been developed and it was applied in the field of environmental analysis.

1.4 Carotenoids

Carotenoids are strongly apolar prenyl lipids. They possess a C₄₀ carbon skeleton built up from 8 isoprene unit forming a linear molecule. They are lipid antioxidants in human, plant and in animal organisms and some of them are fat soluble provitamins (Jauregui, et al. 2011). β -carotene and its derivatives contain unmodified β -ionone end groups, and they serve as precursors for vitamin A and are therefore essential dietary components for humans and for mammals in general (Thorne-Lyman and Fawzi 2012). Plants are a natural source of colourful carotenoids. Pigmentation is caused by the chromophore part, the conjugated double bond system of the molecule. The higher the number of the conjugated double bonds is, the higher the absorbance and the longer the wavelengths of the absorbed light will be, causing a more reddish hue of carotenoids. Carotenoids can be divided into two major groups: carotenes and xanthophylls. Carotenes consist of only carbon and hydrogen atoms (e.g., α -, β - and γ - carotenes and lycopene), while xanthophylls are oxygenated derivatives of carotenes containing hydroxyl-, keto-, epoxy- and methoxy- groups. The number of known natural carotenoids is over 700 and carotenoid content of different plants have a high variability (Gomez-Garcia and Ochoa-Alejo 2013). HPLC-UV-VIS or HPLC-ECD methods applying C₁₈ or C₃₀ stationary phases are generally used for the determination of these compounds (Gulyas-Fekete, et al. 2013; Horvath, et al. 2010). In mass spectrometric studies of such non-polar analytes as carotenoids APCI ion sources are dominant, but the softer ESI ionization is also capable for their analysis (Agocs, et al. 2007; Bijttebier, et al. 2013; van Breemen, et al. 2012).

2. Aims

2.1 Steroid measurements

1. Development of a simple, fast, sensitive and relatively inexpensive method for the simultaneous detection and quantification of DRO, LNG and PRG from freshwater samples.
2. Development of rapid and sensitive methods for the determination of E2 and EE2 from water samples as environmental pollutants and from blood and brain tissue (E2 only) as endogenous hormone.
3. Survey of the endogenous brain E2 levels of mice including regional differences.
4. Providing reliable data about the actual level of contamination regarding these steroids in Hungarian and other freshwaters

2.2 Structural elucidation of carotenoids

5. Identification of β -cryptoxanthin-epoxides extracted from Red Mamey (*Pouteria sapota*)

3. Instrumentation, Materials and Methods

3.1 Instruments

A Q-Exactive quadrupole-orbitrap hybrid mass spectrometer (Thermo Fisher) was used in all direct infusion-MS, and LC-MS experiments. It is a high resolution mass spectrometer with a maximum resolving power of 140000. This resolution is given in full width at half maximum (FWHM). The scan range of the instrument is from 50 to 6000 m/z. Positive and negative mode detection or parallel running alternate mode detection are available. It is not a three dimensional iontrap, thus fragmentation is possible only to the depth of the first daughter ions (MS/MS, but no MSⁿ). There are other limitations. The upper threshold of the scan range cannot be larger than 15 times the lower threshold. The relatively high scan speed of the instrument enables a useful feature for the simultaneous analysis of more than one compounds in complex matrices, which is called multiplexing. Multiplex analysis in the case of an orbitrap mass spectrometer means parallel running and saving the data of several scan events. Such analysis must always be planned carefully, because in multiplexing the scan speed, as it is divided between the scan events, may not be high enough for the simultaneous detection or quantification of all components. A compromise must be found between the number of scan events, resolution and ion package size so that each component of interest shall have enough measured data point to draw and later process its chromatographic peak. The higher the resolution is set the slower the data scanning will be. I applied 35000 (FWHM) or higher resolution. FWHM must be given at a specific mass to charge value, which in metabolomics is often 200 (m/z). Best values of automatic gain control target and the aimed size of ion packages are depending on peak width and intensity therefore they have been adjusted to chromatography individually. Dionex Ultimate 3000 UHPLCs (Thermo Fisher) were used in LC-MS experiments. These HPLCs are called UHPLCs, but their upper pressure limit is 600 and 1000 bar only. Pressure values during my methods were typically 350-500 bar. Chromatographic flow rate can be chosen from 1 μ l/min up to 10 ml/min, but the ion source of the mass spectrometer enabled 1-1000 μ l/min flow rate only. Considering these pressure and flow rate limitations conventional LC columns with 2.1 mm internal diameter have been used. All the columns were packed with simple or modified C18 core-shell materials. These column and flow rate settings enabled separation with peak widths of 6-20s. Narrower

chromatographic peaks would have spared analysis time, but they also would cost a reduction in number of data points to integrate in data processing and a raise in column backpressure.

A Fusion 100 T syringe pump (Thermo Fisher Scientific, Germany) was used to achieve direct infusions to the mass spectrometer.

3.2 Mass spectral calibration and blanks

In order to maintain mass spectral accuracy and keep mass tolerance at minimum the mass analyser was calibrated at least fortnightly according to the manufacturer's directions using a mixture of caffeine, MRFA peptide, and Ultramark for positive ionization mode or a mixture of MRFA peptide, Ultramark, SDS, and sodium taurocholate for negative ionization mode. Mass accuracy was tested with Agilent's ESI-L Low Concentration Tuning Mix as a custom standard mix of hexamethoxyphosphazine (m/z 322.048121), hexakis(2,2-difluoroethoxy) phosphazine (m/z 622.028960), hexakis (1H, 1H, 3H-tetrafluoropropoxy) phosphazine (m/z 922.009798), and hexakis (1H, 1H, 4H-hexafluorobutyloxy)phosphazine (m/z 1221.990637) also. Calibrations showed mass tolerance typically between 0.5 and 2 parts-per-million (Figure 1).

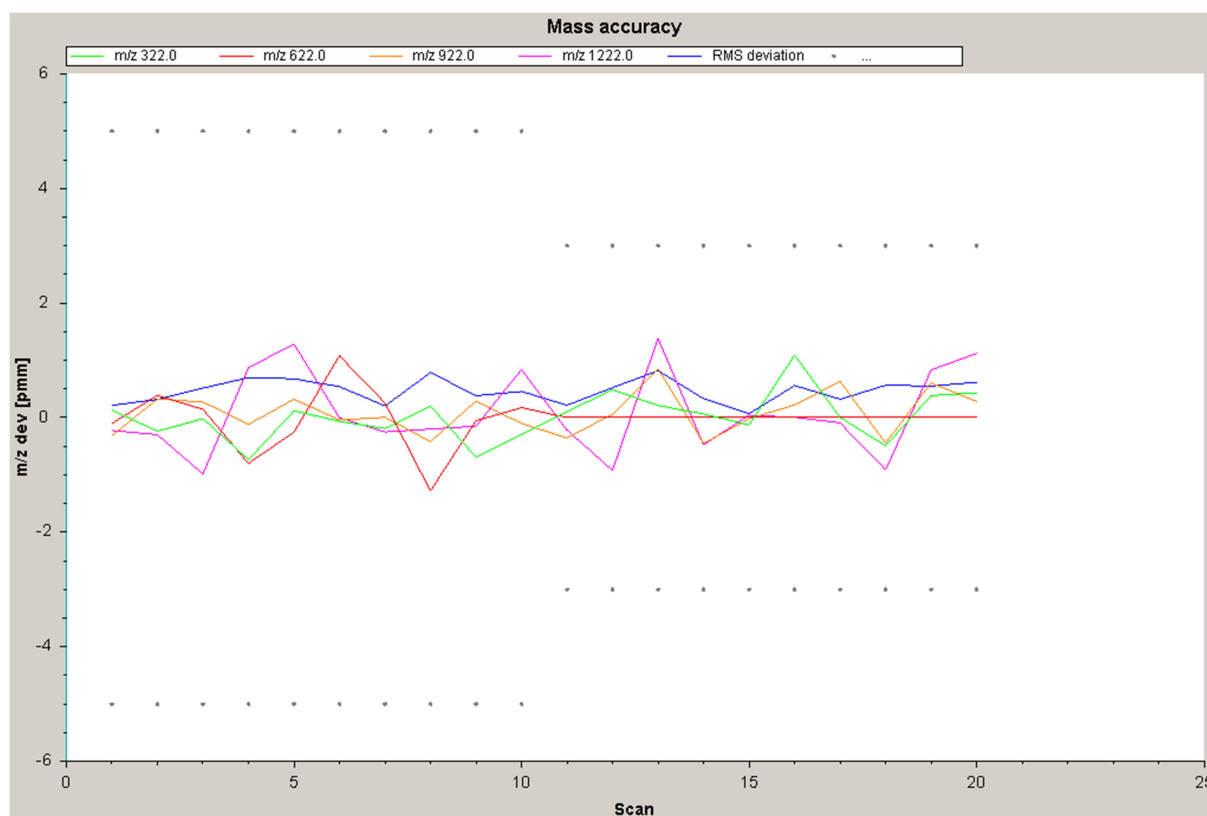


Figure 1.

Typical mass accuracy of the Q-Exactive orbitrap mass analyser after spectral calibration

Solvent blanks, containing no detectable compounds, have been used throughout all the studies as follows. Solvents, which were used to dissolve samples have been injected as empty blanks as the starter run and as the last run of each sequence and also after each triplicate of each sample.

Procedure blanks have been used as tests for background noise during method development and one procedure blank was made and run with each batch of derivatisation. No background correction was applied, if a blank showed detectable compound(s) the concerned files have been excluded from the analysis, and I investigated the mistake or source of contamination. After I resolved the cause of mistake, the affected samples were reanalyzed if it was possible.

3.3 Materials and standards

LC-MS grade methanol, acetonitrile and distilled water were used, all were obtained from VWR (Debrecen, Hungary). Dulbecco's Phosphate Buffered Saline (PBS) was obtained from Sigma-Aldrich (Budapest, Hungary). Tert-Butyl methyl ether (TBME) was HPLC grade, and it was from ScharLab (Debrecen, Hungary). Formic acid and hydrochloric acid (LC-MS grade) were purchased from LGC Standards (Wesel, Germany). Ethyl acetate (HPLC grade), E2, EE2, DRO, LNG and PRG analytical standards for quantitative measurements were purchased from Sigma-Aldrich. Drospirenone-d4 (DRO-d4) was ordered from Biozol (Eching, Germany). Oestradiol-d3 (E2-d3) was from Isosciences (King of Prussia, USA). Progesterone-d9 (PRG-d9) and Norgestrel-d6 (LNG-d6) were from TRC (Toronto, Canada). β -cryptoxanthin was from CaroteNature (Ostermundigen, Switzerland). Glass microfiber filters were obtained from Whatman (Maidstone, UK). The Strata C18-E SPE cartridges (55 μm , 70 \AA), Kinetex C18 (2.6 μ , 100 \AA , 100*2.1mm), Kinetex EVO C18 (5 μ , 100 \AA , 150*2.1mm) and Kinetex XB-C18 (2.6 μ , 100 \AA , 150*2.1mm) HPLC columns were ordered from GenLab (Budapest, Hungary).

Calibration solutions of DRO, LNG and PRG were prepared by weighing and dissolving solid standards in acetonitrile and diluting them with water and acetonitrile so that they reached 30v/v% (acetonitrile/water). E2 and EE2 standard solutions were prepared by weighing and dissolving one oestrogen at a time in methanol. E2 and EE2 calibration standards were derivatised at seven concentration levels covering the range from 0.005 $\mu\text{g}/\text{mL}$ to 0.1 $\mu\text{g}/\text{mL}$. Dansyl derivatisation was carried out with 100 μL from each standard solution similarly to water samples. Carotenoid samples were dissolved in pure LC-MS grade methanol.

3.4 Sample preparation

By means of different origins I had to work with four types of samples. This would mean 4 different matrices, but I describe only 3 of them in details, those which were my own work.

City canal water and freshwater samples (i) were both extracted on solid phase, with the only difference that in the case of city canal water a smaller volume was collected and analysed. Difficulties lay in the extraction of blood (ii) and brain (iii) tissues, by which I had to apply

different liquid/liquid extractions. The extraction and isolation of carotenoids from plant tissue (iv) were achieved by the carotenoid research group of our institute. Natural carotenoids have been extracted from 500g red mamey (*Pouteria sapota*) in several steps using open column chromatography and semi-preparative chromatography. I performed only the mass spectrometric analysis after these steps. Further details can be found in the following publications: (Gulyas-Fekete, et al. 2013; Turcsi, et al. 2015)

3.4.1 Environmental studies

Sampling of rivers, watercourses and Lake Balaton

We collected samples in Slovenia and in Hungary, from Lake Balaton, from six Central-European rivers (Danube, Drava, Mur, Sava, Tisza and Zala), from smaller watercourses and from a city canal in the urbanized area of the city of Pécs. Samples have been collected from the upper 30 cm surface layer of freshwater within 2 m distance from the bank unless otherwise described with coordinates. Two main environmental studies are described in the thesis. The first study was achieved in July 2014. In this earlier study progestins have been investigated in freshwater at 53 sampling points in the catchment area of Lake Balaton, Hungary. In the second study, which was achieved in July 2015 the occurrence of oestrogens (E2 and EE2) has been investigated in all the large rivers of Central-Europe (21 sampling points). Samples in main experiments have been analysed without filtration. Filtration was applied in supplementary experiments, which aimed to deliver information about the proportion of the dissolved and the suspended amount of residues in the samples. In these experiments water samples have been filtered and analytes have been extracted from the two parts. At first the filtrate was extracted on solid phase, then the filter paper itself underwent an extraction procedure separately. The volumes of the analysed samples have been: 770 ml in the first study (progestins), 1000 ml freshwater in the second study (oestrogens) and only 500 ml in the case of Pécsi víz city canal water (PV) in the second study. Sampling locations are visualized on the following two maps (Figure 2 and 3).

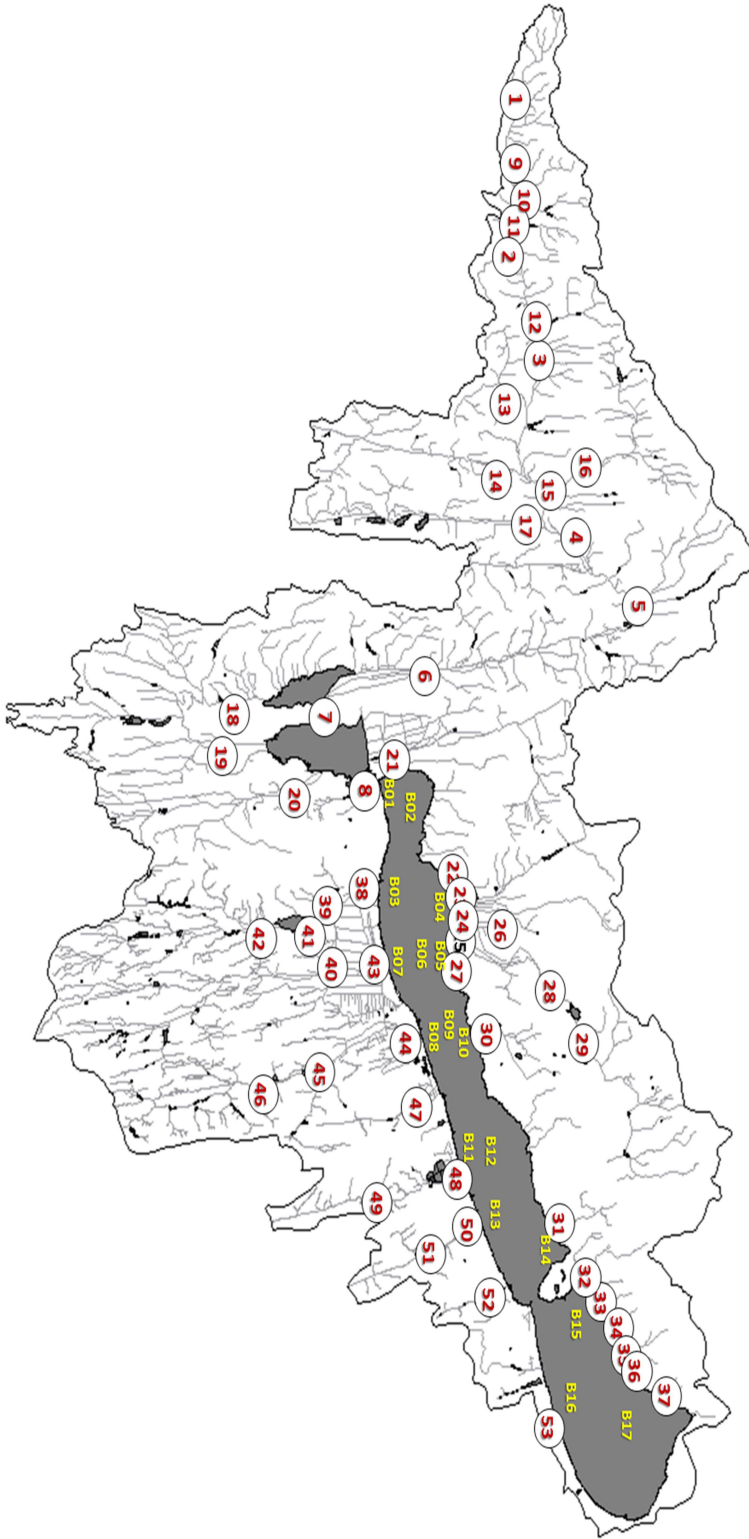


Figure 2.

Sampling locations (red numbers in circles) in the catchment area of Lake Balaton; July, 2014; in the progestin study

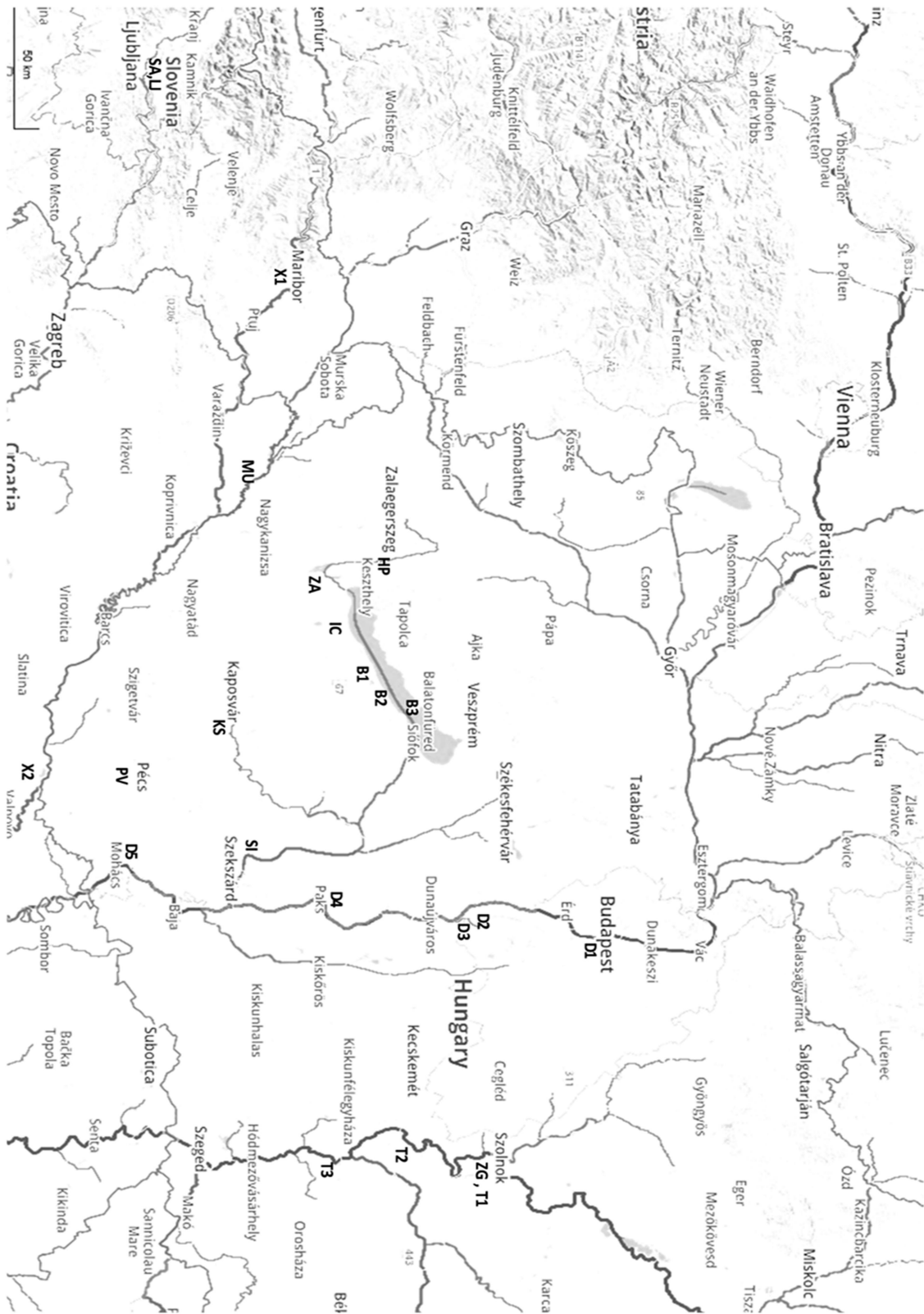


Figure 3.

Sampling locations (black, two capital letters or a capital letter and a number) used in July, 2015; in the oestrogen study

Solid phase extraction

Drug residues from water samples were concentrated on Strata C18-E (1g/20 mL) SPE cartridges. A vacuum dividing system has been fabricated from plastic vacuum tubes and glass T and Y elements, and I set and, if it was necessary, adjusted the vacuum manually using an opening slot and a Mohr clamp. The speed of the extraction was adjusted to 1-2 drop/second, which meant 3-4 hours extraction time for a 1000 mL freshwater sample, but fortunately up to four sample could be extracted at the same time. I conditioned the SPE cartridges with 15 mL methanol, equilibrated them with 20 mL LC-MS grade water, loaded them with water sample, and washed them with 20 mL LC-MS grade water. Elution was achieved with 15 mL methanol. Methanol has been evaporated with a rotary evaporator, at 40 °C bath temperature. Extracts were reconstituted in 750 µL methanol. The 750 µL methanol was concentrated to dryness with an Eppendorf Concentrator Plus at 30°C, in V-AL (vacuum-alcoholic solutions) mode. After that in the case of progesterin measurements extracts were reconstituted in 50 µL methanol. In the case of oestrogen measurements derivatising agent (dissolved in acetone) was added to them and they were derivatised.

3.4.2 Mouse brain and blood E2 study

Animals

Adult female mice (C57BL6) were bred and housed at the University of Pécs. The animals were maintained under conditions of 12-h light, 12-h dark cycle (lights on at 0800 h) with food and water available *ad libitum*. All protocols and procedures were approved and performed in accordance with approved protocols (University of Pécs; BA02/2000-15024/2011). Control and ovariectomized (OVX) animals have been used. The latter were bilaterally ovariectomized under isoflurane anesthesia and used for experiments 2 weeks later. Animals were sacrificed with decapitation, blood was collected in heparinized Eppendorf tubes and brains were removed and dissected in a sagittal axis, which resulted in 500 µ thick coronal sections. Numbering of the sections starts with No. 1 at the nasal end of the brain, numbers are increasing to the caudal direction.

Liquid/liquid extraction

Several solvents (Acetonitrile, ethyl acetate, methanol TBME) have been compared in order to find the optimal extraction method for the extraction of E2 from blood and from brain samples. TBME was found to be the best for the extraction of plasma. In the final method 25 μL plasma was extracted 3 times with 200 μL TBME. The best analytical parameters of extraction of brain tissue samples could be reached if the procedure was achieved with ethyl acetate in PBS buffered milieu. Animal brains were placed into constantly cooled isotonic buffer solutions. Brain sections were collected either with manual operation or with a microtome working in the buffer solution. Selected regions have been cut, and replaced to glass manual homogenizers. 200 μL (or at least four times the weight of the tissue) PBS and reference solution was added to each sample and they have been homogenized thoroughly. Homogenates were placed to Eppendorf tubes and extracted with 200 μL ethyl acetate 3 times. They have been vortex-mixed and centrifuged. The organic phase (supernatant) was taken to Eppendorf tubes and concentrated to dryness with an Eppendorf Concentrator Plus for later analysis.

3.4.3 Preparation of dansyl-derivates

E2 and EE2 standards and dried sample extracts were treated similarly. 50 μL 0.2M sodium bicarbonate (in LC-MS grade water) and 50 μL 1 mg/mL dansyl chloride (in acetone) were added to each of them. These mixtures were incubated in a thermomixer (65°C, 300rpm) for 10 minutes. Then they were cooled down on ice (2 minutes) and transferred to the autosampler of the HPLC, which was kept at 4°C. Theoretically dansyl-chloride could bind in the derivatisation reaction to one or to both OH groups (3-OH, 17-OH) of E2 or EE2. The m/z value of E2-DS and EE2-DS is independent of the position of dansyl-derivatisation, but the nucleophilic substitution on the phenolic hydroxyl group is preferred due to the electron withdrawing effect of the aromatic ring. The introduction of the basic nitrogen containing group to the oestrogen molecule (E2, EE2) enhances positive mode ionization under acidic conditions through decreasing pKa of the 3-OH (after derivatisation: 3-O-dansyl) group (Figure 4). E2-(DS)₂ and EE2-(DS)₂ have not been used.

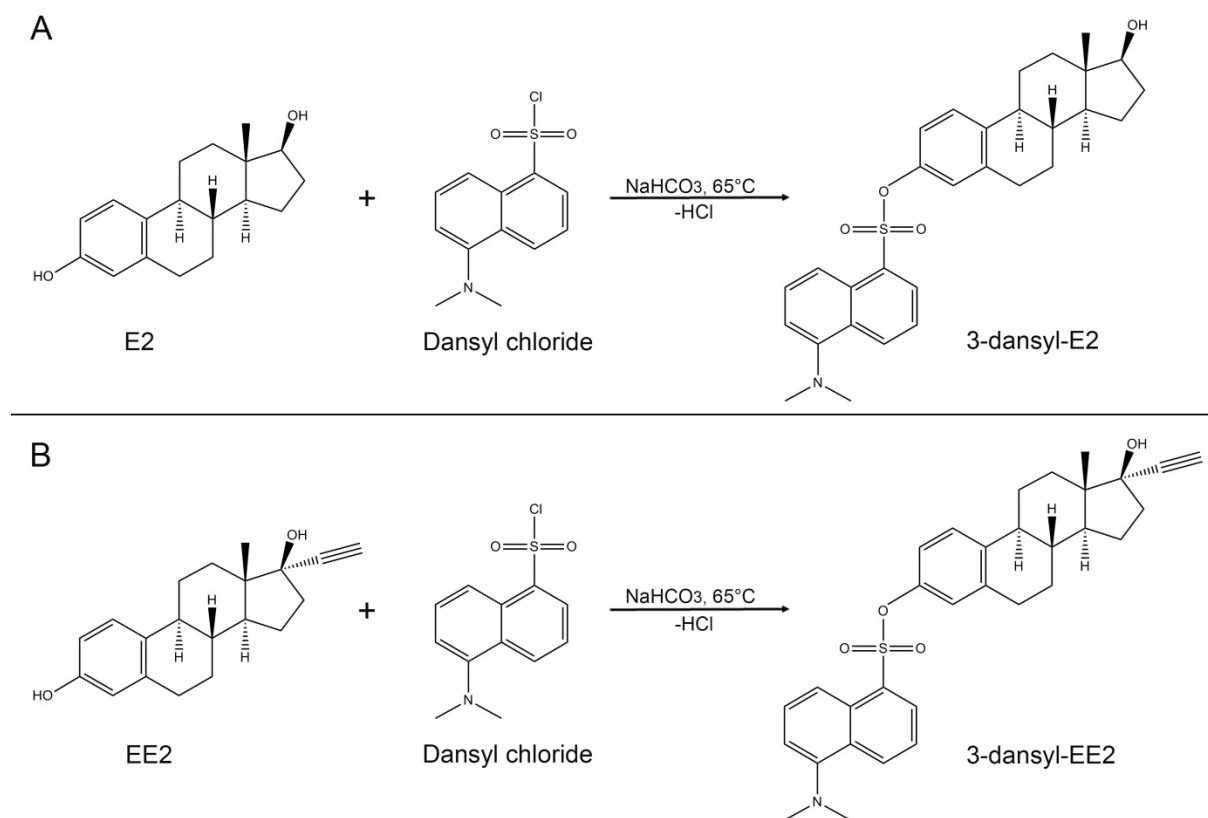


Figure 4.

A: Derivatization of E2 with dansyl chloride

B: Derivatization of EE2 with dansyl chloride

3.5 HPLC-MS/MS methods

3.5.1 Analysis of DRO, LNG and PRG as environmental contaminants

5 μL was injected 3 times from each concentrated sample into the HPLC-MS system. Liquid chromatographic separation was carried out on a Kinetex 2.6 μ C18 100 \AA HPLC column (100*2.1 mm) maintained at 40°C . The mobile phase consisted of solvent A (0.01 v/v% formic acid in water) and solvent B (0.01% v/v% formic acid in acetonitrile). The flow rate was 300 $\mu\text{L}/\text{min}$. Initial gradient conditions were set to 35% B and held for 3 min then B was increased linearly reaching 55% at 12 min, then initial conditions were reached in 0.2 min and the column was equilibrated for 7.8 min.

The mass spectrometer was equipped with a heated electrospray ion source which was

operated in positive ion mode. Spray voltage was set to 4.0 kV. Capillary temperature was 300 °C. The probe heater temperature was 50 °C. S-lens RF level was set to 70. Different HCD (Higher-Energy Collisional Induced Dissociation) cell energies were applied for the fragmentation: 55% by DRO and 45% by PRG and LNG. Data analysis was carried out with the software Thermo Xcalibur (version 2.2 SP1.48).

The observed ions were accepted if their m/z value were within the following limits: DRO - MS1: 367.21-367.24, MS2: 97.06-97.08, retention time: 5.2±0.25 min; LNG - MS1: 313.20-313.23, MS2: 109.05-109.07 and 245.18-245.20, retention time: 5.7±0.25 min; PRG - MS1: 315.21-315.24, MS2: 97.06-97.08 and 109.05-109.07, retention time: 9.8±0.25 min. ICIS peak detection was achieved using Xcalibur (area noise factor: 5.0, peak noise factor: 10.0 and minimum peak heights 3.0 (S/N)). For quantitative analysis five point calibration curves were used in the concentration range of 0.2 to 16 ng/L. Correlation coefficients (R^2) of the calibration curves were over 0.95. The limit of detection (LOD) calculated from standard mixture was 0.01 (DRO), 0.01 (LNG) and 0.003 (PRG) ng/L while the limit of quantification (LOQ) was 0.11, 0.09 and 0.03 ng/L, respectively. Recoveries of 100 µg spiked standards were (in %) 75.81±4.94 (DRO), 80.54±1.67 (LNG) and 95.28±1.69 (PRG).

3.5.2 Analysis of E2 and EE2 as environmental contaminants

10 µL was injected three times of each derivatised sample. Liquid chromatographic separation was performed on a Kinetex 2.6u XB-C18 100Å HPLC column (150x2.1mm) maintained at 30°C. The mobile phase consisted of solvent A (0.01% v/v formic acid in water) and solvent B (0.01% v/v formic acid in acetonitrile). Flow rate was set to 400 µL/min. The initial composition contained 10% B and it was kept constant for 1 minute. Percentage of eluent B was raised to 25 in 2 minutes. Then it was raised further to 70% in 2 minutes, then to 75% in 10 minutes. After that the column was washed and equilibrated for 15 minutes. The chromatographic peak of E2 was observed at 13.09-13.17 min, EE2 was eluted at 13.66-13.73 min. Mass detection was carried out in positive mode. Spray voltage in the heated electrospray ion source was set to 4.0 kV Capillary temperature was set to 380°C, while the probe heater temperature was 300°C. RF of the S-lenses was set to 60. Sheath and auxiliary gas flow rates were set to 60 and 20 arbitrary units respectively. No sweep gas was applied. The energy in the higher-energy collisional induced dissociation (HCD) cell was set to 50%

by E2 and 49% by EE2. Automatic gain control was set to $1e6$ by MS1 and $2e5$ by MS/MS scans. tSIM scan ranges were 506.0-506.5 and 530.0-530.5 m/z. By targeted-MS/MS scans we applied a 0.4 m/z isolation window and 506.24-171.10 (E2) and 530.24-171.10 (EE2) transitions (m/z) have been used.

3.5.3 Analysis of endogenous E2 in blood and in brain tissue

25 μL was injected three times of each derivatised sample. Liquid chromatographic separation was performed on a Kinetex EVO 5u C18 100Å HPLC column (150*2.1mm) maintained at 30°C. The mobile phase consisted from the same A and B solvents which were described by the environmental oestrogen method. Flow rate was set to 300 $\mu\text{L}/\text{min}$. The initial composition contained 75% B and it was kept constant for 1 minute. Percentage of eluent B was raised to 80 in 2 minutes. Then it was raised further to 99% in 2 minutes, and then composition was kept constant for 5 minutes. After that the column was washed and equilibrated for 15 minutes. Settings of the mass spectrometer were the same as applied by environmental oestrogen measurements. Brain tissue samples have been spiked with either 50 pg EE2 prior to homogenization, EE2 was used as internal standard in data processing.

4. Results and discussion

4.1 Steroid measurements

4.1.1 Environmental analysis of steroids

4.1.1.1 DRO, LNG and PRG

In progestin measurements less than 1ng/L LOQ (limit of quantitation) has been reached from less than 1 L freshwater. The analysis was achieved in a relatively environmentally friendly way, because it did not require chemical derivatisation. Using our sensitive method we could detect and quantify DRO, LNG and PRG from real freshwater samples. Identification of the progestins was based on three analytical parameters: retention time, exact molecular mass and their characteristic fragments. DRO was observed at the retention time of 5.2 min, LNG at 5.7 min and PRG at 9.8 min (Figure 5).

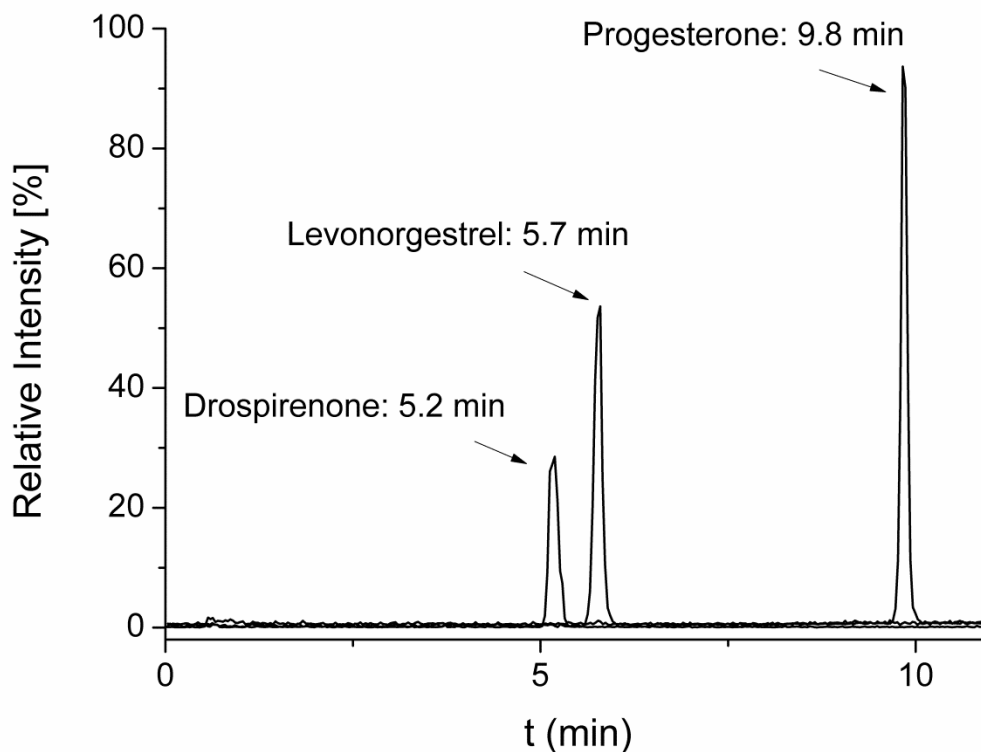


Figure 5. Separation of Progestins

The following precursor-fragment transitions $[M+H]^+ \rightarrow [F+H]^+$ were monitored for identification in mass to charge ratio $[m/z]$: DRO 367.23 \rightarrow 97.07 (Figure 6A,B), LNG 313.21 \rightarrow 109.06 and 245.19 (Figure 6C,D) as well as PRG 315.23 \rightarrow 97.07 and 109.06 (Figure 6E,F).

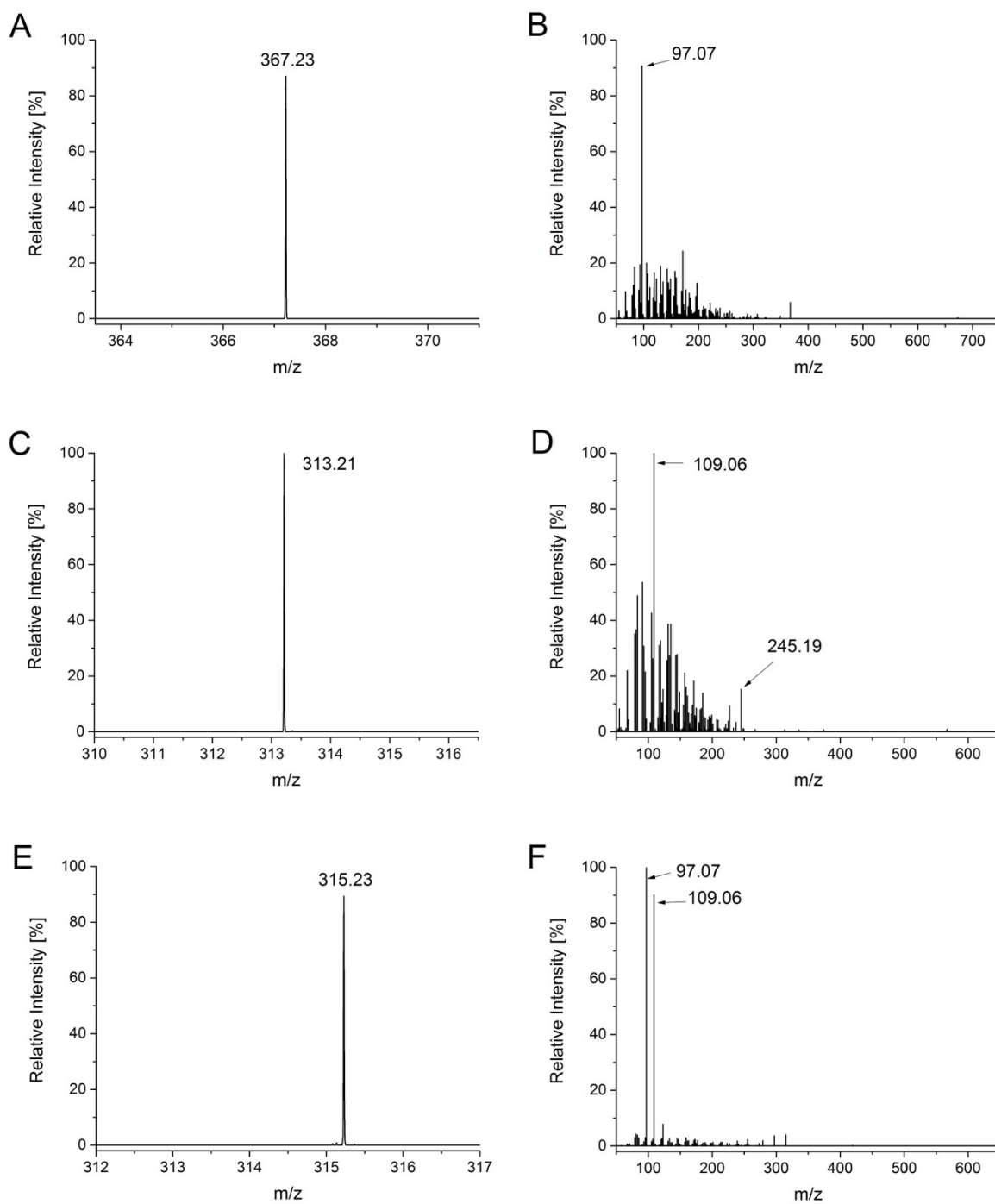
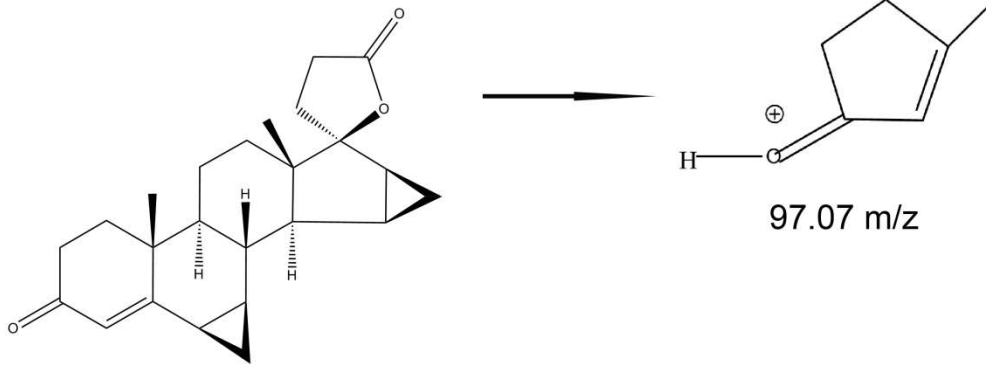


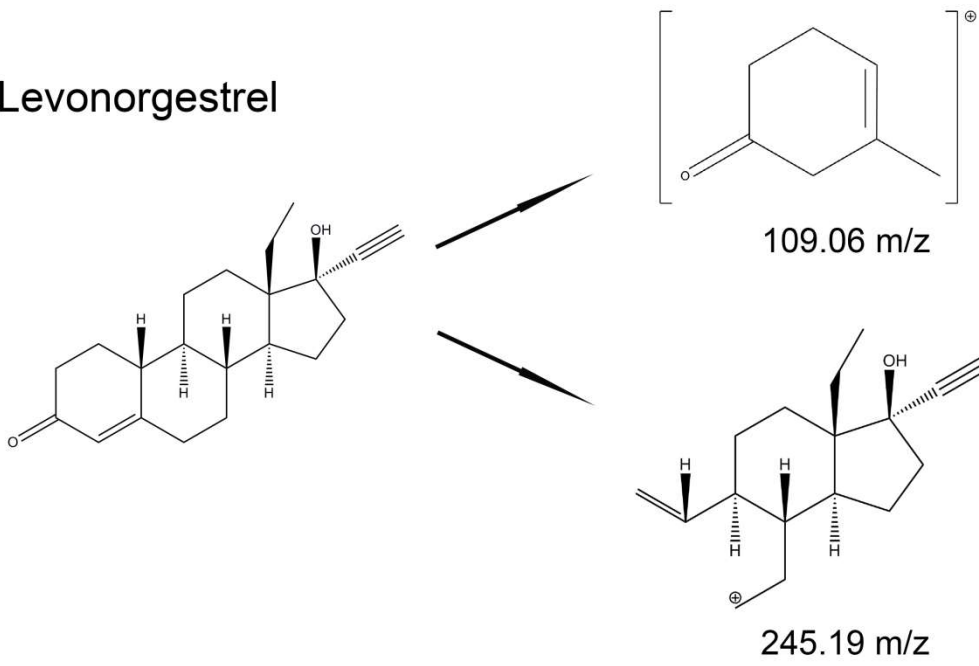
Figure 6. Spectra of precursor → fragment transitions of DRO, LNG and PRG

Characteristic fragments were determined from direct infusion experiments and confirmed by literature data (Figure 7).

Drospirenone



Levonorgestrel



Progesterone

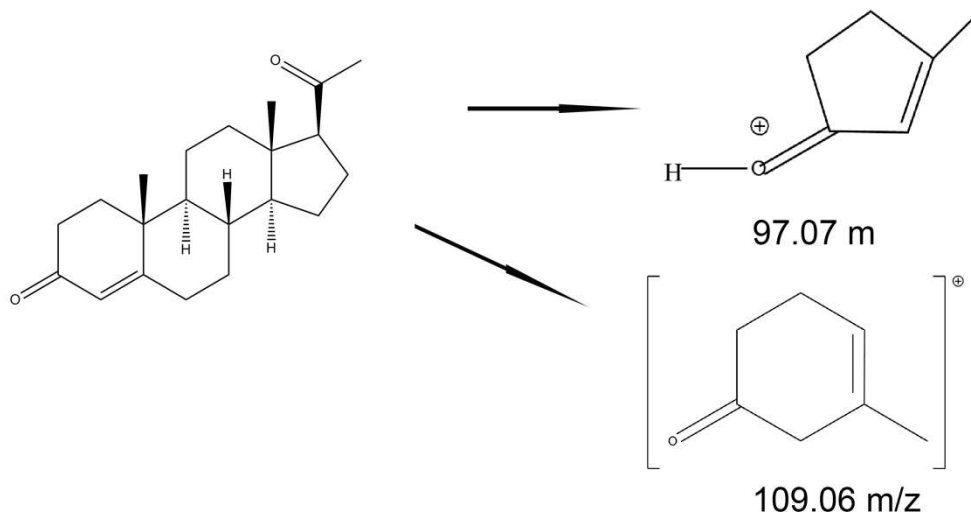


Figure 7. Proposed formula of characteristic fragments of DRO, LNG and PRG

Quantification was based on peak areas of precursor ions in single ion monitoring. MS/MS experiments were used for confirmation only. Precursor ion peaks which were not coeluting with the corresponding fragment peak(s) have been excluded.

Progestin concentrations were variable among the 53 collecting places around Lake Balaton. 23 of the freshwater samples did not contain measurable amount of contamination (<LOQ, not shown), however at 21 collecting places we observed steroids (Table 1). Out of this 21 freshwater sample in 20 PRG was detected. LNG was found at 6 (No 1, 5, 11, 12, 20, 21) and DRO was found at 5 places (No 1, 5, 11, 19, 21). We observed 4 places (No 1, 5, 11, 21) where all the three steroids were present. Measured progestin concentrations were found [in ng/L] 0.26-4.30 (DRO), 0.85-3.40 (LNG) and 0.23-13.67 (PRG).

Map Code	Watercourse	Sampling site	Coordinates	DRO	LNG	PRG
1	River Zala	Őriszentpéter	N46.83666, E16.43392	1.85 (0.04)	3.40 (0.45)	3.25 (0.59)
2	River Zala	Zalalövő	N46.85013, E16.62666	NF	NF	2.74 (0.12)
4	River Zala	Petőhenye	N46.88008, E16.92030	NF	NF	2.92 (0.33)
5	River Zala	Zalabér	N46.97823, E17.02623	NF	NF	2.81 (0.25)
7	River Zala	Balatonhídvég	N46.63705, E17.18369	1.54 (0.03)	1.29 (0.09)	10.17 (1.17)
9	Denke stream	Pankasz	N46.83737, E16.49827	NF	NF	1.47 (0.16)
10	Szentjakabi stream	Felsőjánosfa	N46.84740, E16.55136	NF	NF	0.52 (0.04)
12	Szélvíz stream	Zalaszentgyörgy	N46.86587, E16.69467	NF	NF	0.73 (0.14)
14	Felső-Válicka canal	Zalaegerszeg	N46.84334, E16.86976	NF	NF	4.91 (0.24)
15	Sárvíz stream	Zalaszentiván	N46.89049, E16.89153	NF	NF	4.41 (1.31)
21	Hévíz-Páhoki canal	Alsópáhok	N46.76038, E17.20494	1.62 (0.05)	1.26 (0.01)	2.28 (0.34)
22	Edericsi stream	Balatonederics	N46.80127, E17.38797	NF	0.85 (0.07)	NF
27	Egervíz stream	Szigliget	N46.80661, E17.45916	NF	NF	1.81 (0.03)
28	Egervíz stream	Hegyessd	N46.91531, E17.52500	NF	NF	1.88 (0.07)

29	Egervíz stream	Monostorapáti	N46.92733, E17.55878	NF	NF	2.37 (0.03)
31	Örvényesi-séd stream	Örvényes	N46.91394, E17.81708	NF	NF	0.23 (0.02)
32	Aszófői-séd stream	Balatonfüred	N46.93233, E17.85875	NF	NF	1.00 (0.01)
37	Kéki stream	Balatonalmádi	N47.04098, E18.02046	NF	NF	2.27 (0.10)
42	Boronkai stream	Marcali- Boronka	N46.58641, E17.44498	0.26 (0.01)	NF	2.07 (0.01)
*	Sári canal	Marcali	N46.58546, E17.44281	NF	2.14 (0.04)	1.38 (0.03)
43	Imremajori canal	Balatonfenyves	N46.70956, E17.47938	4.30 (0.30)	2.45 (0.01)	13.67 (0.31)

Table 1. Progestins found in the catchment area of Lake Balaton standard deviations are indicated in brackets,

NF not found

* not on map

In order to test accuracy of the progesterin method we purchased deuterated standards. Samples were collected at those sampling points, where the contamination was measured in July. They were spiked with deuterated standard mix so that the concentration of each reference material reached 10 ng/L and analysed in August, 2014. Found concentrations are summarized in Table 2.

Map Code	Watercourse	Sampling site	Coordinates	DRO	LNG	PRG
1	River Zala	Őriszentpéter	N46.83666, E16.43392	NF	NF	1.08 (0.13)
3	River Zala	Zalaszentgyörgy	N46.86938, E16.71139	NF	NF	0.43 (0.07)
32	Aszófői-séd stream	Balatonfüred	N46.93233, E17.85875	NF	<LOQ	NF
41	Boronkai stream	Marcali-Boronka	N46.63344, E17.44251	0.27 (0.03)	NF	4.59 (0.06)
42	Boronkai stream	Marcali-Boronka	N46.58641, E17.44498	NF	NF	0.47 (0.02)
43	Imremajori canal	Balatonfenyves	N46.70956, E17.47938	NF	NF	0.52 (0.15)

Table 2. Method test using deuterated standards. Standard deviations are indicated in brackets,

NF not found

<LOQ concentration is under the limit of quantification

4.1.1.2 E2 and EE2

E2 and EE2 without derivatisation can only be detected with poor LOQs. Achieving negative mode measurements, I found the following LOQs: 7.5 µg/L (E2) and 20 ng/L(EE2). Applying derivatisation we reached 0.05 ng/L (E2), and 0.001 ng/L (EE2) LOQs. These methods are also fast and simple, however, it is less environmentally friendly than the progestin method. In environmental measurements both E2 and EE2 were found in river samples. EE2 was less abundant than E2, but it was also present in almost all of the samples. 0-5.8 ng/L E2 (Table 3) and 0-0.7 ng/L EE2 concentrations (Table 4) were measured. A relative high amount of EE2 was found in River Zala (0.7ng/L) and in Hévíz-Páhoki canal (0.5ng/L), which are both in the catchment area of Lake Balaton. The presence of E2 could be confirmed with MS/MS transitions in 12 out of 23 samples. The presence of EE2, due to poor (0.2ng/L) LOQ value, could only be confirmed with MS/MS transitions at two sampling sites (Hévíz-Páhoki canal; HP and River Zala; ZA). In the case of five samples we repeated the measurements to determine, how much oestrogen can be extracted from filtered samples and from the filter papers separately. From the extracted amounts I calculated an estimation of proportionality of E2 and EE2 between suspended and dissolved phase. Smaller amounts (17-33% E2 and 0-5% EE2) could be extracted from the suspended phase and larger amounts from the liquid phase, which according to literature data is unexpected, most steroids show the opposite proportionality (Figure 8).

E2 in samples							
Symbol	Watercourse	sampling site	coordinates	MS1 ng/L	RSD %	MS2 ng/L	RSD %
D1	Danube	Budapest	N47.46925, E19.06509	2.970	30	3.132	25
D2	Danube	Dunaföldvár	N46.80948, E18.93039	0.028	7	NF	
D3	Danube	Solt	N46.81653, E18.95233	0.039	24	NF	
D4	Danube	Paks	N46.61934, E18.86086	0.018	14	NF	
D5	Danube	Mohács	N45.99042, E18.69697	0.224	5	0.291	12
X1	Drava	Maribor	N46.55668, E15.66338	0.580	17	0.626	5
X2	Drava	Drávaszabolcs	N45.78609, E18.19699	0.052	39	NF	
SA	Sava	Ljubljana	N46.08725, E14.53171	5.202	1	5.818	6
LJ	Ljubjanica	Ljubljana	N46.05081, E14.50602	2.046	5	2.242	10
MU	Mur	Murarátka	N46.45150, E16.67819	0.198	38	0.452	1
ZA	Zala	Balatonhídvég	N46.63705, E17.18369	0.550	6	0.298	7
HP	Hévíz- Páhoki canal	Alsópáhok	N46.76038, E17.20494	0.245	17	0.263	13
IC	Imremajori canal	Balatonfenyves	N46.70956, E17.47938	0.128	7	0.247	8

SI	Sió	Szekszárd-Palánk	N46.38072, E18.70058	0.085	4	NF	
KS	Kapos	Kaposvár	N46.35208, E17.79119	0.024	12	NF	
ZG	Zagyva	Szolnok	N47.17556, E20.20497	NF		NF	
T1	Tisza	Szolnok	N47.17253, E20.20494	LOQ		NF	
T2	Tisza	Tiszkécske	N46.93626, E20.10907	0.103	4	NF	
T3	Tisza	Csongrád	N46.68519, E20.19844	0.450	2	0.540	8
B1	Lake Balaton	Balatonlelle	N46.79180, E17.69445	0.076	3	NF	
B2	Lake Balaton	Balatonszárszó	N46,83336, E17.82887	0.233	6	0.319	4
B3	Lake Balaton	Tihany	N46.91427, E17.89253	0.129	4	NF	
PV	Pécsi víz	Pécs	N46.05507, E18.21152	7.320	8	6.980	21

Table 3. E2 found in waters of the Carpathian basin

NF: not found

RSD%: relative standard deviation in percent

EE2 in samples							
Symbol	Watercourse	sampling site	coordinates	MS1 ng/L	RSD%	MS2 ng/L	RSD%
D1	Danube	Budapest	N47.46925, E19.06509	0.124	14	NF	
D2	Danube	Dunaföldvár	N46.80948, E18.93039	NF		NF	
D3	Danube	Solt	N46.81653, E18.95233	NF		NF	
D4	Danube	Paks	N46.61934, E18.86086	NF		NF	
D5	Danube	Mohács	N45.99042, E18.69697	0.005	57	NF	
X1	Drava	Maribor	N46.55668, E15.66338	0.006	25	NF	
X2	Drava	Drávaszabolcs	N45.78609, E18.19699	NF		NF	
SA	Sava	Ljubljana	N46.08725, E14.53171	0.002	44	NF	
LJ	Ljubjanica	Ljubljana	N46.05081, E14.50602	0.003	54	NF	
MU	Mur	Murarátka	N46.45150, E16.67819	0.008	17	NF	
ZA	Zala	Balatonhídvég	N46.63705, E17.18369	0.678	1	0.620	17
HP	Hévíz-Páhoki canal	Alsópáhok	N46.76038, E17.20494	0.521	12	0.436	20
IC	Imremajori canal	Balatonfenyves	N46.70956, E17.47938	0.018	9	NF	

SI	Sió	Szekszárd-Palánk	N46.38072, E18.70058	0.097	2	NF	
KS	Kapos	Kaposvár	N46.35208, E17.79119	NF		NF	
ZG	Zagyva	Szolnok	N47.17556, E20.20497	NF		NF	
T1	Tisza	Szolnok	N47.17253, E20.20494	NF		NF	
T2	Tisza	Tiszaújváros	N46.93626, E20.10907	0.099	3	NF	
T3	Tisza	Csongrád	N46.68519, E20.19844	0.143	3	NF	
B1	Lake Balaton	Balatonlelle	N46.79180, E17.69445	0.133	3	NF	
B2	Lake Balaton	Balatonszárszó	N46.83336, E17.82887	NF		NF	
B3	Lake Balaton	Tihany	N46.91427, E17.89253	NF		NF	
PV	Pécsi víz total	Pécs	N46.05507, E18.21152	0.175	31	NF	

Table 4. EE2 found in waters of the Carpathian basin

NF: not found

RSD%: relative standard deviation in percent

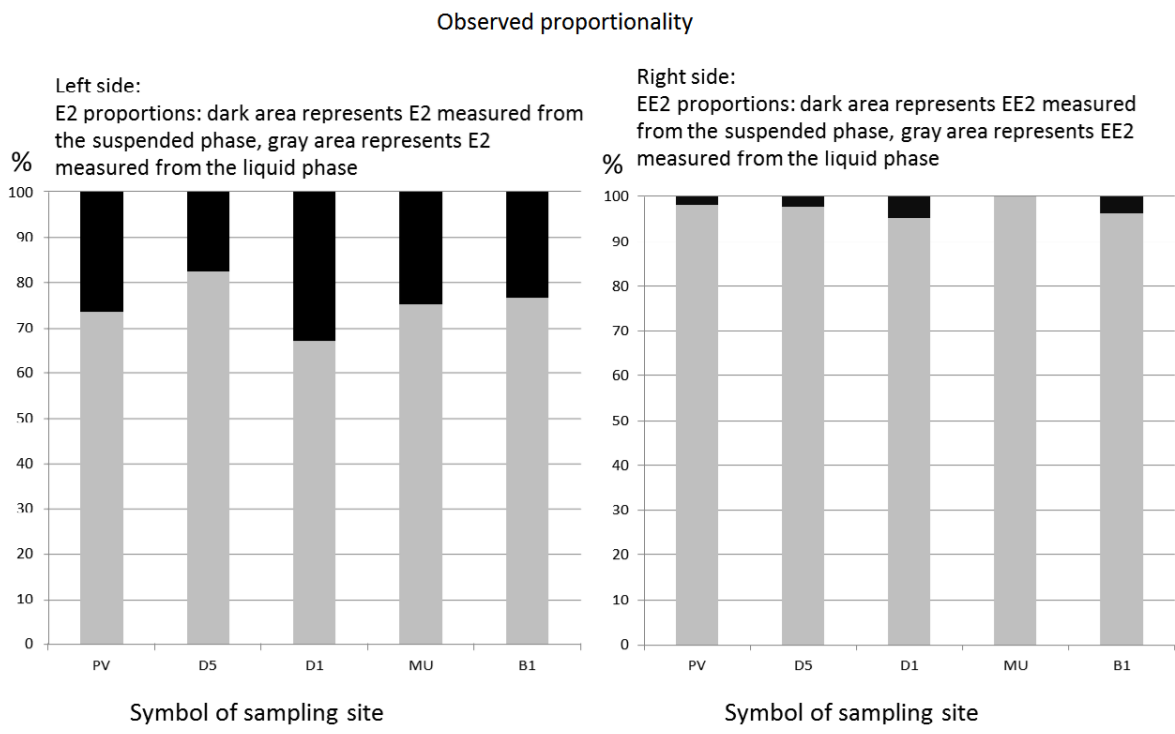


Figure 8. Observed proportionality of oestrogens. Black colour indicates the proportion extracted from the suspended phase, grey colour indicates the proportion extracted from the liquid phase. E2 proportionality: left side, EE2 proportionality: right side

In order to provide information about the reliability of the E2 and EE2 method complementary measurements have been achieved. Four freshwater samples were collected and analysed with (quantitation with internal standard), and without E2-d3 (external standard only). E2-d3 eluted at 13.00-13.15 (MS1:m/z=509.25-509.27, MS/MS transition: 509.26→171.10). The use of deuterated standards confirmed the identity of the analytes. Calculated E2 and EE2 concentrations were 7-40% higher compared to results of external standard measurements if I used internal standards.

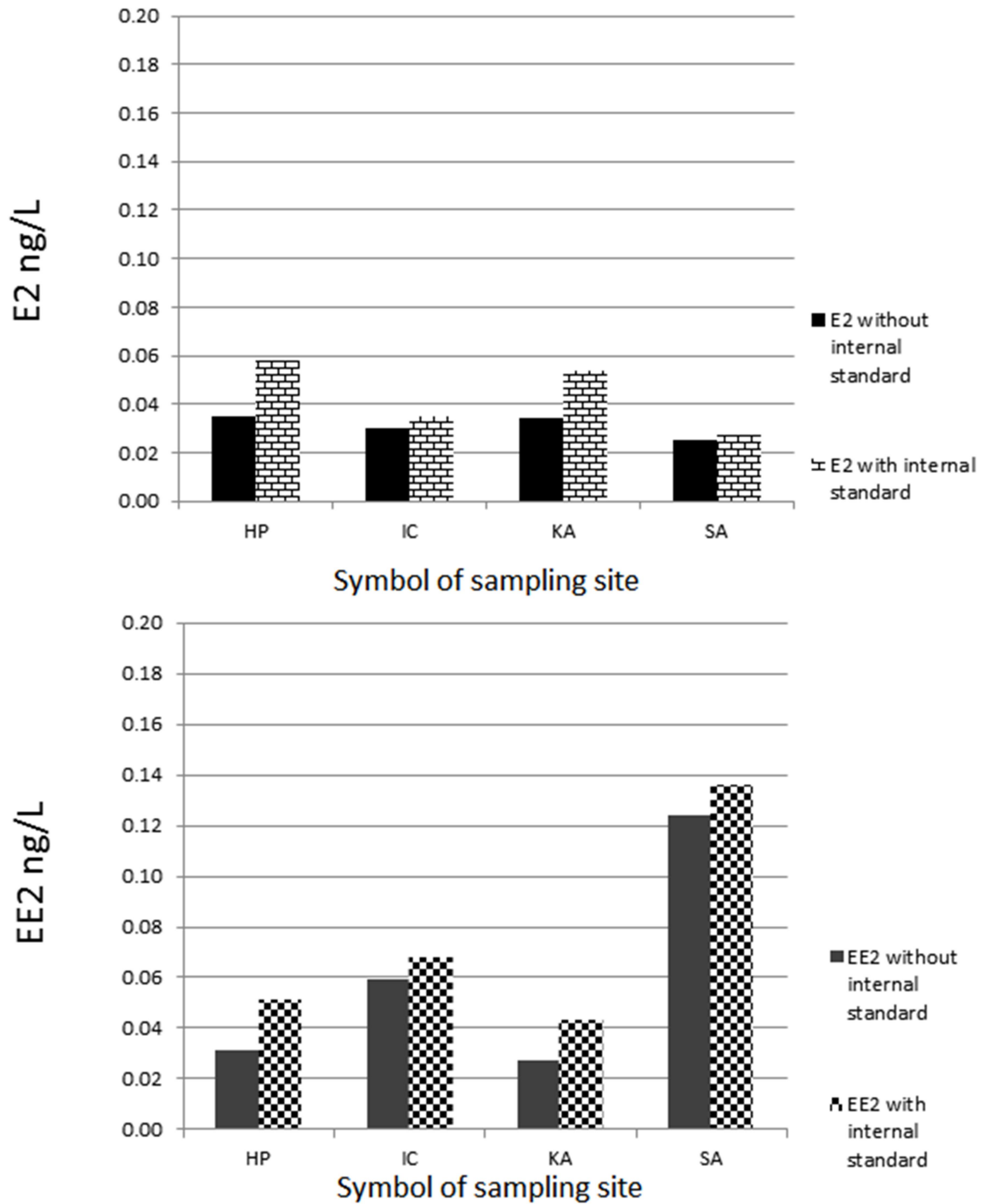


Figure 9. Comparison of external and internal standard measurements at four sampling site.

Due to the intensive use and high demand of estrogens, the occurrence of E2 and EE2 in natural waters is a major concern. According to data published between 2003 and 2013, scientists found more than 1 ng/L E2 at least at one of their sampling sites in 13 out of 18 freshwater investigations worldwide. EE2 could be detected over 1 ng/L with a 50% chance

(Aris, et al. 2014). No detectable amount of E2 and EE2 was found in surface waters at the Mediterranean Spanish coast (with LODs=0.06 ng/L and 0.02 ng/L, respectively)(Ripolles, et al. 2014). Neither E2 nor EE2 was found in River Tiber and River Aniene (in Italy, LODs=10 ng/L) (Patrolecco, et al. 2015). In Dutch surface waters, both analytes were detected mostly under the limit of quantification (<0.1 ng/L) (Belfroid, et al. 1999). In river samples in the Paris area (France), 1.4 to 3.0 mean E2 and 1.1 to 2.9 mean EE2 were found (Cargouet, et al. 2004). Hungarian scientists reported contamination in the River Danube earlier. In 2009, E2 was found neither in local WWTP effluents nor in River Danube (LOD=8.6 ng/L) (Sebok, et al. 2009), but there were n.d.-0.45 ng/L E2 and n.d.-1.16 ng/L EE2 found at nearby locations in 2011-2012 (Andrasi, et al. 2013). This study used derivatisation in order to improve sensitivity (LODs not available), and the occurrence in the suspended phase was also studied. In that phase of Danube water, n.d.-0.46 ng/L EE2 and no E2 was detected. Changing amount of E2 (low ng/L, only graphical data is available) and no EE2 was detected in Yangtze Estuary (China), where their occurrence was followed over four seasons (Nie, et al. 2015). In rivers of the UK, 1 ng/L to almost 50 ng/L E2 was measured and EE2 could also be detected, but not quantified (Desbrow, et al. 1998). Later, in 2003 in River Nene and River Lea (UK), 0.9 ng/L mean E2 and 0.7 ng/L mean EE2 were measured (Williams, et al. 2003). Moreover, 0.15 to 3.6 ng/L E2 and 0.1 to 5.1 ng/L EE2 were found in German rivers in 2001 (Kuch and Ballschmiter 2001). In 2010, 1.1 ng/L mean E2 and no EE2 (LOD=0.14 ng/L) were found in the Ebro basin on the Iberian Peninsula (Gorga, et al. 2013). High concentrations have been found in the Klang Valley in Malaysia (average E2= 20ng/L) (Ismail Ahmad 2007), in Venice lagoon (175 ng/L E2 and 34 ng/L EE2) (Pojana, et al. 2007), and in the Piracicaba River in Brazil (137 and 194 ng/L) (Torres, et al. 2015).

EU regulation has put E2 and EE2 on the watch list of emerging pollutants in 2013 and in this year they established maximum acceptable detection limits for them. These limits are 0.035 ng/L for EE2 and 0.4 ng/L for E2 (European Parliament 2015). These contaminants in an average equipped laboratory using multiresidue analysis without derivatisation can only be determined with poor detection limits (Liu, et al. 2011b; Loos, et al. 2010). Our methodology with dansyl derivatisation is cheap, quick and simple enough to be considered for use in monitoring studies in the future. The use of 1-methylimidazole-2-sulfonyl adducts may be another possibility, but it must be tested (Li and Franke 2015). Compared to the international dataset, E2 and EE2 contamination in rivers of the Carpathian basin and Lake Balaton is

generally moderate (Table 5). Even the concentrations measured at capital cities (3.0 ng/L E2 and 0.1 ng/L EE2 at Budapest and 5.2 ng/L E2 at Ljubljana) seem to be tolerable.

Earlier published E2 and EE2 freshwater contaminations				
Location	Reference	Country	E2 ng/L	EE2 ng/L
Paris area rivers	Cargouet et al. 2004	France	1.4 - 3.0	1.1 - 2.9
Danube River	Andrasi et al. 2013	Hungary	n.d. - 0.4	n.d. -1.16
UK rivers	Desbrow et al. 1998	UK	1-50	n.a.
UK rivers	Williams et al. 2003	UK	0.9 mean	0.7 mean
German Rivers	Kuch and Ballschmiter 2001	Germany	0.15-3.6	0.1 - 5.1
Ebro River	Gorga et al. 2013	Spain	1.1	n.d.
Klang River	Ismail Ahmad 2007	Malaysia	20	n.a.
Adriatic See	Pojana et al. 2007	Italy	n.d. - 175	n.d. -34
Piracicaba River	Torres et al. 2015	Brasil	n.d. - 137	n.d. - 194

Table 5.

n.d. not detected

n.a. data not available

Though it must be taken into account that very limited data is available, that concentrations can raise dramatically if passage flow decreases, and that most toxicological experiments are achieved during a few weeks or months, thus long-term effects are very hard to discover. This low level presence of E2 and EE2 in natural waters is just a small contributions to the overall oestrogenicity of waters (Wise, et al. 2011), which at its actual level can cause local problems in the wildlife but probably means minimal risk to public health.

4.1.2 Mouse brain and blood E2 study

Original fast and simple LC-MS methods have been developed for the measurement of E2 from blood and brain samples. I continue to work toward better analytical features in forthcoming applications, but this technology has already been successfully applied considering both matrices. E2 content of dissected brain cortex regions of adult female mice and plasma has been measured. Plasma concentration have been compared with oestrus phases determined via vaginal cytology. Measured plasma E2 concentration were well-fitted with oestrus phases. E2 plasma concentrations of intact and OVX mice have been compared. The bilateral removal of the ovaries ablates the gonadal E2 production that way brain concentrations can be investigated minimising the influence of circulating E2. (Adrenal glands still fulfil their functions.) Plasma E2 concentrations of OVX animals after two weeks of the surgery were found detectable (1-3 pg/mL), but 10-75 fold lower than the normal level.

E2 could be quantified from less than 5 mg brain tissue sample. As an average mouse brain is approximately 350-450 mg the technique allows a limited mapping the E2 content of mice brains. The limit of the quantification (LOQ) of local concentration of the hormone in this assessment is 0.05 pg E2 on column or 0.2 pg E2 in sample. It is equivalent with 0.04 pg/mg if we have 5 mg brain tissue, but of course we have a lower pg/mg limit if we analyse a larger tissue section and a higher limit by the analysis of a smaller tissue section. Hypothalamus, hippocampus and cortex sections have been analysed (n=9). I have measured the following average E2 concentrations: hypothalamus: 0.20-50 pg/mg, hippocampus: 1.0-155 pg/mg, cortex: 0.25-6.2 pg/mg. In the case of cortex sections left and right sides have been investigated separately (Table 6). Estradiol concentration in the left side and in the right side were compared. Proportionality (left side E2 concentration/right side E2 concentration ratio) was found smaller than 0.5 in 1 brain, between 0.5 and 1.5 in 4 brains and over 1.5 in 4 brains.

Mouse	E2 in the left side of the cortex (pg/mg)	E2 in the right side of the cortex (pg/mg)	left/right proportion
1 st	0.94	3.36	0.28
2 nd	2.78	4.79	0.58
3 rd	6.32	1.93	3.27
4 th	5.46	6.89	0.79
5 th	0.59	0.50	1.18
6 th	3.20	1.60	2.00
7 th	0.90	0.30	3.00
8 th	0.31	0.21	1.48
9 th	0.90	0.40	2.25

Table 6. E2 concentrations in the mouse brain cortices

Left and right sides of five hippocampi have also been investigated separately. (Mice brains numbered from 5th to 9th). By these five hippocampi left/right proportionality was always smaller than 0.5 or larger than 1.5 and it was never between 0.5 and 1.5 (Table 7).

Mouse	E2 in the left side of the hippocampus (pg/mg)	E2 in the right side of the hippocampus (pg/mg)	left/right E2 proportion in the hippocampus	E2 concentration in the plasma (pg/mg)
5 th	76	1.5	50.67	63
6 th	0.7	1.6	0.44	12
7 th	1.4	8	0.18	155
8 th	1.5	155	0.01	33
9 th	1.9	1	1.90	186

Table 7. E2 concentrations in the mouse hippocampi and plasmas

Coronal brain sections were cut and local E2 concentrations were investigated in separate samples (mice numbered from 10th to 14th) on a sagittal (nasal to caudal) axis of mouse brain

cortices also (n=5) (Figure 10). These samples showed similar average E2 concentrations to concentrations measured in mice 1st-9th, but large local differences could also be observed.

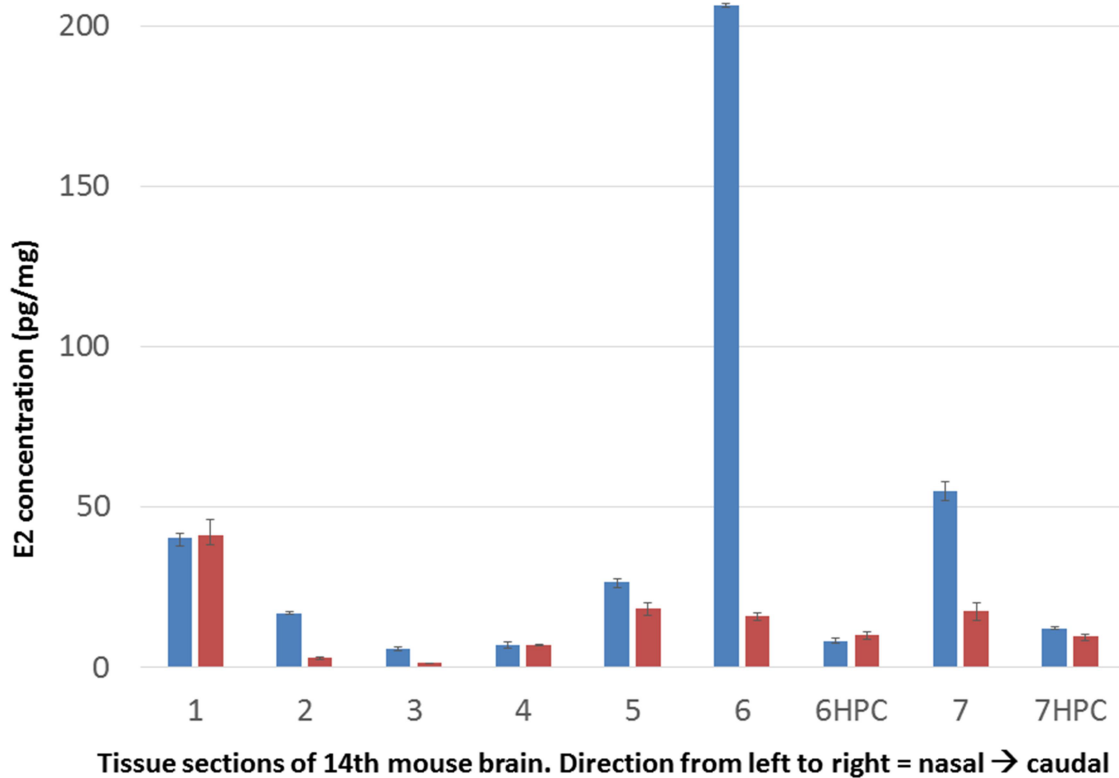


Figure 10. Local E2 concentrations in the sections of a mouse brain (Mouse No. 14th). Numbers 1-7 mean cortices of brain section 1-7. Section No. 1 is the nasal end of the cortex, section No. 7 is the caudal end of the cortex. 6HPC and 7 HPC are hippocampus sections cut from brain sections 6 and 7.

E2 plasma concentrations are frequently measured by immunoassays. Commercially available RIAs are able to detect and quantify 2.5-9 pg/ml or higher plasma/blood concentrations of E2, but a comparison revealed, than measured values are not always in a good correlation with each other or with a golden standard mass spectrometric (GC-MS) method (Haisenleder, et al. 2011). We reached 0.2 pg/sample sensitivity, and overcome the difficulties of the immunoassays with the developed LC-MS method with the help of derivatisation. Endogenous E2 levels relate to many processes in molecular and cellular level. They determine sensitivity of response to oestrogen therapy (HRT) and they are associated with cognitive function (Li, et al. 2013). The total number of investigated mice (n=14 normal

brain, n=5 normal plasma, n=1 OVX plasma) does not enable us to claim well established conclusions about the observed phenomenon, that E2 concentration were not the same in the right and left sides of hippocampi and cortices, but it raises the question whether differences are significant. If E2 concentrations differ on the left and right sides of brain structures many results of previous experiments become more problematic to comprehend. Studies where the two hemisphere of brain tissue are used for different type of analysis, for example one hemisphere for immunohistochemistry and the other hemisphere for molecular biological analysis must be planned more carefully. We continue this study in order to answer this and several further questions.

4.2 Structural elucidation of carotenoids

The carotenoid research group of our institute provided me two chromatographic fractions. Both were identified as β -cryptoxanthin epoxides based on their UV-VIS spectra. The component collected from the chromatographic separation at the retention time 14.5 min had the mass 568.43 (m/z). That m/z matches with the calculated theoretical mass of a β -cryptoxanthin epoxid. Fragmentation of this m/z even with relatively low fragmentation energies produced a large number of ions. The fragmentation profile was very similar to earlier reported fragmentation of β -cryptoxanthin. Characteristic ions were 553.40 ((M-15) or β -cryptoxanthin), 488.36 (M-C₆H₈), 476.36 (M-C₇H₈), 461.34(β -cryptoxanthin-C₇H₈), 415.30 (M-153, loss of the hydroxylated ring with cleavage at the 7,8 carbon-carbon bond from the protonated molecule) and several fragment ions corresponding to cleavages of the polyene chain.

The other component, collected from the chromatographic separation at the retention time of 17.5 min had the same mass 568.43 (m/z). The fragmentation of this compound seemed to be almost identical to the fragmentation of the other compound with the retention time 14.5 min, it contains the same high abundant ions, however not the same ions are the most intensive ones. To tell the position of the epoxidation (Figure 11) the theoretical masses of two ions (169.12340 and 171.13769) (Figure 12) have been calculated, which both contained the six membered ring, four methyl groups, the epoxide and the hydroxyl groups as they all together could only occur in the tandem spectra if the position of the epoxidation is 5,6. All the tandem spectra were analysed using mass isolation ranges 169.11-169.13 and 171.13-171.15. These two ions were only observed in the spectra of the compound with the retention time 17.5 min Their intensities applying 25% HCD cell energy were 3 and 6 % of the intensity of the precursor ion (568.43) or 0.01 and 0.02 % of the total ion chromatogram (Figure 13).

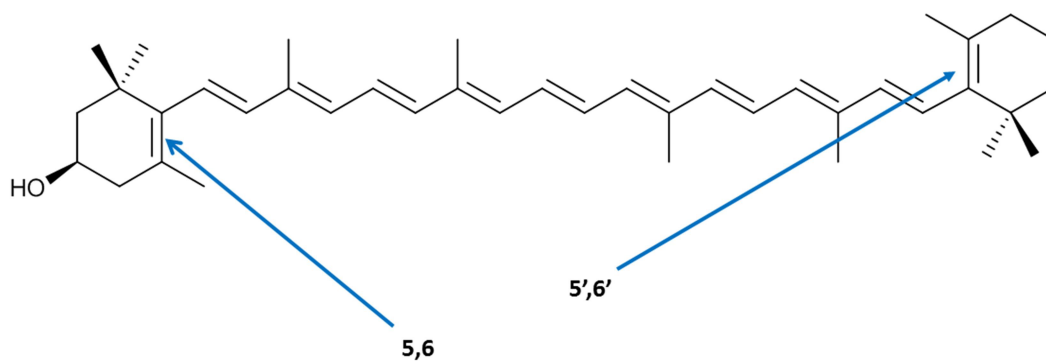


Figure 11. Possible sites of epoxidation on β -cryptoxanthin. Epoxid formation is possible either on the 5,6 carbon-carbon bond or on the 5',6' carbon-carbon bond.

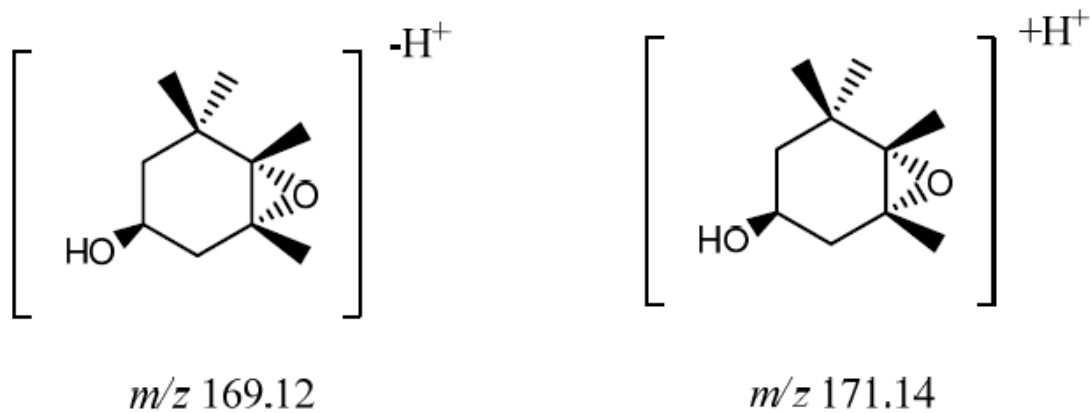


Figure 12. The two ions, 169.12 (left) and 171.14 (right) which were used for the differentiation of 5,6 and 5',6' epoxides.

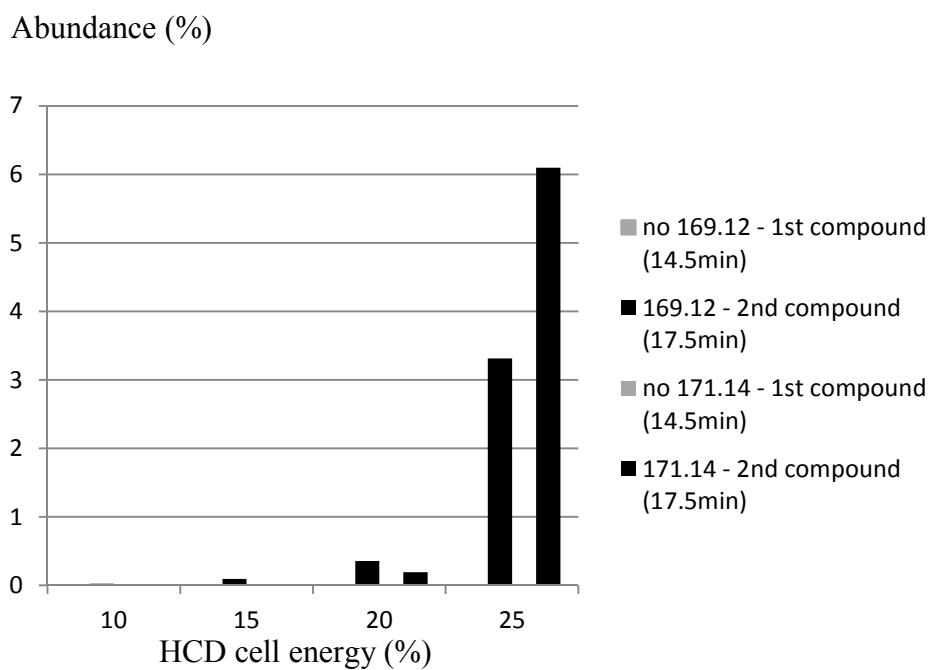


Figure 13. The abundance of differentiating fragment ions at 10,15,20 and 25% HCD cell energy expressed in % of the abundance of the precursor ion (568.43 m/z) Black indicates both ions in the 2nd compound. Grey colour would indicate both ions in the 1st compound, but they are not present.

5. Summary

In the present doctoral thesis new mass spectrometric techniques have been developed and the analysis of steroid lipids and carotenoid lipids was achieved.

Steroid methods yielded quantitative determination of important progestogens and oestrogens and they suited for the analysis of both environmental samples and medical research samples. The occurrence of drospirenone (DRO), levonorgestrel (LNG) and progesterone (PRG) as environmental contaminants have been surveyed in freshwater samples from the catchment area of Lake Balaton. 17β -oestradiol (E2) and 17α -ethinyl oestradiol (EE2) have been determined in Lake Balaton and in all the large European rivers. DRO, LNG and PRG were analysed at 53 sampling points. They could be analysed in their original form. E2 and EE2 were analysed in waters collected at 23 sampling points, they had to be derivatised and were detected as dansyl-derivates. Derivatisation was necessary to reach sufficient limits of detection. The concentrations of contaminants showed variability, but they were found generally moderate, in the low ng/L range.

Endogenous E2 levels in mouse brain tissue samples (n=14) have been surveyed to support further research. Average concentrations in full cortices were 0.25-6.2 pg/mg, but great local differences have been observed. The analysis of whole cortices, cortex sections and hippocampi all raised the question whether lateralisation of the brain in the context of different hemispherical E2 levels exist.

The qualitative analysis of carotenoid extracts proved, that ESI-MS is suitable for the structural elucidation of such apolar molecules. The position of epoxidation of β -cryptoxanthin was successfully identified.

Results have demonstrated that LC-MS is an excellent tool for determining low ng/L environmental contaminants in freshwater, low pg/ml E2 in plasma and low pg/mg E2 in brain tissue. Preconcentration due to low analyte concentrations is indispensable in both environmental and medical research methods. Solid phase extraction has shown great potential in preconcentration of water samples while effective liquid/liquid extractions have been developed for tissue and plasma preconcentration.

6. Novel findings

1. Original, sensitive and fast methods based on SPE and LC-MS have been developed for the determination of the following environmental contaminants: DRO, LNG, PRG, E2 and EE2.
2. A significant steroid hormone contamination including quantitative data was measured in the catchment area of Lake Balaton and in several Central European rivers.
3. A sensitive method has been developed for the determination of E2 from brain tissue, physiological concentrations in the mouse brain have been surveyed.
4. Using MS/MS experiments the identity of 5,6 and 5',6' β -cryptoxanthin-epoxides was successfully confirmed and I could tell them apart.

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8. List of Publications

8.1 Thesis related publications

Communications in scientific Journals

- **Avar P**, Zrínyi Z, Maász G, Takátsy A, Lovas S, G.-Tóth L, Pirger Z (2016)
β-estradiol and ethinyl-estradiol contamination in the rivers of the Carpathian Basin
Environ Sci Pollut R, DOI: 10.1007/s11356-016-6276-2 IF: 2.828
- **Avar P**, Maász G, Takács P, Lovas S, Zrínyi Z, Svigruha R, Takátsy A, G.-Tóth L,
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HPLC-MS/MS analysis of steroid hormones in environmental water samples Drug
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Lectures

- **Avar P**, Maász G, Takács P, Lovas S, Zrínyi Z, Svigruha R, Takátsy A, G.-Tóth L,
Pirger Zs (2015)
Szteroid szennyezők tömegspektrometriás vizsgálata a Balaton vízgyűjtő területén
XII. Környezetvédelmi analitikai és technológiai konferencia, Balatonszárszó,
Hungary
- Pirger Z, Takács P, Bévárdi N, Svigruha R, Maász G, **Avar P** (2014)
Humán eredetű szteroid terhelés és annak lehetséges élettani hatásai a Balaton és a

Zala vízgyűjtőjén IV. Ökotoxikológiai Konferencia, Budapest, Hungary

- Pápai Z, Maász G, Schmidt J, **Avar P**, Márk L (2014)
Lipidek időbeli és térbeli változásainak feltérképezése a korai embriogenezis során
III. Interdisciplinary Doctoral Conference, Pécs, Hungary

Posters

- Pálhalmi J, **Avar P**, Ábrahám M. I (2016)
Single molecule imaging of AMPA receptor in live neural network of adult mouse brain, International Brain Research Organization (IBRO) Workshop, Budapest, Hungary
- **Avar P**, Maász G, Zrínyi Z, Takátsy A, Pirger Z (2015)
Quantitative measurement of 17 β -estradiol and Ethinyl estradiol in some Central-European rivers, European Meeting on Environmental Chemistry (EMEC 16), Turin, Italy
- **Avar P**, Maász G, Takács P, Svigruha R, G.-Tóth L, Pirger Z (2015)
HPLC-MS/MS analysis of steroid hormones in environmental water samples, International Symposium on Separation Sciences (ISSS), Ljubljana, Slovenia

8.2 Other publications

Communications in scientific Journals

- Antus Cs, Radnai B, Dombóvári P, Fónai F, **Avar P**, Matyus P, Rácz B, Sümegi B, Veres B (2015) Anti-inflammatory effects of a triple-bond resveratrol analog: Structure and function relationship Eur J Pharmacol, 748: 61-67. IF: 2.532
- Kuzma M, Fodor K, Maász G, **Avar P**, Mózsik G, Past T, Fischer E, Perjési P (2015) A validated HPLC-FLD method for analysis of intestinal absorption and metabolism of capsaicin and dihydrocapsaicin in the rat. J Pharmaceut Biomed, 103: 59-66. IF: 2.867
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III. Interdisciplinary Doctoral Conference, Pécs, Hungary
- **Avar P**, Pápai Z, Kuzma M, Maász G (2013)
HPLC-MS analysis of capsaicin, dihydrocapsaicin and their metabolites
12nd Internal Doctoral Workshop on Natural Sciences, Pécs, Hungary

Poster

- **Avar P** (2015)
Cell energetics with HPLC-MS
33rd Informal Meeting on Mass Spectrometry, Szczyrk, Poland

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