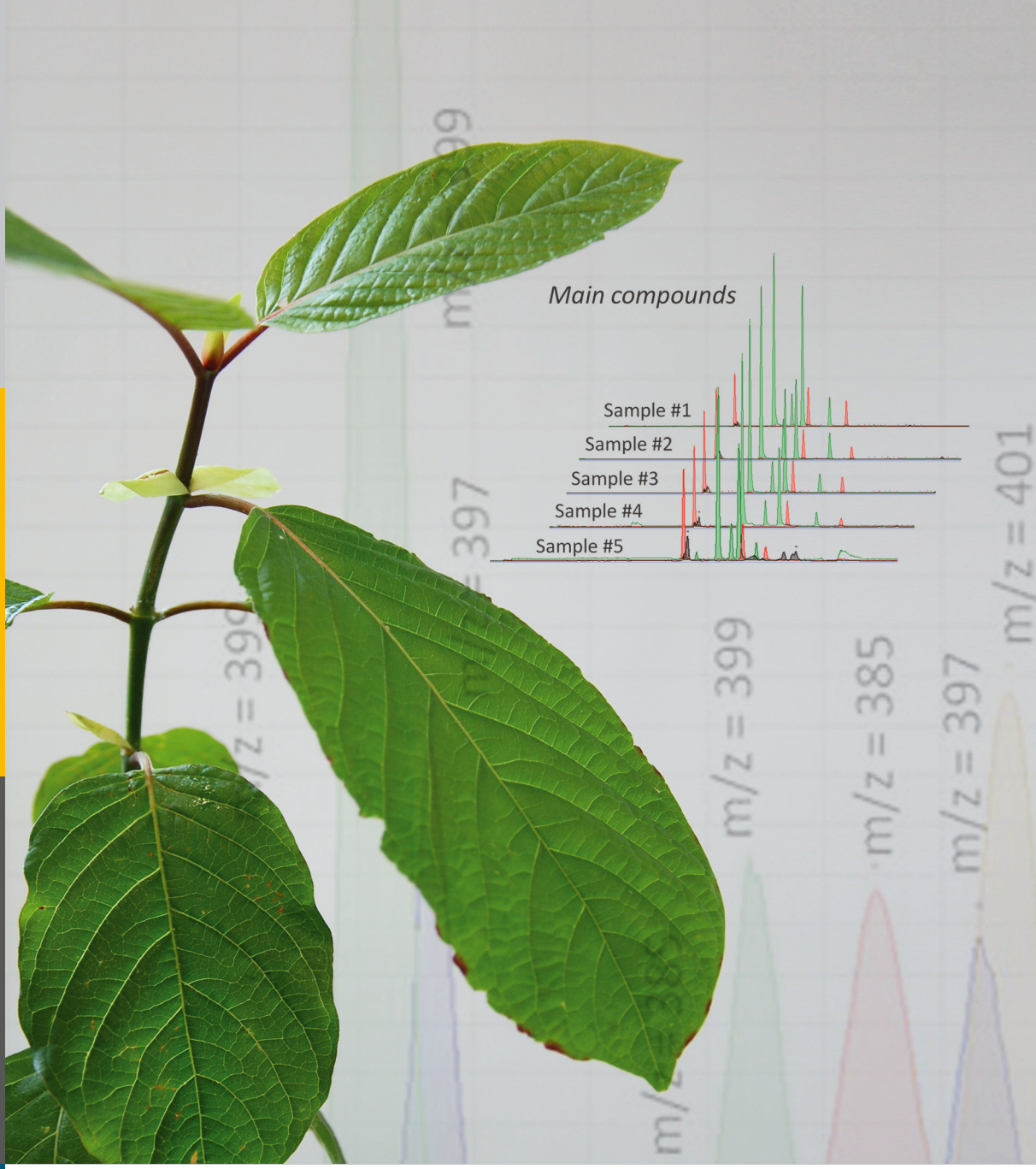

Implementation of capillary electromigrative separation techniques coupled to mass spectrometry in forensic and biological science

Tjorben Nils Posch

Member of the Helmholtz Association

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LIST OF ABBREVIATIONS

5-MeO-N,N-DMT	5-methoxy-*N,N*-dimethyltryptamine
ACN	Acetonitrile
AM	Amphetamine
ANOVA	Analysis of variance
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photo ionization
BGE	Background electrolyte
BKA	German Federal Criminal Police Office
BMBF	Federal ministry of education and research
BPC	Base peak electropherogram
BPE	Base peak electropherogram
C4D	Capacitively coupled contactless conductivity detection
CCD	Central composite design
CD	Cyclodextrin
CE	Capillary electrophoresis
CEC	Capillary electrochromatography
CE-MS	Capillary electrophoresis-mass spectrometry
CHES	*N*-cyclohexyl-2-aminoethanesulfonic acid
CI	Counterion
CMC	Critical micelle concentration
CTAB	Cetyl trimethylammonium bromide
DAD	Diode array detection
DBT	Dibenzothiophenes
DEA	Drug enforcement agency
DHPT	Dihydrophenanthrothiophenes
DNA	Deoxyribonucleic acid
DOE	Design of experiments
EACA	ε-amino caproic acid
EDTA	Ethylenediaminetetraacetic acid

EIC	Extracted ion chromatogram
EIE	Extracted ion electropherogram
EOF	Electroosmotic flow
EPO	Erythropoietin
ESI	Electrospray ionization
EtG	Ethyl glucuronide
EZE	Eigen—Zundel—Eigen
FASS	Field amplified sample stacking
GBL	γ-butyrolactone
GC-MS	Gas chromatography-mass spectrometry
GCxGC	Comprehensive gas chromatography
GHB	γ-hydroxybutyric acid
GTFCh	Society of Toxicological and Forensic Chemistry
HEPES	4-(2-hydroxyethly)-1-piperazineethanesulfonic acid
HIS	Histidine
HPLC	High performance liquid chromatography
HRMS	High resolution mass spectrometry
ICP	Inductively coupled plasma
ITP	Isotachophoresis
IUPAC	International Union of Pure and Applied Chemistry
LC	Liquid chromatography
LC-MS	Liquid chromatography-mass spectrometry
LE	Leading electrolyte
LIF	Laser induced fluorescence
LKA NRW	Criminal Institute of North Rhine-Westphalia
LLE	Liquid-liquid extraction
LOD	Limit of detection
LOQ	Limit of quantification
LSD	lysergic acid diethylamide
LSFER	Linear solvation free energy relationship
MA	Methamphetamine
MALDI	Matrix assisted laser desorption ionization

MAO	Monoaminooxidase A
MBE	Moving boundary equation
MC	Mitraciliatine
MDMA	3,4-methylenedioxymethamphetamine
MEEKC	Microemulsion electrokinetic chromatography
MEKC	Micellar electrokinetic chromatography
MES	2-(*N*-morpholino)ethanesulfonic acid
MFE	Molecular feature extractor
MG	Mitragynine
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MS^n	Multiple stage mass spectrometry
N,N-DET	*N,N*-diethyltryptamine
N,N-DMT	*N,N*-dimethyltryptamine
NACE	Non-aqueous capillary electrophoresis
NFS	Nebulizer-free sprayer
NPD	Nitrogen-phosphorus detector
NPS	New psychoactive substances
PASH	Polycyclic aromatic sulfur heterocycles
PAY	Paynantheine
PCA	Principle component analysis
PFT	Partial filling technique
QqQ-MS	Triple quadrupole-mass spectrometry
QTOF	Quadrupole-time-of-flight-mass spectrometry
RP-LC	Reversed phase-liquid chromatography
RSD	Relative standard deviation
RSM	Response surface methodology
SC	Speciociliatine
SDS	Sodium dodecyl sulfate
SG	Speciogynine
SPE	Solid phase extraction
THBNT	Tetrahydrobenzonaphthothiophenes

THC	Tetrahydrocannabinol
THDBT	Tetrahydrodibenzothiophenes
TOF-MS	Time-of-flight mass spectrometry
TRIS	Tris(hydroxymethyl)aminoethane
UPLC	Ultra performance liquid chromatography
UV/Vis	Ultraviolet-visible spectroscopy

SCOPE OF THE THESIS

The overall scope of this thesis is to investigate the manifold application possibilities of electromigrative separation techniques in the field of forensic science, especially for the analysis and the subsequent profiling of biogenic drugs and preparations thereof, to gain comprehensive information on strategic (e.g. origin of samples) and tactical intelligence (attribution of samples to a certain batch) [1].

To achieve this overall goal, this thesis will start to answer the question "What is the current role of electromigrative separation techniques in forensic science?" to provide a suitable scientific background about the potentials, the advantages and the limitations of these techniques for the following chapters in form of a critical literature review (**see Chapter** 1). In contrast to a classical review, presenting what has been done in the past, this review is merely a summary of illustrative studies and on the authors experience in the field, rather than a complete survey. It shows where these techniques offer significant advantages over more traditional methods and provide orthogonal up to comprehensive information.

This thesis will also demonstrate the latest state of the art of electromigrative separation techniques, as well as highlight open questions and further potentials, as the investigations made in this thesis go beyond the current state of the art and will provide fundamental research, especially in the field of separation mechanisms, and aid to solve some of the open questions. Additionally, the implementation of electromigrative separation techniques for the solution of complex problems in fields where the analytical techniques applied so far provide only unsatisfactory results is exploited.

The abuse of "Kratom" (Mitragyna speciosa) as a so called "legal high" is relatively new in Germany [2] and the biogenic drug is not listed within the German narcotic act, but a request to include it has already been made. Consequently, it is important to establish analytical methods for the analysis, as a matter of prudence. The first central question that has to be answered within this thesis is consequently: "Is it possible to establish a whole analytical workflow for the forensic profiling of the biogenic drug "Kratom"?" This

task is very complex as Kratom represents a very complex mixture of structurally closely related alkaloids [3] and systematic scientific investigations of this drug are scarce [4]. Especially the high number of diastereoisomers present in Kratom makes the analysis very challenging, as the main alkaloids exhibit at least three but in some samples more than ten diastereomers or isoforms. Analytical techniques for this complex sample therefore usually rely on the analysis of only some analytes [5] or using a sum parameter, which is unsatisfactory for establishing a forensic profiling. Consequently, an analytical strategy implementing a high performance separation technique and chemometric data evaluation has to be established and validated. To achieve this, the individual working steps need to be developed, optimized and validated, starting with the sample preparation, the analytical method, the data evaluation and further chemometric analysis of the results for a comprehensive view on the whole process (**see Chapter** 2). To ensure the significance of the optimization, it is necessary to understand the underlying working principles of the individual steps, especially of the separation technique. Also the coupling of capillary electrophoresis to mass spectrometry is often practiced, but the effects from the detection on the separation and vice versa or the possible interactions are rarely investigated. This thesis will therefore present such investigations in a chemometric method development and an in-depth study of the interactions and effects of the coupling of capillary electrophoresis to mass spectrometry, to rule out possible biases arising from the hyphenation of both techniques, but also to identify the key parameters affecting and determining robust separation and detection.

This deeper understanding of the separation principles allows for an easy adaptation of established method towards new analytes and sample classes (**see Chapter 3**). Together with different cooperation partners, it was therefore investigated in detail whether the developed capillary electrophoretic separation techniques can be applied broadly and thus deliver additional information on a large variety of samples, e.g. psychoactive plant materials, biogenic drugs but also crude oils and extracts thereof (**Chapter 4**).

When it comes to the analysis of large sample cohorts for a forensic profiling, CE still suffers from problems originating from the poor automation in sample and buffer

handling for non aqueous methods and the lack of automated software tools with accurate peak picking and data extraction, which are otherwise widely available for LC-MS analysis [6]. Therefore, this thesis will study the possibilities of implementing software, originally developed for high-throughput clinical studies using LC-MS [7-9]. It will be tested for the extraction of CE-MS data from drug seizures of Kratom, opium and heroin, focusing on the major cationic alkaloids, as well as cationic side compounds. Profiling with automated data extraction and completion with chemometric data evaluation will be performed for Kratom and the whole supply chain of the heroin production in a single, universally applicable, comprehensive workflow, which has not been achieved before (**see Chapter 5**).

For comprehensive profiling, it is desirable to analyze as many analytes and characteristics of the sample as possible. However, so far in forensic science mostly cationic analytes (the active compounds) were considered, but small organic compounds and acids can deliver additional relevant information, especially as marker substances in biogenic samples (from the citric acid cycle or the shikimic acid pathway, etc.) or from colorants in drug charges [10]. But how do you analyze these substances reliably? The analysis of small acids is a complex task, which has not been satisfactorily solved yet. The major problem in this case is not the separation, which can generally be achieved with several techniques, but the detection. These small analytes are usually inaccessible via UV/Vis-detection. If identification is desired, mass spectrometry has to be employed; however, due to the very high polarity of these substances they do not reside at the outer surface of the droplets in electrospray ionization and thus evade the ion-evaporation. Poor ionization efficiency results with high detection limits and poor robustness. So how do you avoid this problem? An immediate solution would be a change of the ionization source or an optimization especially with regard towards these analytes. But wouldn't it be more interesting and elegant to make the quantitative determination of these analytes simply independent from ionization efficiency? Isotachophoresis coupled to mass spectrometry has the potential to solve these questions for its unique separation principle, where the quantitative information is absolutely independent from the signal height and purely relying on the length of the signal. Although ITP is the oldest electromigrative

separation technique and is especially useful for the separation of acids, the potential of this method can be multiplied by employing negative mass spectrometric detection, which has not been achieved before the beginning of this thesis. This interesting approach will be tested and established, although several, so far unknown problems (dramatic influence from H^+- ions migrating from the sheath liquid, countermigrating ITP and other) are connected with this coupling. However, in this thesis one of the first successful couplings of isotachophoresis with mass spectrometry worldwide is represented. Many confounding parameters like the mobility of the ions in the sheath liquid or the establishment of local pH-shifts during the separation can be expected to present and have to be identified and discussed together with potential solutions. As most of the problems were suspected to be associated with the ion source, the question arose whether more advanced interfaces could help for their solution. A recently designed and validated micro-flow-through vial junction at the tip interface [11] will be tested (**see Chapter 6**).

Consequently, in order to present a comprehensive investigation of biogenic drugs and other forensic samples, the feasibility of the combined application of non aqueous capillary electrophoresis-mass spectrometry and the hyphenation of isotachophoresis with mass spectrometry to achieve a powerful separation and detection technique for analytes relevant to forensic profiling will be presented.

In summary, this thesis deals with the specific basic separation principles of different modes of electromigrative separation techniques. This experience will form the basis for method optimization and adaptations of the methods to different forensic applications. Robustness will allow analyzing larger sample cohorts and include further cutting agents or other substances artificially added to drug batches. For profiling, advanced data extraction tools are to be implemented and the suitability of recent commercial data extraction tools for CE-MS analysis have to be tested. We aim at establishing a comprehensive analytical workflow for the profiling of biogenic drugs, starting from the basic sample preparation, method development and validation up to the chemometric data evaluation strategies. The insights gained into separation principles and influences from detection on the separation systems will allow for a deeper understanding of the underlying principles and will help in future method

developments. The isotachophoretic methods, coupled to mass spectrometry, represent an innovative approach to solve old problems with creative ideas. With the methods developed within this thesis, the toolbox for the holistic analysis of all charged, extractable analytes from various samples matrices will be extended.

1. CHAPTER I: ELECTROMIGRATIVE SEPARATION TECHNIQUES IN FORENSIC SCIENCE: COMBINING SELECTIVITY, SENSITIVITY AND ROBUSTNESS

Based on: T.N. Posch, M. Pütz, C. Huhn, submitted for publication in Analytical and Bioanalytical Chemistry

1.1. CHAPTER SUMMARY

In this review chapter the authors want to introduce the advantages and limitations of electromigrative separation techniques in forensic toxicology. We here thus present a summary of illustrative studies and our own experience in the field together with established methods from the German Federal Criminal Police Office rather than a complete survey. We here focus on the analytical aspects of analytes' physicochemical characteristics (e.g. polarity, stereoisomers) and analytical challenges such as matrix tolerance, separation from compounds present at large excess, sample volumes and orthogonality). For these aspects we want to demonstrate the specific advantages over more traditional methods. Both, detailed studies as well as profiling and screening studies were taken into account. Care was taken to nearly exclusively document well validated methods outstanding for the mentioned analytical challenge. Special attention was paid to aspects being exclusive to electromigrative separation techniques such as the use of the mobility axis, the potential for on-site instrumentation as well as the capillary format for immunoassays. The review concludes with an introductory guide to method development for different separation modes presenting typical buffer systems as starting points for different analyte classes. This review wants to give an orientation for those users in separation science considering using capillary electrophoresis in their laboratory in the future.

1.2. INTRODUCTION

A forensic scientist is faced with multiple problems: Unambiguous identification and quantification of chemical substances is needed with greatly varying matrices (such as tablets, raw materials, biofluids, wipe samples, etc.) and concentrations (from main components to traces), sometimes from only a small amount of sample available and of course in a short time frame. Nowadays, additional challenges arise from the new psychoactive substances, sold as “research chemicals” “bath salts” or “legal highs” [12], originating from the rapid development of designer drugs and the ongoing interplay of reconfiguration and prohibition by federal laws [13]. However, the “common” drugs are also subject to changes and developments e.g. via new synthetic routes including changes of the matrices. This demands a permanent update of established as well as the fast development of new methods with the inclusion of new compounds used as adulterants or to cover side-effects (e.g. nausea) by medicinal drugs. But also the medicinal drugs may not be neglected in forensic science, as they are distributed via the grey market with a high potential of abuse and as they may illegally be introduced into the country. Consequently, all this demands a deep knowledge of multiple separation techniques and detection systems by the forensic scientist. Also, the employed techniques have to fulfill the requirements for high robustness and reliability.

Especially with regard to the new psychoactive substances it is desirable to have a separation technique, which allows for fast method development, which is basically unlimited in terms of polarity of the analytes, does not need a lot of sample preparation due to its high matrix tolerance and is technically applicable to any possible analyte. For this reason, the article will cover literature without special regard to a certain time frame, but instead presents excellent and/or illustrative examples. To allow for an easy reproduction and overview of the discussed methods, a table, summarizing all relevant information on the experimental parameters at a glance, is provided at the end of the chapter in Table 2, sorted in alphabetical order according to the analyte classes. For information about the advances in capillary electrophoresis in forensic sciences in a regular, updating format, the authors recommend the interested reader to the reviews of the Tagliaro group [14-17]. All in all, this article is intended for readers who want to

get a first glimpse into the possibilities of electromigrative separation techniques and may thus be ideal for analytical chemists and forensic toxicologists who are up to now more/only familiar with chromatographic techniques.

1.3. ADVANTAGES AND LIMITATIONS

1.3.1. FLEXIBILITY IN SEPARATION AND DETECTION MODES

Similar to chromatographic techniques, a wide variety of separation and detection modes may be employed for electromigrative separation techniques with typical, commercial equipment. Common capillary electrophoresis equipment can be very flexibly applied to all modes. Except for capillary electrochromatography, only a change in the buffer composition is necessary to switch modes. This is in contrast to HPLC, where a large set of different stationary phases is required to address specific separation problems and even different equipment, e.g. for ion chromatography However, in this section we will show that the flexibility of capillary electrophoresis instrumentation is higher as most separation modes do not require special or further equipment, like e.g. HPLC vs. ion chromatography. All, what is needed for a change of the separation mode are-in most cases-just some milligrams of inexpensive chemicals.

1.3.1.1. SEPARATION MODES

One of the most striking arguments for using electromigrative separation techniques is their ability to operate in many different modes without having to buy new hardware or larger amounts of expensive chemicals. An overview on modes and terms is given in the IUPAC recommendations by Riekkola et al. [18]. For those readers without basic prior knowledge of electromigrative separation techniques we recommend the “Brief introduction to capillary electrophoresis” [19] or the Primer “High performance capillary electrophoresis” [20] for a fast overview. In addition, there are many good text books on the basics of electromigrative separation techniques (e.g. [19,21]), some of them with dedicated chapters to the use of CE in forensic science as well as reviews on this topic [14-17,19,22-44]. Capillary electrophoresis with aqueous and non-aqueous [45-52] buffers as well as with additives for chiral separation [53,54] are the most common modes applied and are well suited for the analysis of small molecules up to proteins

with the only requirement that a charge (mostly depending on pH) is present on the analytes to enable electrophoretic migration upon an external electric field. Most of this review deals with CE, NACE and chiral separations, so in this section we focus more on less widely applied electromigrative separation techniques.

This limitation of the necessity of charge on the analyte can be surmounted when charge is introduced by so called separation carriers, either complexing agents (e.g. sulfated cyclodextrins, also applicable for chiral analysis) or charged detergents to also target neutral analytes [55]. In micellar electrokinetic chromatography (MEKC), a surfactant, mostly sodium dodecyl sulfate is used with a concentration above its critical micelle concentration. The micelles create a micro phase which may be charged and thus migrates through the separation column, serving as a pseudo-stationary phase to induce chromatographic separation: As in classical chromatography, the separation is based on the equilibrium distribution of the analytes in the free buffer solution as mobile phase and within the micelles as pseudostationary phase. These effects can freely be combined with additives (e.g. organic solvents or urea) changing the relative polarities of the two phases and their interaction, with evoking secondary equilibria (e.g. cyclodextrins) or modifying the electroosmotic flow velocity and thus the relative velocity of the mobile phase (e.g. calcium ions, coatings). A fully optimized MEKC method is theoretically capable of separating any couples of analytes, but limits the applicable detection systems, as the surfactants are usually not MS-compatible, though some solutions have been presented [56] (see also Section 1.3.2.2).

A further development is microemulsion electrokinetic chromatography, where a microemulsion is established, serving as a pseudostationary phase [57-59]. Here, the applicability of MEKC is broadened towards highly unpolar compounds, but also comparatively polar compounds may be analyzed. MEEKC has an increased solubilization capacity and can exhibit a highly efficient mass transfer of solutes between the pseudostationary phase and the organic/aqueous phase [60,61]. It also offers an even higher potential for fine tuning and flexibility than MEKC, allowing for an enlargement of the separation window and faster analysis, originating from the more complex buffer composition, while being easy and cheap to use [62,63]. The first MEEKC method and buffer system was introduced by Watarai in 1991 [57] consisting

of water/SDS/1-butanol and heptane (89.3/3.3/6.6/0.8). This system is still most frequently applied, but up to now, hardly used in forensic toxicology.

The employment of real stationary phases is possible, either by filling the capillary with chromatographic material or synthesizing monolithic stationary phase material directly inside the column, giving rise to capillary electrochromatography [64-66]. Here the eluent is transported via the electroosmotic flow induced on the charged stationary phase surface (e.g. via sulfate groups). The opinion about CEC is diverse among experts, as some regard this technique being a combination of the advantages of CE with its high separation efficiency and of HPLC with stable retention times, high sample capacity and the possibility to handle thermally labile and neutral substances [67], while others regard it to be the combination of all the disadvantages of both techniques (low reproducibility, poor limits of detections, possibility of cross contamination, sensibility to matrix compounds due to the stationary phase). So far, the use of CEC is mostly demonstrated in model applications, most likely due to the limited commercial availability of column hardware and instrumental extras being necessary such as an LC pump. However, an undeniable advantage of CEC lies in the high compatibility of the mobile phases with MS-detection. Promising studies with remarkable separation efficiency are available [67,68] and will be discussed in Section 1.3.2.3.

Isotachophoresis, where charged analytes are separated according to differences in their effective electrophoretic mobilities as in CE, though using a discontinuous buffer system [69-71] is the oldest electromigrative separation technique. Today it is mostly applied as a sample preconcentration technique, but can advantageously also be used in a column-coupling format for very high sensitivity and increased matrix tolerance [72]. Further modes of electroseparation, like gel electrophoresis and isoelectric focusing, are well known for DNA sequencing and protein analysis, but have not been used for drug analysis to our knowledge. During the last years some publications dealt with the transfer of immunoassays into capillary formats to enhance identification and thus selectivity. An example is shown is Section 1.3.8.

Some modes or methods require the fused silica capillary to be coated in order to reduce adsorption phenomena (of analytes and/or matrix components) or to change

the magnitude of the electroosmotic flow. The latter can be used to enhance the speed of analysis or to enable the analysis of anions with high electrophoretic mobilities. Coatings may also help to increase the repeatability of a specific method, e.g. when working with background electrolytes at intermediate pH value close to the pK_a-value of the fused silica surface or when working with high matrix loads, where a renewal of the capillary surface is frequently required [73].

For switching between modes or methods, only the background electrolyte and most likely the separation capillary (costs ca. 10 €/m, length typically 20-80 cm) have to be exchanged. This can be achieved within minutes, as conditioning procedures for new capillaries are typically 30 min long (rinsing with 0.1 mol/L NaOH and BGE). This very fast method switching option has been highlighted in a number of previous reviews [17,40,74]. An excellent example for a very fast and efficient transfer of methods even using a single capillary is the work of Lurie *et al.* [73] who used different aqueous buffers and dynamic capillary coatings for the analysis of β-phenylethylamines, cocaine, oxycodone, heroin, lysergic acid diethylamide, opium alkaloids, ingredients of psychoactive mushrooms, γ-hydrodxybutyrate and γ-butyrolactone together with their respective, individual side compounds. Using commercial buffer solutions with or without addition of different cyclodextrins or detergents allowed baseline separation for all analytes within usually 10 min total run time; all separation procedures were validated for routine forensic drug analysis.

1.3.1.2. MANIFOLD DETECTION POSSIBILITIES

Most of today's commercial CE instruments are fabricated with integrated UV-detection or a diode array detection system. They are suitable for classical photometric detection, where the absorbance of the analyte molecule is measured, or for indirect detection, where an absorbing compound, the probe ion, is added to the background electrolyte. In this case the reduced concentration of the probe ion is recorded, when it is displaced by the analytes in the peak for reasons of electroneutrality. The direct detection is suitable for any molecule exhibiting π-bonds, while the indirect method is usually applied for small organic or inorganic ions or carbohydrates without a suitable chromophore. Examples are given in Section 1.3.4.2. Laser induced fluorescence (LIF)

detection is also widely available and is known for its extremely high sensitivity as well as selectivity and has been used in combination with CE as direct [75,76] or indirect [77-80] detection system, on-column or post-column [81]. In the past, capillary electrophoresis has been conducted with further detection techniques [82] based e.g. on Raman scattering or refractive index changes though they are barely used today and were gradually superseded by the previously mentioned systems [82]. Non-optical post-column detection techniques are potentiometric detection, conductivity detection and amperometric detection. Of these three, only capacitively coupled contactless conductivity detection has prevailed as a universal detection technique [83]. All detection modes can be freely combined, but mostly on-column formats are employed. This is especially useful, when the detection systems are complementary, for example if one delivers the quantitative information and the other is used for identification, like in ITP-C4D-ICP-MS [84] or CE-LIF-MS hyphenation [76].

Like LC, capillary electrophoresis is coupled to mass spectrometry [85] mostly via electrospray ionization, especially in proteomics and metabolomics, but increasingly also in forensic studies. The crucial point in coupling capillary electrophoresis with mass spectrometry is the interface: although many partially very specialized ion sources were developed for CE-MS coupling (summarized in [86]), including fast atom bombardment [87], laser vaporization [88] sonic spray [89] or offline coupling with matrix assisted laser desorption ionization [90] up to “pseudo”-online MALDI using a rotating ball interface [91,92], only electrospray ionization has prevailed and is used regularly with a commercial interface, the triple tube sheath liquid interface. However, as capillary electrophoresis typically delivers only some nanoliters per minute as effluent or has even no solvent net flux, the stability of the ionizing electrospray is problematic. There are basically two approaches to solve this problem: the sheath-less and the sheath-liquid assisted interfaces [93]. In the sheathless approach, the end of the capillary is usually etched until it becomes porous and analyte ions can directly evaporate from the surface and thus be introduced into the mass spectrometer [94]. The sheath-liquid assisted interfaces use a make up liquid (typically a water/isopropanol mixture containing 1% formic or acetic acid) to yield a robust electrospray that is independent from the efflux from the CE-capillary. The

commercially available interface uses a pneumatically assisted electrospray, thus having a sheath-liquid flow rate of about 4 µL/min. Although only correct for some applications, this additional flow rate is widely perceived as a dilution of the analyte solution, impairing detection limits. However, this additional liquid can be used to directly enhance the ionization efficiency of the analytes e.g. adapting pH-values and surface tension. Likewise, buffer systems, unfavorable for ionization can be diluted. Then, even a sensitivity enhancement is possible compared to sheath-less interfaces. Mass calibrants for the mass spectrometer can be added to the sheath liquid and chemical reactions within the ionspray (e.g. in-source decay) can be introduced or suppressed. However, to circumvent or to reduce the dilution, new developments in the sprayer design make it possible to drastically reduce the flow rates (<0.1 µL/min) of the sheath-liquid [93,95]. These new interfaces do not need pneumatic assistance anymore and are actually using a real electrospray for ionization.

Beside ESI, also atmospheric pressure chemical ionization and atmospheric photo ionization sources have been utilized for coupling CE with mass spectrometry, increasing the number of detectable analytes in terms of polarity and ionization efficiency. For a more detailed overview of capillary electrophoresis mass spectrometry, some excellent reviews are recommended [85,93,96-98].

1.3.2. SEPARATION EFFICIENCY, BROAD APPLICABILITY AND PROFILING

High separation efficiency is desired for all analytical separation techniques, especially in combination with high separation selectivity and thus high peak capacities and resolution. In electromigrative separation techniques, these characteristics are further combined with short analysis times of mostly below 15 min for CE and 20 min for MEKC. A high sample throughput can thus easily be reached with no or only few overlapping signals for most analytical applications using simple photometric detection. This has led to a number of publications on profiling strategies where a large number of structurally related analytes at trace or minor component level have been separated within only one analytical procedure to yield a characteristic signature of the analyzed materials. Likewise, many different electromigrative separation techniques have been

optimized to be employed as universal screening methods for an impressive range of analytes.

1.3.2.1. BROAD APPLICABILITY OF CAPILLARY ELECTROPHORESIS

Already in 1993 Chee & Wan [99] explored the implementation of capillary electrophoresis with UV-detection for a forensic-toxicological application, namely the analysis of 17 psychotropic alkaloids of various drug classes including a.o. amphetamine, methamphetamine, procaine, lidocaine, codeine and methaqualone. Impressively, baseline separation (permitting undisturbed quantification with UV detection) was achieved for all analytes within 12 min by simply employing 5 mM sodium dihydrogenphosphate, titrated to pH = 2.35 with phosphoric acid as BGE in an uncoated bare fused silica capillary. The analysis of the drugs in spiked human urine and serum was demonstrated after liquid-liquid extraction (chloroform/isopropanol, pH 10.5) and redissolution in BGE after drying for sample preparation. This publication demonstrates the ease of applying CE despite different sample matrices while being fast and highly efficient. However, the method showed only limited robustness with a strong dependence of resolution on small pH variation (0.05 pH units) in the BGE.

Today, more and more groups apply CE-MS for drug profiling and screening purposes. Already in 1997 Unger et al. [100] presented a generic approach for the analysis of plant alkaloids. Indole alkaloids, benzophenanthridine alkaloids, ß-carboline alkaloids and isoquinolines were separated in a 1:1 mixture of 100 mM ammonium acetate in water (pH 3.1) and acetonitrile. In total, 37 different analytes could be separated using one generic buffer composition, showing the general applicability of this hyphenated technique for the separation and identification of alkaloids from plant extracts. This method was improved later on by Unger & Stöckigt [101] adding a field-amplified sample stacking procedure for preconcentration (BGE: 200 mM ammonium acetate in a 1:1 mixture of water and methanol). A similar but non-aqueous BGE of 60 mM ammonium formate in a mixture of acetonitrile and glacial acetic acid (100:6) was used by Posch et al. [102,103] for a large variety of indole alkaloids and plant samples (see also Section 1.3.3.2). From this and further publications it is obvious that a screening of cationic analytes, especially protonated indole alkaloids in complex sample matrices

can be very well attained using NACE: also opium alkaloids and amphetamines were separated very quickly and precisely as demonstrated in a series of excellent publications by Bjørnsdottir and Hansen [104-106], where the underlying separation mechanisms of NACE were also investigated. They compared an MEKC procedure [107] for the separation of five opium alkaloids to the results of NACE [104] and additionally cross validated both procedures with a validated HPLC procedure [105]. All three methods performed at a similar level. In 1999, they developed a very fast NACE procedure for the separation of 16 drugs of forensic interest (amphetamine, cocaine, heroin, etc.) in less than 2 min using a short end injection (injection from the outlet end) with only 8.5 cm effective capillary length (total length: 32.5 cm, i.d.: 25 µm) [106]. The BGE was composed of 25 mM ammonium acetate and 1 M acetic acid in a mixture of acetonitrile, methanol, glycerol, and water (75:15:8.5:1.5). Retrospectively, regarding the employed short effective capillary length, this article clearly shows the power of a miniaturized separation system, which is reflected in recent intensive studies on microfluidic devices.

Due to the high separation efficiency of capillary electrophoresis in general and the complemented high sensitivity and selectivity of MS detection, CE-MS is also well applicable for the screening of drugs of abuse together with their (structurally related) Phase I metabolites. An illustrative example was presented by Kohler et al. [108] for drug screening in diluted urine samples with CE-MS after preconcentration using pH-mediated sample stacking. The developed procedure, using a simple 1 mol/L formic acid solution (pH 1.8) as BGE, is remarkable, as a very fast screening approach was obtained with full to partial separation of 33 drugs of abuse in a small time window of 4-7 min (in total 15 min including rinsing protocols). Unambiguous identification and quantification via selected reaction monitoring in a triple-quadrupole mass spectrometer was possible with the same method as verification of positive results from the screening approach. Clearly reduced analysis times for screening were thus obtained compared to corresponding chromatographic techniques, like the recently developed multidrug-screening approaches using UPLC-TOF-MS [109] (52 common pharmaceuticals in 17 min chromatographic run time) or UPLC-MS/MS [110] approaches (29 drugs of abuse in 20 min chromatographic run time). For their CE-MS

procedure, Kohler et al. [108] also performed a full validation according to the guidance of the Food and Drug Administration. Their results disprove many prejudices towards electromigrative separation techniques showing that well optimized methods with good performance can be obtained: very good robustness, linearity over a wide range (10-1000 ng/mL for cocaine, 21-1000 mg/L for methadone) and accuracy was demonstrated. In the authors' opinion, such methods could be powerful tools for establishing new libraries for automated CE-MS analysis interpretation, similar to LC-MS or GC-MS, where commercial and self-built libraries [111] are widely distributed and used.

1.3.2.2. UNIVERSAL SEPARATION MODES MEKC AND MEEKC

In MEKC, the range of polarities encompassed can be optimized by means of the type and concentration of the pseudostationary phase (the type and concentration of the micellar detergent) and the relative polarity of the mobile phase by addition of organic solvents and other additives (see also Section 1.3.3.1). Relatively polar and medium hydrophobic analytes can be covered by MEKC, whereas MEEKC can be used for very hydrophobic analytes. For a detailed explanation of MEKC and MEEKC the interested reader is referred to the book by Pyell [112] or the publication from Terabe explaining how to influence selectivity in MEKC [113].

Already in 1991, Weinberger & Lurie developed a simple MEKC-UV method for the forensic screening of illicit drug substances [114]. Their screening system is composed of an 85 mM SDS, 8.5 mM phosphate and 8.5 mM borate buffer (pH 8.5) with 15% acetonitrile. The analysis of heroin and cocaine including products of decomposition in samples from police seizures was presented. In this method most of the common illegal drugs of abuse (heroin, cocaine, psilocybin, Cannabis ingredients, LSD, methamphetamine, etc.) were included within one analytical run. Beside the impressive separation, the authors compared their method to standard HPLC methods while explaining the separation principle, making this publication an easy entry for an experienced HPLC operator, who wants to gain some information at a glance on MEKC. In 2004, Lurie and co-workers presented two very robust and reliable methods for the forensic screening of heroin [115]: a CE-UV method for heroin's basic adulterants and impurities and an MEKC-UV method for the determination of neutral,

acidic and weakly basic adulterants, showing the broad coverage of MEKC with regards to analyte characteristics. Both methods are in routine use in heroin signature programs of DEA laboratories [115]. The authors also show that CE and state-of-the-art LC show comparable performance. Excellent run-to-run precision (RSD < 0.9%) and a wide linear range (0.0035-0.454 mg/mL) were obtained by MEKC using a 32 cm, 50 µm capillary, dynamically coated with a double-layer coating (using the polycationic coating (CElixir®) followed by dodecylsulfate in the run buffer as dynamic coating). The migration time precision was improved significantly by reducing the flushing/recoating steps between runs to a partial recoating step only. The total analysis time was below 15 min.

MEEKC methods used in forensic science are scarce. However, the technique has demonstrated its power in the analysis of heroin as shown by Wen et al. [116]. Using a classical MEEKC system (see Section 0) as buffer, 17 analytes encompassing heroin, amphetamine and impurities therein were separated within 12 min using a 40 cm (i.d.: 75 µm) bare fused silica capillary. The method is characterized by low RSD's (0.11-1.45%) for relative retention times, linearity from 1-600 µg/L and limits of detection suitable for routine control of drugs of abuse (1.0-1.5 µg/L). One of the few other applications of MEEKC was presented by the Tagliaro group for the analysis of caffeine in different beverages and in smart drugs [62].

1.3.2.3. NOT YET IN ROUTINE: CAPILLARY ELECTROCHROMATOGRAPHY

CEC has been investigated as a potential alternative to GC-MS or HPLC-MS for the fingerprinting of cannabinoids in 1998 by Lurie et al. [67]. The study includes a short investigation of the impact of the pH-value, acetonitrile concentration, phosphate concentration, column length and the type of stationary phase on the separation giving a nice introduction to CEC and method optimization. A 25 mM phosphate buffer (pH 2.57) with 75% acetonitrile was finally used in a commercially available 49 cm (i.d.: 100 µm) 3 µm Hypersil C18 column. After correction of retention times and peak areas using cannabinol as internal standard, high precision in relative retention times (RSD < 0.1%) and relative peak areas (RSD < 4.6%) were obtained while achieving low limits of detection (500 ng/mL for d9-tetrahydrocannabinol, d9-THC) using a high-sensitivity

cell with extended detection path length. Also, separation efficiency was high (200 000 plates/m) and the linear detection range was significantly improved by the utilization of the high sensitivity cell. The method was applied to the separation of concentrated hashish and concentrated marijuana extracts. The final procedure resolved about 50% more peaks compared to a gradient HPLC procedure with similar run time. In the same year, Lurie and co-workers presented an additional study, comparing CEC with CE, MEKC and HPLC for the analysis of 15 organic compounds of forensic interest [117]. For CEC a 3 µm Hypersil C8 column (i.d.: 100 µm, length: 34 cm, 25 cm bed length, Hewlett-Packard) was used with a 25 mM phosphate buffer (pH 2.5) in 75% acetonitrile containing 2 µL/mL hexylamine to suppress silanophilic interactions. The specialty of this CEC method was its capability to separate strongly basic, weakly basic, neutral, weakly acidic and strongly acidic compounds within 33 min in a single run using a step gradient. Although CE achieved higher plate numbers than CEC, the overall resolution was better in CEC, which was explained by the authors as the consequence of a mixed mode separation with a combination of a free zone separation mechanism and chromatography via the packed bed. In comparison to MEKC, CEC was less prone to injection artifacts. When compared to HPLC, CEC showed only small advantages: impurities from the buffer system (hexylamine) created extra peaks in chromatography since a full gradient had to be employed, while they were absent in the CEC system since a step gradient was sufficient for the analysis. However, a clearly different selectivity especially for weakly and strongly basic analytes was observed. In conclusion, the authors pointed out that each technique has its distinct advantages and that a combination of multiple techniques can achieve a more comprehensive view for drug screening.

CEC has been optimized to allow for the extremely fast analysis of six opiates in self-packed octadecyl silica packed capillaries (length: 28 cm, i.d.: 75 µm, particle size: 1.5 µm), exhibiting plate numbers up to 444 000/m (for oxycodone) [68] using a 10 mM Tris, 50 mM SDS buffer (pH 8.3) in 20% acetonitrile. A very detailed and well executed study can be found in the work of Aturki et al. [118], who used a self packed cyano stationary phase in CEC to establish a validated procedure for the analysis of ten drugs of abuse in urine yielding a very robust separation system, which sustained more than

300 runs. Their buffer system, consisting of 20 mM sodium phosphate in a 4:1 mixture of water and acetonitrile (pH 2.5), separated the analytes in spiked urine samples within 18 min in a 33 cm long capillary (i.d.: 75 µm, effective length: 24.5 cm, packed length 23 cm) packed with CN-modified 3 µm particles. Good reproducibility of retention times (RSD < 5%), even in column-to-column experiments (RSD < 7%) was found. The detection was sensitive (LOD 5-15 ng/mL; LOQ 10-30 ng/L) and a linear working range of 100-1200 ng/mL was obtained, employing field amplified sample stacking as online preconcentration technique. The publication also included detailed investigations on the effect of different packing materials, of the acetonitrile content, of buffer pH values and concentration as well as the effect of sample injection techniques on sensitivity. In 2010, the same group adjusted the procedure for MS compatibility [119] by switching to 25 mM ammonium formate (pH 3) in a water-acetonitrile mixture (7:3) as mobile phase. The implementation of MS as detection system lowered the LODs to 0.8-3 ng/mL but, as often observed in MS detection, repeatability of peak areas was reduced (RSD < 16.3%) and the calibration range was shifted towards lower concentrations (2.5-100 ng/mL).

1.3.2.4. COLUMN-COUPLING ISOTACHOPHORESIS FOR PRE-CONCENTRATION AND MATRIX REMOVAL

Except being the older method to CE, ITP is hardly applied as a stand-alone method in forensic science. Transient ITP may be used as an on-line preconcentration technique [120,121] with a high matrix tolerance [120], leading to improved detection limits. Additionally the column-coupling mode ITP/ITP or ITP/CE (commercial instrumentation is available) were utilized with impressive preconcentration capabilities and matrix removal in various applications. A non-forensic example is the chiral analysis of tryptophan in urine with only dilution as sample preparation step [122].

However, these coupling techniques have a very high potential as high sensitivity techniques [123]: Figure 1shows an electropherogram from the analysis of harmala alkaloids using column-coupling ITP/CE-LIF. With the possibility of injecting 30 µL of a sample into the first ITP column of the two-dimensional setup, unprecedented detection limits of 20 pmol/L for norharman and harman were obtained. These two examples however, can only show the capabilities of the method; its real use in forensic science

has not yet been demonstrated. One of the conceivable forensic applications of this technique could be the trace detection of common drugs of abuse and their decomposition products in wipe and suction samples from surfaces (e.g. from hollows in vehicles that are suspected to have been used for drug smuggling).

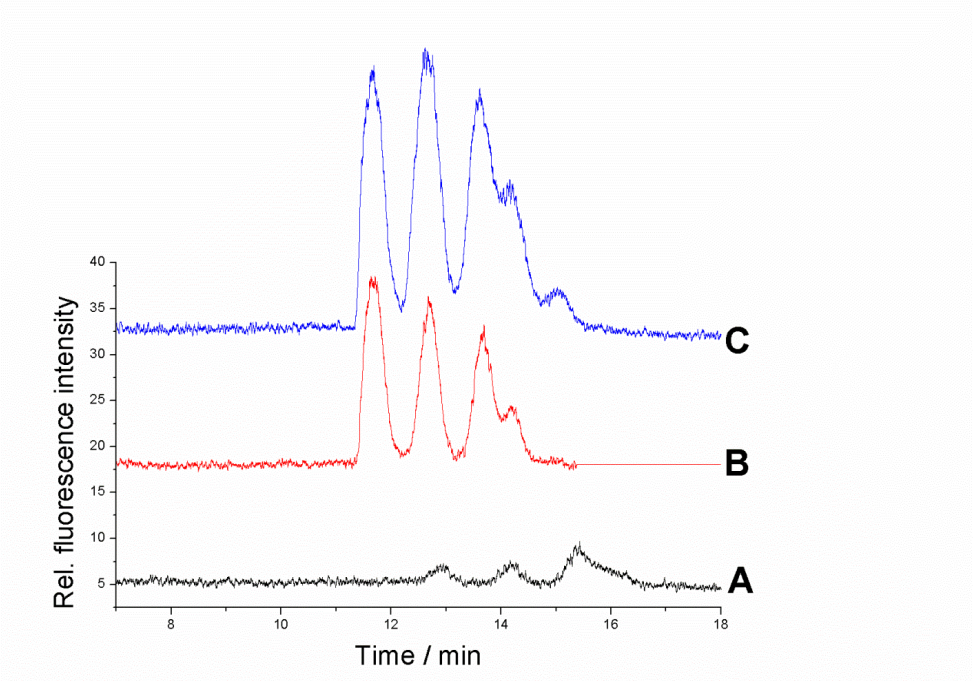


Figure 1: High sensitivity analysis of harmala alkaloids using ITP/CE-LIF: analyte concentration A) 0.0001 µmol/L, B) 0.001 µmol/L, C) 0.01µmol/L. LE: 10 mM ammonia solution (titrated to pH 4.7 with acetic acid), TE: 20 mM acetic acid, BGE: 20 mM NaH_2PO_4, pH 2.4 (titrated with HCl) (with kind permission from the author [123])

1.3.3. ANALYTES WITH SPECIAL PROPERTIES

CE and other electromigrative separation techniques have the reputation to be "specialized" techniques for analytes which are difficult to analyze with routine HPLC or GC methods. And indeed, there are several applications where only CE succeeded in

analyzing samples where analytes exhibited a high charge, were highly polar or highly hydrophobic or a mixture of enantiomers.

1.3.3.1. ANALYTES WITH HIGH POLARITY/CHARGE

Extremely hydrophobic or highly polar or even charged analytes are a challenge for routine chromatographic applications, different liquid chromatographic stationary phases like reverse phase vs. hydrophilic interaction chromatography are applied. For unpolar analytes, GC is employed or analytes are derivatized to meet the requirements of the method. In contrast, for CE polarity is not critical for separation as long as the analyte is sufficiently soluble in the BGE. Using MEKC the polarity range can easily be broadened via the concentration and type of the surfactant and BGE additives.

Highly charged compounds such as sulfonic acids and sulfates are ideal analytes for CE providing high charge also at very low pH. An illustrative example is the colorant analysis within drug profiling of heroin and Ecstasy pills (typically containing amphetamine derivatives). These food colorants employed are usually azo- or triarylmethane compounds with at least one sulfonate-group [10] Chromatographic analysis with MS detection is possible, but requires specialized stationary phases with a higher polarity to ensure sufficient retention [124,125]. The advantage of CE for these analytes is that the low pK_a of the sulfonate moiety can be utilized to achieve extremely high selectivity of the method [10] without the need for further sample clean-up: the food colorants in heroin samples and in Ecstasy tablets were separated at very low pH (pH 2.2 in 200 mM formic acid buffer) employing negative polarity for the CE separation. Only the target analytes with a negative charge at this low pH migrate towards the detector under these conditions, giving rise to very clean electropherograms even in case of matrix-loaded samples such as direct heroin extracts (all contained opium alkaloids and heroin itself are protonated and migrate to the cathode and thus are not detected). Selective detection is possible either with UV detection in the visible range or with MS detection for unambiguous identification. The method was used for the differentiation of Ecstasy and heroin seizures via the colorants. Typical cutting agents for heroin consist of caffeine, paracetamol and a mixture of several food colorants to create a brown color (comparable to the color of uncut heroin) to conceal the extent of cutting. A combination of different concentrations

of the azo dyes E 102, E 110 and E 151 is often used for that purpose, allowing establishing links between different seizures of heroin based on the colorant composition. Ecstasy tablets are also frequently colored with a range of different food colorants to increase the value of brand recognition together with the tablet logo and to shade unfavorable color effects caused by synthesis impurities. It is interesting to note that the separation of the positional isomers of quinoline yellow (E 104) was successfully performed also in real samples (yellow colored Ecstasy tablets), using MS/MS spectra to differentiate between the isomers (Figure 2). Thus, Ecstasy pills can be linked on the level of the tableting process based on colorant composition, even if only one colorant is contained, in this case by the relation of the proportions of the two positional isomers present in commercially available E 104 food colorant.

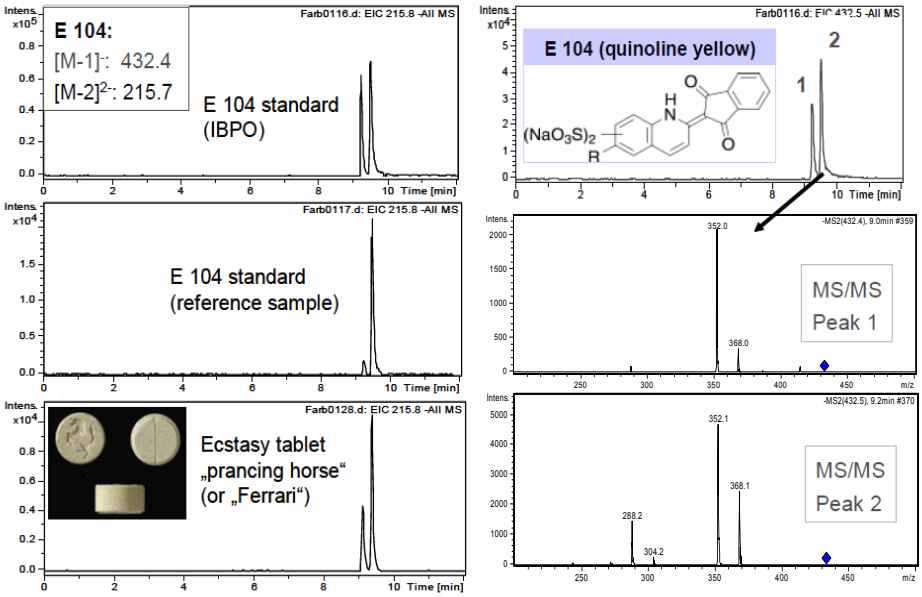


Figure 2: CE-MS analysis of two positional isomers of quinoline yellow (E 104) in two different reference materials and in a yellow colored Ecstasy tablet. BGE: 200 mM formic acid (pH 2.2), capillary: length: 82 cm, i.d.: 75 µm.

Another good example for the suitability of CE for the analysis of highly polar compounds in the field of forensic toxicology is the quantification of γ-hydroxybutyric acid in samples of so-called “liquid ecstasy”. Although structurally not related to the amphetamine derivatives typically contained in Ecstasy pills, GHB and its corresponding internal ester γ-butyrolactone have been frequently abused as recreational drugs. Furthermore, GHB unfortunately has gained a significant level of awareness because of its misuse as a “date-rape-drug”, meaning the use for drug facilitated sexual assaults, by pouring the drug into the beverage of the victim. GHB is typically consumed in the form of aqueous solutions of its sodium or potassium salt and, consequently, it is reasonable to also analyze GHB in its anionic form at high pH to prevent conversion to GBL. Quantification of γ-hydroxybutyrate can easily be achieved by a very rapid CE procedure using a simple sodium tetraborate buffer (20 mmol/L) at pH 10 and UV-detection at 206 nm. To attain high reproducibility, potassium sorbate was used by Pütz et al. (unpublished results) as an internal standard for normalization. For eight consecutive runs the precision of the migration time was 0.08 % and that of the peak area 0.8 % for GHB after normalization (Figure 3). This procedure is routinely applied for GHB quantification in the forensic laboratory of the BKA (German Federal Criminal Police Office) and has been successfully cross validated by participation in round robin tests.

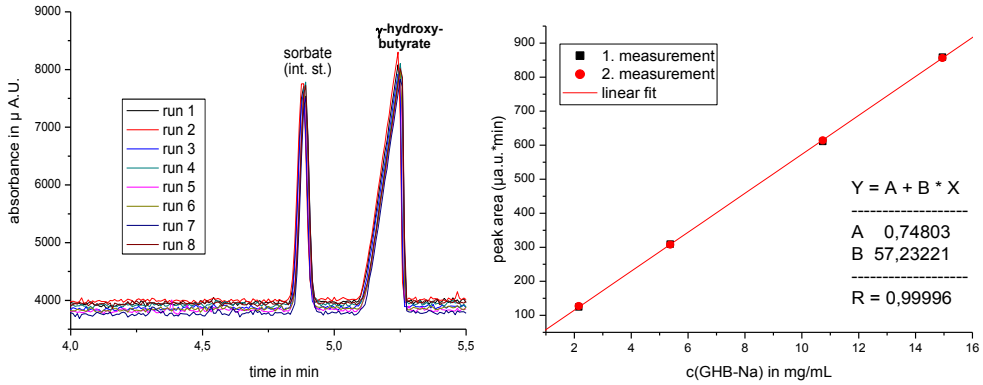


Figure 3: Eight consecutive CE runs for the quantification of GHB using sorbate as i.s. (left) and calibration plot with duplicate determination of four weighed portions of sodium γ-hydroxybutyrate (right); tetraborate buffer (20 mmol/L) at pH 10 and UV-detection at 206 nm.

Beside the separation of permanently charged and highly polar analytes, also very hydrophobic compounds, even those insoluble in water, as e.g. the hydrophobic steroidal alkaloids present in plants like *Solanum elaeagnifolium* or *Splanum sodomaeum* are analyzed in buffer systems containing high amounts of organic solvents or being purely non-aqueous. Cherkaoui and coworkers [126] analyzed extracts from these plants using NACE-MS, achieving a high resolution and thus very pure spectra for the unambiguous identification and reliable quantification using 25 mM ammonium acetate and 1 M acetic acid in a mixture of MeOH:MeCN (1:4).

MEKC together with MEEKC can cover an impressive range of polarities, with only a small set of buffer combinations to be tested. The classical MEKC buffer with borate pH 9.2 and 60 mmol/L SDS is well suited for relatively polar analytes. The polarity range can be enhanced to medium polar analytes by addition of organic solvents, mainly methanol and acetonitrile or with a combination of urea and acetonitrile for aromatic analytes in sassafras oils with octanol-water partition coefficients of up to 5 [127]. Selectivity changes are possible via changing the micellar pseudostationary phase just employing another detergent. For the choice of a suitable detergent, the

linear solvation free energy relationship model as applied to MEKC by Poole's group [128-130] can aid as it is based on possible interactions and thus physicochemical properties of the analytes with the mobile vs. pseudostationary micellar phase. The characteristics of most micellar phases are well described. The main underlying interactions are hydrophilic/hydrophobic in nature or stem from hydrogen bonds. Furthermore electrostatic interactions or Van-der-Waals interactions occur. Ionic interactions can be found mainly at the outer sphere of the micelle, at the charged head groups. In the core of the micelle also steric hindrance can influence and determine the allocation of a compound. For a general discussion on MEKC see Section 1.3.2.2.

1.3.3.2. STRUCTURALLY CLOSELY RELATED ANALYTES

A) Diastereomers: Especially with regard to mass spectrometric detection, diastereomers and positional isomers have to be baseline resolved by the separation technique, as being isobars, their individual mass spectra and usually also their fragment spectra cannot be differentiated. This task is not trivial due to near- identical hydrodynamic radii and charge of the molecules relevant for electromigrative separation techniques leading to very similar migration behavior in CE. Posch et al. [102,103] used a design of experiments for the analysis of the biogenic drug "Kratom" (made from the leaves of *Mitragyna speciosa*) in a combined approach to (I) assess the robustness, (II) to optimize an NACE-MS set-up and (III) to gain insight into the separation principles and interacting parameters in NACE [102]. The optimization of the method led to the following conditions: 60 mM ammonium formate in a mixture of acetonitrile and glacial acetic acid (100:6) served as background electrolyte, while a mixture of isopropanol, water and acetic acid (62/33/5) was used as sheath-liquid at a rate of 7 µL/min. Although 167 analytical runs had to be conducted for the method development, this experimental setup can still be considered a fast method development, as the results allowed insight into the separation principle and the relevance of all significant parameters. The basic separation procedure with its relevant tuning parameters is now known so that adaption to new forensic applications can easily be performed. The broad applicability of this NACE-MS procedure was further investigated for the analysis of various psychoactive plant materials and proven to be

nearly generic for indole alkaloids [103]. A possible explanation for the good separation efficiency for diastereomers in NACE is the absence of a large hydration shell in the non aqueous environment with reduced analyte-solvent interactions, pronouncing small differences in Stokes radii of the diastereomers, which would otherwise be masked by the attached water molecules in aqueous solution. The high selectivity is further explained by a different separation mechanism in NACE, where ion-pairing, homo- and heteroconjugation of the analyte or of the analyte-ion-pair complex with the ammonium ion from the BGE is the dominating separation principle [103]. Resolution optimization is thus possible via the ammonium formate concentration. More work regarding the separation principles in NACE is still required to further broaden its applicability.

B) Constitutional isomers: a very good visualization of an easy and fast method development and optimization of the separation of positional isomers of 1-(chlorophenyl)piperazine was presented by Pütz and Martin [10], which is of forensic relevance, as only the 1-(3-chlorophenyl)piperazine is scheduled in most controlled substance acts. As shown in Figure 4, baseline separation of the three positional isomers (1-(2, 3, 4-chlorophenyl)piperazine in CE-MS was achieved simply via adding 10 mmol/L hydroxypropyl-β-cyclodextrin to the run buffer. Cyclodextrins are complexing agents with a bowl-like shape to incorporate small organic compounds. Isomers clearly differing in their three dimensional structure fit into the relatively hydrophobic cyclodextrin cavity to a different extent but also exhibit different numbers and strengths of hydrogen bonds to the cyclodextrins OH group at the rim of the cavity. However, ideally, near-equal signal intensity would be obtained for each isomer when injected at like concentration. As visible from Figure 4, this is clearly not the case. The higher the migration time, which is correlated with a stronger interaction with the neutral cyclodextrin, a distinct quenching effect of the analyte CE-MS signal is visible, the ionization efficiency of the analyte-CD complex is clearly lowered compared to the free, uncomplexed analyte.

With the knowledge of separation principles of several electromigrative separation techniques, it seems logical, that the high separation potential of NACE was also

applied for the separation of positional isomers no longer necessitating the addition of chiral selectors. This was demonstrated by Cherkaoui et al. [131,132] in the manipulation of the separation of the constitutional isomers littorine and hyoscyamine for the analysis of a plant extract. They were able to inverse the migration order of both isomers upon addition of the strong ion-pairing reagent trifluororacetic acid to the BGE, demonstrating that the counter ion plays a significant role in NACE and enables impressive tuning possibilities. They used 25 mM ammonium acetate and 1 M acetic acid (or 1M TFA) in acetonitrile in a 64.5 cm (effective length: 56 cm), 50 µm bare fused silica capillary for the separation of extracts made from *Datura candida* and *Datura aurea.*

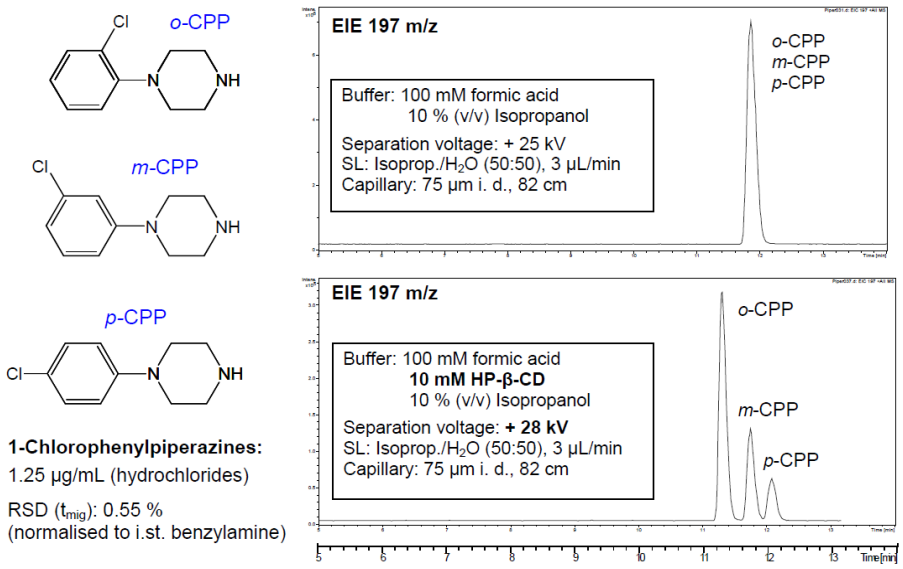


Figure 4: Baseline separation of the three positional isomers of 1-(chlorophenyl)piperazine by CE-ESI-MS utilizing the chiral selector 2-hydroxypropyl-β-cyclodextrin. (with kind permission from the authors [10])

C) Positional and cis-trans isomer separation with MEKC or NACE is possible: good separation of stereoisomers including e.g. cis-trans isomers of internal double bonds

can be achieved in MEKC. Selectivity in MEKC stems from various interactions of analyte and micelle with different dominating interactions at the surface of the micelle (mostly ionic interactions), the inner core (mostly hydrophobic interaction such as London forces, dipoles and induced dipoles) and hydrogen bonding in the interim of the shell, where water is present to a high extent as visualized in Figure 5.

Amphiphilic molecules may be incorporated between the detergent molecules of the micelle taking advantage of both hydrophilic and hydrophobic interactions. The high selectivity for structural isomers stems from steric effects influencing these interactions and differences in dipole moment and polarizability. The good separation is further aided by MEKC's high separation efficiency, which is due to a negligible resistance in mass transfer as the micelles are dynamic entities with life times of 100-1000 ms [133] leading to high plate numbers with longitudinal diffusion as the major source of band broadening. Resolution and peak capacity may be enhanced by an optimization of the micellar vs. the electroosmotic mobility, increasing the migration time window and thus resolution [133]. Good examples for the separation of cis-trans isomers with MEKC can be found in the work of Lucangioli et al. [134] and Huhn et al. [135]. Lucangioli and coworkers analyzed sertraline in a 20 mM sodium borate, 50 mM sodium cholate, 15 mM sulfated-ß-cyclodextrin and 5 mM hydroxypropyl-ß-cyclodextrin buffer (pH 9) in a 60 cm (effective length: 53 cm, i.d.: 50 µm.) bare fused silica capillary. They achieved a low limit of detection (0.2 µg/mL) and good repeatability. The method was also applied to the analysis of the bulk drug containing 2 mg/mL sertraline hydrochloride and was able to determine small (0.1%) impurities like R-(-)-mandelic acid. Comparison of their procedure to an HPLC procedure showed good agreement. The separation of cis- and trans-isoeugenol, together with several other analytes present in sassafras oils, containing safrole as an important precursor chemical for methylenedioxyamphetamine derivatives, was part of the work of Huhn et al. [135]. Separation was performed in 1.875 mM sodium tetraborate, 60 mM SDS buffer with 15% acetonitrile in a 27 cm (20 cm effective length, 50 µm i.d.) bare fused silica capillary.

In NACE, separation of cationic cis-trans isomers results from different formation equilibrium constants with anionic chiral counterions as investigated by Bjørnsdottir et al. [136]. This publication also featured a theoretical model for the separation principle

and is highly recommended as an introduction. Hansen et al. [137] also used NACE for the separation of cis-trans isomers without employing any surfactants, cyclodextrins or complexing agents in the analysis of several drug substances exhibiting isomerism. This publication is also an excellent example for the analysis of minor compounds in the presence of excessive amounts of similar compounds (see Section 1.3.4.2). The separation is presumably based on ion-pair formation likely to be present in the non-aqueous buffer system of acetonitrile, methanol and ammonium acetate.

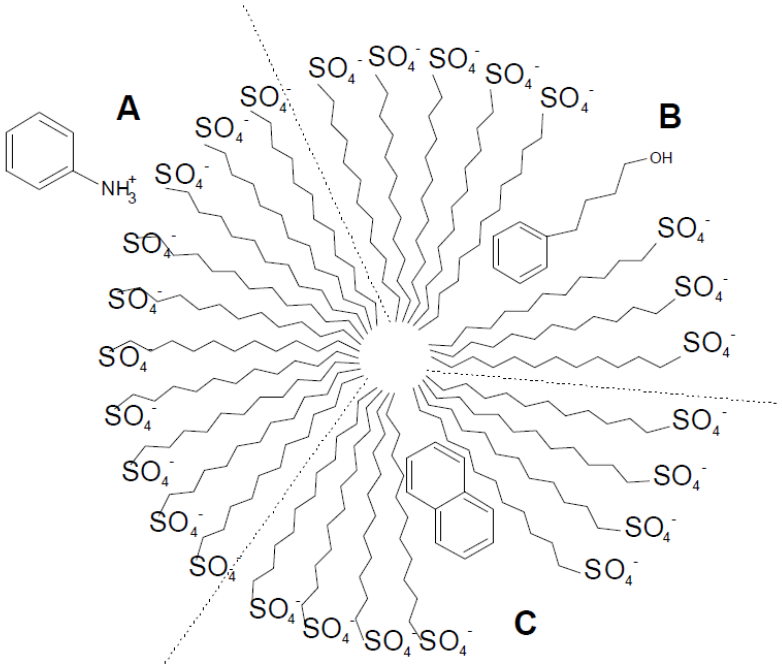


Figure 5: Different allocations of charged, polar and hydrophobic compounds in a dodecyl sulfate micelle. A: ionic interactions at the micelle's outer sphere, B: bridging via hydrogen bonds between the compound and the Stern-Layer, C: hydrophobic interactions in the core of the micelle (taken with kind permission from the author from [123]).

D) Chiral analytes: chiral separation is an important tool for forensic drug profiling, as the relative concentration of the enantiomers can provide information on the applied synthesis pathway, the precursor substance and the origin of a sample which is

especially relevant for profiling of clandestinely produced methamphetamine ("crystal meth") [138]. Therefore chiral analysis forms a link between drug batches, production sites, precursor chemical supply chains and distribution networks among others [139,140]. In addition, chiral separation is vital, e.g. if one enantiomer has health beneficial properties, while the other one might cause severe health impacts (e.g. Thalidomide), or if the potency of enantiomers differs significantly (as e.g. for (levo-)methadone and many amphetamine derivatives). As the identification and differentiation of enantiomers cannot be performed by mass spectrometry or UV-spectra alone, comparison of the migration time with a standard in combination with standard addition is usually employed. Consequently, this demands highly reproducible and stable migration times of the analytes, a feature where CE is mistakenly still recognized to perform badly. Several excellent reviews with a focus on enantioselective capillary electrophoresis can be found [141-144] and are recommended to the interested reader. A large number of chiral selectors have been developed, though cyclodextrins are still the most popular chiral additives [145]. The great advantage of chiral CE over other chiral separation methods, especially chromatography, is the ease of method development. This is facilitated as, in contrast to chromatography, the chiral selector and the analyte are in the same phase, that is, both are dissolved in the BGE. As can be deduced from Equation 1, the separation is thus based both on the differences in complexation constants as well as on the differences in the effective electrophoretic mobilities of the evolving diastereomeric complexes [146] (whereas the velocity (and thus the velocity difference) of the diastereomeric complexes in LC is always zero). For a very detailed study on the influence of the nature, the structure and the pH-dependence of complexation equilibria of analytes with cyclodextrins, the interested reader is referred to the work of the Scriba group [146-152].

$$\mu_{\text{eff}} = \frac{\mu_f + \mu_c K[CD]}{1 + K[CD]}$$

Equation 1 with μ_{eff} being the overall effective electrophoretic mobility, μ_f being the effective electrophoretic mobility of the free (uncomplexed) analytes, μ_c being the effective electrophoretic mobility of the cyclodextrin-analyte complex, K being the complexation constant and [CD] being the molar concentration of the cyclodextrins (from Sabbah et al. 2001 [146])

From Equation 1 it is possible to understand the optimization possibilities for chiral CE: Beside the type of the chiral selector and thus the complexation constant and the mobility of the diastereomeric complexes, the concentration of the chiral selector can be optimized to enhance chiral resolution. Especially in case of oppositely charged ligands and analytes, very high resolution can be obtained up to cases where the two stereoisomers migrate in opposite direction as was shown for methadone, venlafaxine, tramadol and fluoxetine separations with highly sulfated cyclodextrins [153] achieving infinite resolution. In many cases also mixtures of chiral selectors have been employed, especially when the analytes largely differed in size and in the number of possible hydrogen bonds to be established with the chiral selector.

Another yet often underestimated advantage of chiral CE is the ease of changing the migration order of enantiomers in order to facilitate quantification of the enantiomer present in traces, for example by reversal of the electroosmotic flow (EOF), type and concentration of the chiral selector, pH value, temperature, etc. In chiral LC, a different chiral column has to be employed as the optical antipodes of the stationary phase are mostly not available.

The work by Iwamuro et al. [154] is a powerful demonstration of how to create robust analytical CE-UV methods for the chiral analysis of methamphetamine and its related compounds by employing a capillary with a modified surface, exhibiting diol groups on the inner surface of the capillary. They separated 18 enantiomers within 9 min, using a 125 mM Tris-125 mM sodium dihydrogen phosphate buffer (pH 6.15) containing 6 mM dimethyl-β-CD and 12 mM β-CD as chiral selectors. RSDs of migration times for standards were 0.09% but 0.14% for crude urine samples due to matrix effects.

Comparable stability for migration times (RSD < 0.04% after correction with internal standards) was achieved for a CE-MS method for the analysis of amphetamine, methamphetamine, p-hydroxymethamphetamine and dimethylamphetamine by Iio et al. [155] using a mixture of 3 mM β-cyclodextrin and 10 mM dimethyl-β-cyclodextrin at acidic pH (pH 2.2) for the separation. With this low pH, only a very slow EOF is induced and the authors thus largely prevented the neutral cyclodextrins to enter the mass spectrometer, where it would have lead to severe ion suppression and thus increased

detection limits (compare Figure 4). However, in a later modification of the method [156] the authors switched to the negatively charged heptakis(2,6-diacetyl-6-sulfato)-ß-cyclodextrins in 1 M formic acid (pH 1.8) to further reduce ion source contamination with the cyclodextrins migrating to the inlet. A major further improvement in terms of ease and applicability enabled the direct injection of urine samples after a simple 0.45 µm filtration step, while maintaining high repeatability (RSDs between runs: 0.3% (migration time) and 5.3% (peak area)) [157].

Although the work of Meng et al. [158] is more focused on the development and evaluation of a liquid-liquid microextraction technique for sample preparation, the achieved chiral baseline separation of methamphetamine, 3,4-methylenedioxymethamphetamine and ketamine is worth to be mentioned here, especially since the achieved limits of detection (0.05 µg/L for diacetylmorphine, 0.2 µg/L for methamphetamine based on S/N = 3) are exceptionally low and linearity was achieved from 0.15-6500 µg/L using standard UV-detection. The authors used a simple potassium dihydrogen phosphate/phosphoric acid buffer (pH 3.2) with 20 mM β-cyclodextrin as chiral selector. In terms of fast chiral separations, Lurie et al.'s work [73] has to be cited here again (see Section 0), as they achieved baseline separation of six racemic beta-phenethylamines and d,l-propoxyphene within 4.5 min using a 32 cm long capillary in a CE equipped with a diode array detection system (Figure 6).

With regard to new psychoactive substances present in so-called "legal highs" such as "bath salt" products marketed via internet shops, the work of Mohr et al. [159] shows the separation of 19 cathinone derivatives used as substitutes for the banned mephedrone using a 50 mM ammonium acetate buffer (pH 4.5) with 10% acetonitrile and 20 mM sulfated-β-cyclodextrins in a CE-DAD system. Separations of enantiomers up to 3 min from peak-to-peak within a total run time of 20 min were achieved. Iwata et al. [160] combined a chiral capillary electrophoresis method with mass spectrometric detection for the analysis of amphetamine-type-stimulants using 2.5 mM highly sulfated cyclodextrins as chiral selectors and electrolyte, although it is very likely that formate ions from the sheath-liquid entered the capillary and acted as additional BGE ions. The achieved resolution with more than 20 min between d-ephedrine and l-ephedrine and more than 10 min between d- and l-amphetamine makes the method worth being cited

here, although the high resolution was at the expense of a very high total analysis time of 55 min.

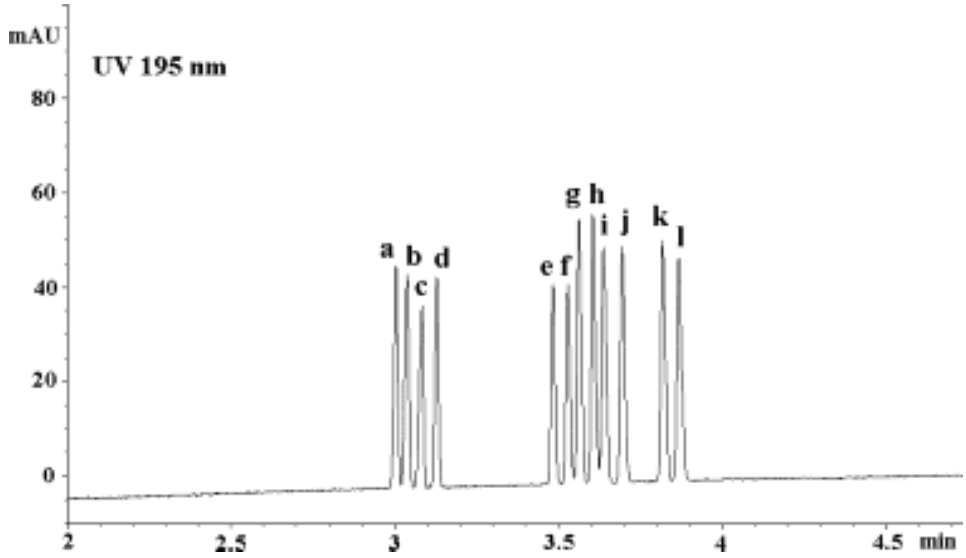


Figure 6: Electropherogram of a standard mixture of (a) l-amphetamine, (b) d-amphetamine, (c) l-methamphetamine, (d) d-methamphetamine, (e) l- or d-n-butylamphetamine, (f) l- or d-n-butylamphetamine, (g) l- or d-MDA, (h) l- or d-MDA, (i) l- or d-MDMA, (j) l- or d-MDMA, (k) l- or d-MDEA, (l) l- or d-MDEA (with permission from WILEY-VCH ([73]))

It has to be noted, however, that chiral identification and identification in general only by migration time and spiking experiments is risky, as the work of Dieckmann et al. [161] demonstrates. To increase the reliability, they combined chiral CE with MS, employing different mixtures of several semi to non-volatile chiral selectors, either charged negatively (1 mM 2,3-diacetyl-6-sulfato-β-cyclodextrins) or neutrally (10 mM 2-hydroxypropyl-β-cyclodextrins), while reducing the electroosmotic flow to nearly zero (BGE was 1 M formic acid, pH ~ 1.9). With this experimental setup, they demonstrated that false positive results may be obtained with UV detection only (see Figure 7): One peak, labeled as (+)-amphetamine in a seizure of an illicit methamphetamine sample showed perfect comigration with reference (+)-amphetamine. However, the absence of (+)-amphetamine was proven by the implementation of the CE-MS method and instead the peak was identified and verified by MS/MS as procaine, a local anesthetic.

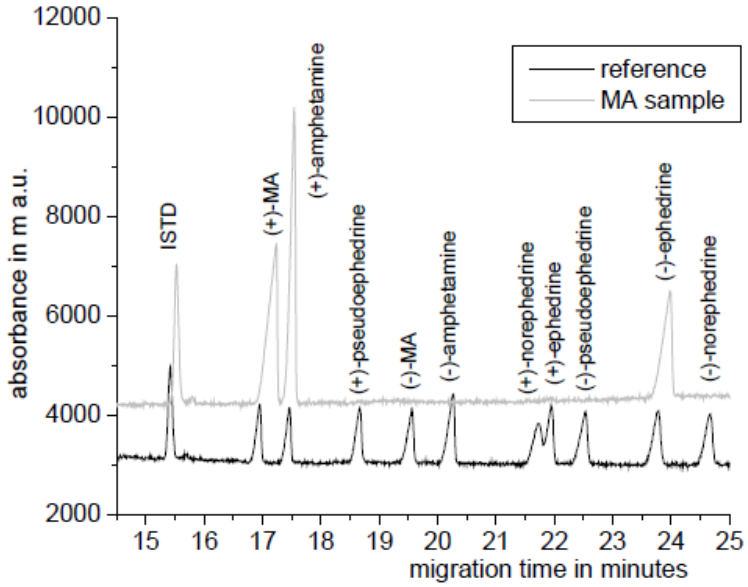


Figure 7: Chiral CE analysis of an illicit methamphetamine sample (MA sample) using CE with UV-detection in comparison to a standard mixture. The peaks are labeled according to the reference standard. Note the perfect co-migration of the peak above the (+)-amphetamine, which was first misinterpreted as (+)-amphetamine, but later identified as procaine by CE-MS. (Figure from [161] with kind permission from the authors)

1.3.4. TOLERANCE TOWARDS SAMPLE COMPONENTS

1.3.4.1. MATRIX TOLERANCE

A striking argument for the implementation of CE in the forensic laboratory is its tolerance towards high matrix load. As can be seen Table 2, summarizing the methods of this review, often only a dilution step is necessary as sample preparation for liquid samples or methanolic extracts. In some cases, additional solid phase extraction or liquid-liquid extraction is necessary, most often, because the sample exhibits a high salt content e.g. in urine samples. The high tolerance towards matrix effects is a result of the very small injected sample volume (a few nL) and of the robustness of the fused

silica capillary as separation column towards aggressive chemicals. Also, only charged matrix compounds with a similar effective electrophoretic mobility in the BGE can interfere with the analyte, neutral compounds remain in the sample plug and are carried towards the outlet without disturbing the separation window, ions with opposite charge are well separated. However, matrix compounds which interact with the capillary surface are problematic for CE as they consistently influence the electroosmotic flow velocity and may accumulate on the capillary wall, if no coatings are applied or adequate flushing procedures after the run are performed. Here, it is of advantage, that the fused silica capillaries can withstand very harsh conditions, allowing flushing with concentrated NaOH or strong acids to remove adsorbed material between runs. Even if in rare cases a capillary is irreversibly contaminated, the exchange by a new capillary is a matter of less than half an hour and by far less expensive (less than 10 EUR per meter fused silica capillary if replaced manually) than the replacement of a separation column in HPLC or UHPLC systems.

A high level of selectivity can already be achieved by the choice of the separation mode, e.g. by excluding all non-charged compounds when using CE instead of MEKC. Additional selectivity can be introduced by the type of the BGE and its pH value, largely influencing the degree of dissociation of the analytes vs. matrix components e.g. having only a few charged at a distinct pH-value. The work of Pütz & Martin [10] on the analysis of sulfonated food colorants at very low pH (see Section 1.3.3.1) is a good example for this approach. Capillary coatings can further be used for the alteration of the EOF, making it possible to exclude slowly migrating analytes in a counter-EOF separation. However, the high tolerance is not without expenses, as high matrix load usually necessitates more intensive and longer rinsing steps for the capillary, coatings might be damaged and require re-coating steps, as well as buffer vials have to be exchanged or replenished more often. Usually, an experienced operator can reduce such effects to a minimum: a good example for a chiral CE method with high matrix tolerance using mass spectrometry for identification was presented by Cherkaoui et al. [162]: they investigated the feasibility of using the partial filling technique (PFT) in combination with mixed charged cyclodextrins to achieve chiral separation and mass spectrometric detection without interference from the chiral selectors. In PFT, a

solution of a chiral selector is injected as a plug into the capillary before the sample plug itself is injected. When the electric field is applied, the chiral selector countermigrates to the inlet, away from the mass spectrometric detection system. The chiral separation occurs when the analytes pass the chiral selector solution, upon depletion of the selector in the capillary, only zone electrophoresis is present. This leads to an undisturbed electrospray with good ionization properties while maintaining the chiral resolution. Serum after a simple liquid-liquid extraction was the matrix for the injection solution in the analysis of methadone, amphetamines, venlafaxine and metabolites and tropane alkaloids. The method was only validated for (-) and (+)-hyoscyamine, but exhibited excellent precision in relative migration times and relative peak areas, which is not trivial for the partial filling technique. Another impressive approach with high matrix tolerance as well as the capability to cope with the presence of excessive amounts of sample components was presented by Iwata et al. [138]. Here, the analytes showed absolutely no interaction with the capillary wall. The absence of tailing proved that no overlapping with closely migrating analytes was present. Impurities in methamphetamine seizures (concentration: 20 mg/mL methamphetamine-hydrochloride) were tentatively identified and quantified without sample pretreatment using a CE-UV method after dissolution of the tablet in water. This enabled the rough classification of methamphetamine seizures in Japan into three groups based on the ratio of (-) ephedrine to (+)-pseudoephedrine which are the most important precursor chemicals for the clandestine production of (+)-methamphetamine.

1.3.4.2. PRESENCE OF COMPOUNDS IN EXCESS

One of the easiest approaches to determine a minor compound in presence of a closely migrating compound present in excessive amounts is of course to detect the minor one first. This is especially true for enantiomeric separations or impurity detection. However, this demands a control of the migration order, which needs in-depth knowledge on the separation principle and how to influence complexation equilibria in case chiral selectors are used. In 1995 Schmitt & Engelhardt [163] named three different approaches to obtain a reversal of migration order in CE experiments (see also Section 0), 1) reversal of the EOF, 2) using cyclodextrins, and 3) using charged cyclodextrins, whose charge depends on the pH of the BGE. They determined

impurities from (+)-ephedrine in (-)-ephedrine, with a BGE containing 2% (w/v) carboxymethylated-cyclodextrins in a 20 mM citric acid buffer at pH 2.5: in their conclusion, they stated that the use of differently charged cyclodextrins was the most elegant method for the reversal of the migration order. This list of migration time reversal is still true and can further be extended to 4) the change of the concentration of the chiral selector and 5) the effective electrophoretic mobility of the chiral selector (e.g. via pH, organic solvents), 6) the binding strength between chiral selector and enantiomer (e.g. via organic solvent additives), 7) induction of additional separation mechanisms (e.g. cyclodextrin-modified MEKC).

Examples for excess compounds in non-chiral analysis are manifold: Jung et al. [164] developed and validated a CE method using indirect-UV detection for the analysis of ethyl glucuronide (EtG), a biomarker to document alcohol abstinence, in serum after a simple protein precipitation step. Consequently, enormous amounts of chloride and formate were present in the sample solution. However uninfluenced detection was possible with an LOD of only 0.25 mg/L in only 15 min run time (see Section 1.3.6). Caslavska et al. [165] presented the confirmation of these experiments via a CE-MS/MS coupling.

Huhn et al. [127] used MEKC coupled to UV and (UV)-LIF detection for the analysis of allylbenzenes in essential oils, which are used as precursors for the clandestine synthesis of amphetamine derivatives that are prevalent in Ecstasy tablets. The method was capable of determining minor components, structurally similar to the main compound present in a molar ratio of 1000:1 using only dilution with methanol and a surfactant as sample preparation (see Figure 8). High inter-capillary repeatability was found despite this large excess using a mobility axis (see also Section1.3.10).

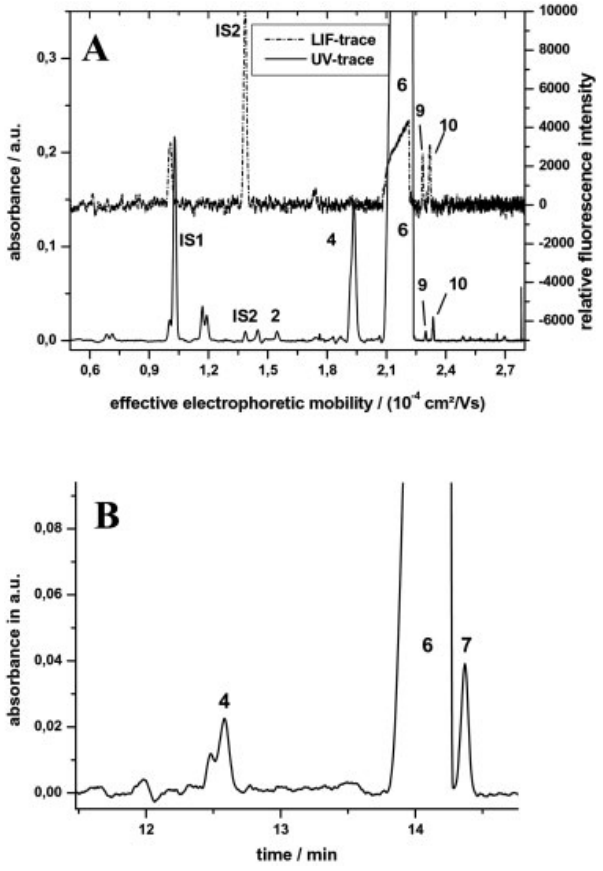


Figure 8: Electropherograms of the analysis of Sassafras albidum oil in different dilutions of the oil in a surfactant/methanol mixture. (A) 1:100 dilution, (B) 1:1000 dilution. The analysis was performed using a separation buffer composed of 60 mM SDS, 7.5 mM sodium tetraborate, 4 M urea and 0.5 mM $CaCl_2$ at a pH value of 9.2 with 20% acetonitrile. Peak identification: (IS1) methyl-4-cyanobenzoate, (IS2) acridone, (2) eugenol, (4) methyleugenol, (6) safrole, (7) myristicin, (9) cis-/trans-isosafrole, (10) anethol. (With permission from: [127])

Several prominent forensic applications are based on the determination of inorganic and small organic anions and cations present in traces besides one excess ion or in complex environmental matrices. An increasingly important application is the determination of typical inorganic cations (e.g. ammonium, potassium, sodium,

calcium, magnesium and strontium) and anions (especially nitrate, chlorate, perchlorate and azide) related to improvised explosives in post-blast residues. Capillary electrophoresis is highly suitable for this application because of its potential for field-suitable analysis directly at the scene of crime using portable systems with capacitively coupled contactless conductivity detection.

In the field of pharmaceutical and illicit drug analysis the determination of the salt form of the main active substance is of general interest as counterfeit pharmaceuticals sometimes contain the active substance in a different salt form compared to the original product. In the case of illicit drugs, the salt form composition can give information about the clandestine manufacturing process, e.g. the presence of methylsulfate in amphetamine sulfate. Figure 9 shows an example of inorganic and small organic ion analysis in a “Mitsubishi” Ecstasy tablet with two indirect photometric CE procedures (study of Pütz et al. as a part of the EU project CHAMP-‘‘Collaborative Harmonisation of Methods for Profiling of Amphetamine Type Stimulants”, 6^{th} framework program of the EC, contract no. 502126). Anion analysis reveals the presence of significant amounts of chloride and sulfate. To ascertain the actual salt form of the main active substance, 3,4-methylenedioxymethamphetamine, an additional cation profile was assessed, showing an unexpectedly large excess of Mg^{2+} as well as small amounts of Ca^{2+}, Na^{+}, NH_4^{+} and the presence of methylammonium as organic trace ion (indicating that a reductive amination synthesis procedure with methyl amine as reactant was performed). Quantification of anions and cations confirmed the salt form of MDMA to be hydrochloride and magnesium sulfate as an unusual cutting agent in the examined Ecstasy tablet.

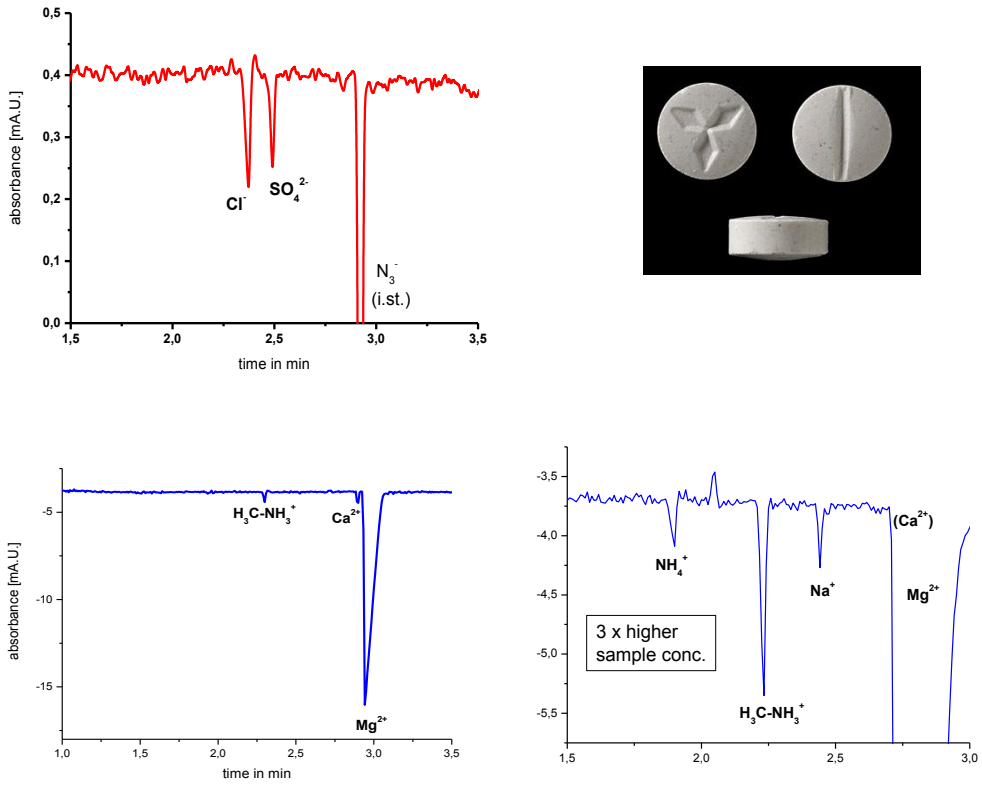


Figure 9: Salt form determination and trace analysis of small anions and cations in an Ecstasy tablet by capillary electrophoresis with indirect photometric detection. Anion analysis: BGE: 2.25 mmol/l sodium hydroxide, 6.5 mmol/l pyromellitic acid (probe ion), 1.6 mmol/l triethanolamine, 0.75 mmol/l hexamethonium hydroxide solution, adjusted to pH 7.7 with NaOH; bare fused silica capillary (i.d.: 50 µm, lengths: 47/40 cm; -28.0 kV, sample injection: 35 mbar/0.1min; UV detection at 254 nm, i.st. sodium azide. Cation analysis: BGE: 10 mmol/l imidazole, 2 mmol/l crown ether 18-crown-6, adjusted to pH 4.0 with sulphuric acid; 50 µm i.d. bare fused silica capillary (57/50 cm); HV:+30.0 kV, sample injection: 35 mbar/0.1min; UV detection at 214 nm.

The assessment of inorganic cation and anion profiles can also be used as a fingerprint to establish links between different seizures of drugs. Figure 10 illustrates such an application for the batch-to-batch comparison of four different types of Ecstasy tablets from seizures in Finland based on CE cation profiles. The relative peak areas

for the four detected ions (K^+, Na^+, Ca^{2+}, Mg^{2+}) are similar for all four tablets and the presence of significant amounts of potassium is unusual. As additionally proven by organic impurity profiling via GC/MS, all tablets originated from the same clandestine tableting lab and the quite uncommon cutting agent gelatin was the cause of the characteristic cation profile.

Another important forensic-toxicological application of cation and anion profiles is the evidence for or the exclusion of a manipulation of foodstuff, especially beverages that are suspected to have been poisoned. In this case the ion profiles of the questioned product and a separately acquired original product (as reference) are directly compared in consecutive CE runs.

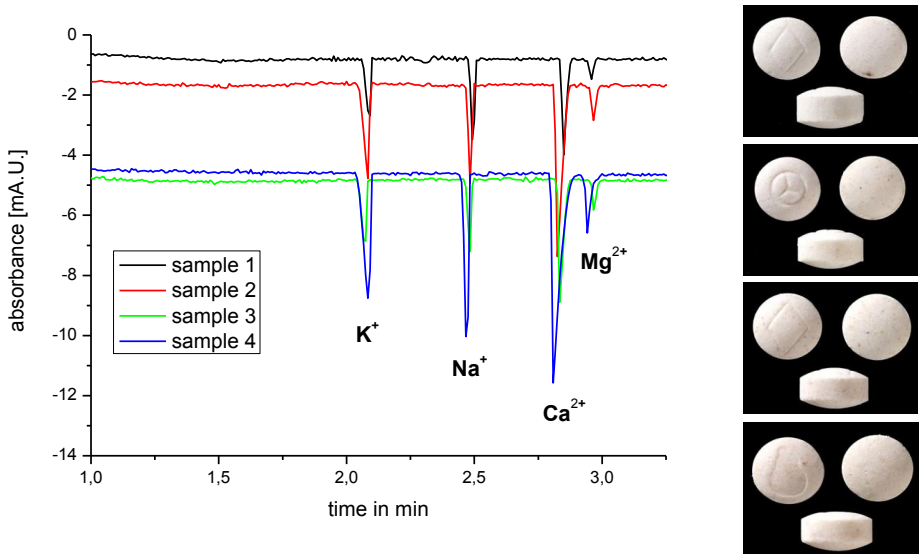


Figure 10: Cation profiles of four Ecstasy tablets with different logo´s via CE with indirect photometric detection (details of procedure, see Figure 9).

1.3.5. FAST METHOD DEVELOPMENT AND OPTIMIZATION STRATEGIES

The development and validation of analytical methods is often very time consuming, labor-intensive and, not to forget, chemical/solvent consuming and therefore expensive. Police seizures of items of evidence that are submitted to forensic analysis often consist of a large variety of different products/materials and come along with a large amount of side-compounds, especially designer drugs, totally different matrices, sometimes with the need for chiral separation. The high separation efficiency (high plate numbers) and high selectivity of electromigrative separation techniques as well as the small volumes needed and the fact that nearly all types of separation-influencing compounds can simply be added to the running buffer, make electromigrative separation techniques very fast, easy and cost effective to optimize [23,24]. The number of possible optimization strategies in capillary electrophoresis is impressive, allowing the operator to quickly tune the different influencing factors of a separation (selectivity, resolution, efficiency) e.g. by differently functionalized capillary surfaces, coatings, complexing agents, surfactants, chiral selectors, pH value, induction of ion-pair formation via addition of organic modifiers, etc. However, mostly several if not all parameters such as resolution, speed, selectivity, efficiency will be simultaneously altered due to changes of equilibria the analyte is involved in (protonation, complexation, ion-pairing, chromatographic interaction) and electroosmotic mobilities.

An interesting example for a method optimization using a one-factor-at-time approach can be found in the work of Iwata et al. from 2002 [166], which also includes a descriptive figure and discussions about the induced separation principles and effects by highly sulfated-γ-cyclodextrins on the chiral analysis of 9 chiral amphetamine type stimulants. They used a short capillary (32 cm) and a high ionic strength buffer (50 mM phosphate, 10 mM highly sulfated-γ-cyclodextrins, pH 2.6), inducing a counter-electroosmotic separation by applying negative polarity. Their method development yielded electropherograms with well resolved analyte peaks (several minutes between enantiomer pairs) and robust results with regard to migration times and peak areas. However, the achieved separation time of 32 min seems to be unnecessarily long, as they only used -12 kV separation voltage (deviation from Ohm's law at higher voltages

and thus effects of excessive Joule heating with resulting peak broadening). Reducing the BGE concentration and/or the use of smaller inner diameter capillaries and capillary cooling would have allowed for much faster separations.

Fast method development does not always lead to a globally optimized method; instead systematic experimental design strategies have to be applied and carried out in a randomized set-up to prevent any systematic errors. Design of experiment strategies are well suited for CE as most parameters of the separation technique can easily be varied. Especially, changing buffers can be realized in CE in a short period of time (approx. 90 s for flushing the capillary), as no pump or column have to be purged or conditioned as e.g. in LC. Therefore, a high number of chemometrically optimized analytical methods can be found in literature.

A good and recent example for such a fast and efficient method development with multivariate data analysis including a comparison to univariatly optimized methods is the work of Ho et al. [167]: They presented the application of a statistical tool using both a Plackett-Burman and a central composite design for the optimization of six parameters for CE-UV method with preconcentration (large volume sample stacking-sweeping) for the analysis of ten common illegal drug substances in urine. The results were compared to a method developed according to the classical one-factor-at-a-time approach. The method optimized by the statistical tools was shown to be superior to the conventional approach in terms of resolution and speed of analysis, providing also a wider linear range for concentration (25-1500 ng/mL for six benzodiazepines, methamphetamine and ketamine and 50-3000 ng/mL for codeine and morphine). Additionally, the method development was found to be more efficient and less time consuming. The final conditions for the statistically optimized system were: 50 mM NaH_2PO_4 buffer with 29% of methanol for the separation (pH 2.3), 10 mM NaH_2PO_4 added to the sample matrix and 150 mM SDS added to sample. Another highly recommended publication in terms of method development is the work of Dahlén & von Eckardstein [168], using an experimental design and simplex optimization in an easy to follow and well explained way. The separation of amphetamine, amphetamine derivatives, cocaine and heroin was optimized (Figure 11). They also described potential alternatives to their method, e.g. to speed it up when a lower resolution of the

analytes is sufficient. The publication includes the validation of the method, as well as the application to street samples. High linearity and low RSD's for peak areas and migration times were demonstrated.

Another well presented method development, chemometric investigation and optimization was performed by Varesio et al [169] for the analysis of amphetamines. By optimizing the analysis time (including shortening of the capillary) while maintaining baseline resolution, they were able to accelerate their separation from a run time of 8 min to a total run time of 1 min only conducting 31 experiments for a full-factorial and a face centered central composite design.

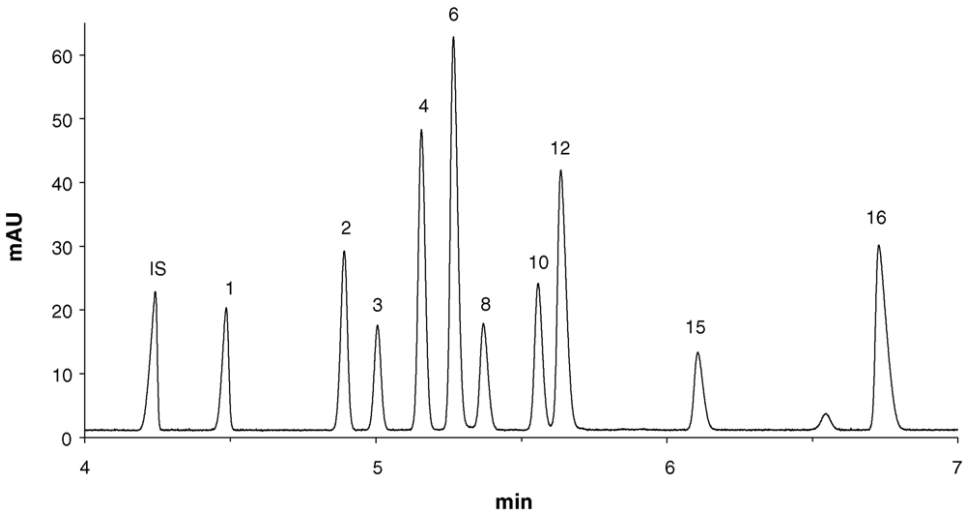


Figure 11: Analysis of a standard mixture of compounds of forensic interest analyzed using a BGE composed of 30 mM Tris/phosphate, pH 2.8 in a 45 cm capillary (eff. length: 37 cm, i.d: 50 µm). (i.st) benzylamine, (1) phenethylamine, (2) amphetamine, (3) methamphetamine, (4) 3,4-methylenedioxyamphetamine, (6) 3,4-methylenedioxymethamphetamine (8) ephedrine, (10) N-methyl-1-(1,3-benzodioxol-5-yl)-2-butamine, (12) 3,4-methylenedioxyethylamphetamine, (15) cocaine, (16) heroin. (adapted from [168] with permission from Elsevier)

1.3.6. COST-EFFICIENCY AND GREEN METHODS

When it comes to the implementation of techniques into routine analysis, it has to be assured that these techniques are validated, sensitive, cover a wide concentration range of the analytes and are cost efficient. The latter is the reason why many excellent methods utilizing e.g. LC-HRMS/MS or GC/GC-MS are not fit for routine analysis, although their intrinsic instrumental properties are excellently suited for the purpose. Additionally one should bear in mind, that developing countries might lack the financial resources to implement expensive high-end equipment, which is not always needed for the analytical task. Also, pure reagents, gases (like pure hydrogen or helium for GC) or even pure water might not always be available. Usually simple GC-MS would be suitable for most of the applications, but what if even a stable electrical supply might not always be present, being potentially harmful for vacuum pumps. Electromigrative separation techniques are absolutely low cost techniques with regard to their maintenance and working costs [170], due to the low consumption of chemicals, only needing typical household electricity, low demands towards chemical purity and water purity. All this makes CE the ideal separation technique for developing countries. In this subchapter we will present some applications, where CE can be employed as low cost alternative.

For the analysis and detection of non UV-active ethyl glucuronide (EtG) a biomarker for recent alcohol consumption, often used as diagnostic tool for the screening of blood samples for chronic alcohol abuse, GC-MS or LC-MS(/MS) have been successfully employed [171]. However, while LC-MS instruments can still be considered expensive, there are cheap benchtop GC-MS instruments available. Nevertheless GC methods usually need derivatization (silylation or perfluoropropionylation) for the analysis of EtG. In contrast to the numerous publications for the analysis EtG by LC-MS, Jung et al [164] developed CE-methods for the analysis of EtG using indirect UV-detection (probe ion: nicotinic acid) [172,173]. An alternative method based on hyphenated ITP/CE was published later on [174]. The latest of the methods [164] used commercially available linear polyacrylamide coated 75 µm capillaries, reaching an LOD of 0.25 mg/L and an LOQ of 0.5 mg/L for EtG after a simple protein precipitation with 200 µL acetonitrile per 100 µL serum as sample preparation (Figure 12). Although SPE was successfully

tested, it was not implemented since it did not significantly improve the results while being more laborious and expensive. The applied BGE was composed of 10 mM nicotinic acid, adjusted to pH 4.65 with ε-amino caproic acid (approx. 11 mM) in a water/acetonitrile mixture (9:1). The method was cross-validated with an enzyme immunoassay, which turned out to be more sensitive, but was also more laborious and required ultrafiltration for sample pre-treatment. In summary, the developed CE method proved to be fast, fit for routine analysis and was shown to be robust in terms of precision and accuracy. Compared to the immunoassay, the consumption of chemicals and thus the costs were negligible. However, the authors recommended that questionable results should be confirmed by a more sensitive LC-MS method to avoid false positives.

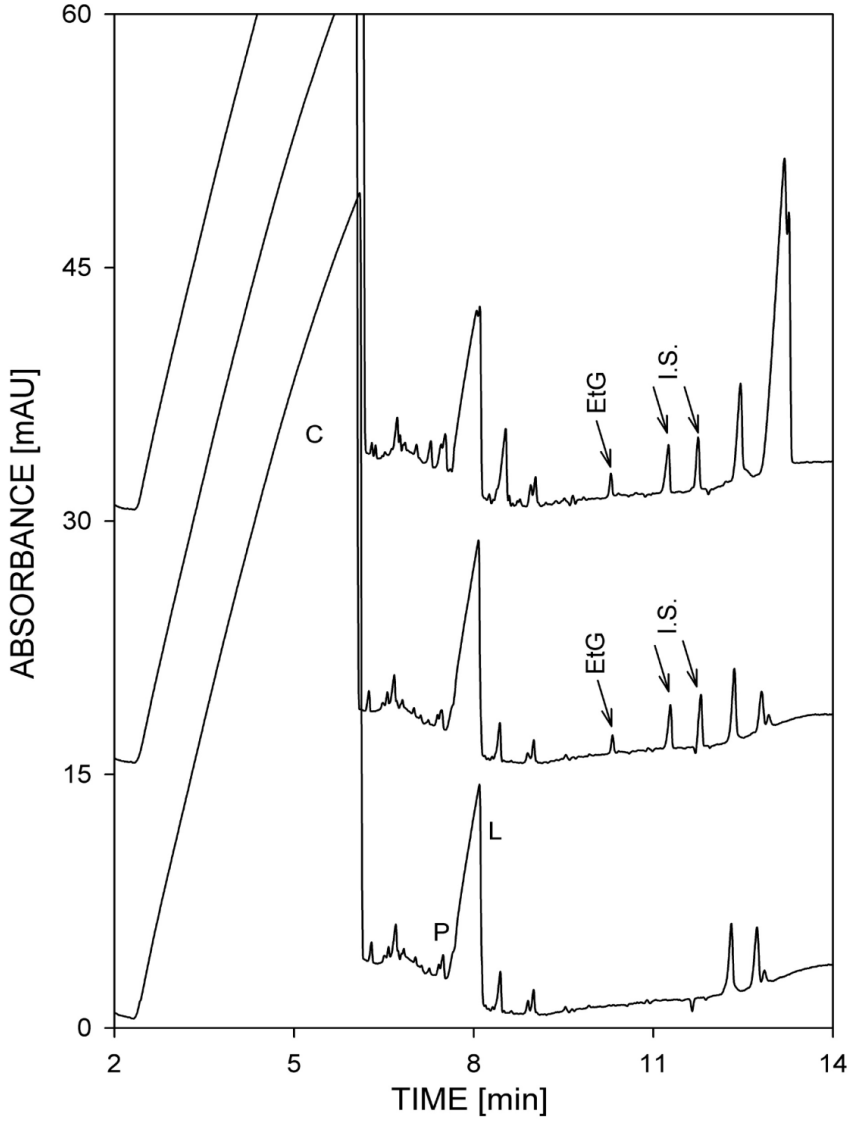


Figure 12: Electropherogram of sera after protein precipitation. From bottom to top: blank serum, blank serum spiked with 4 mg/L ethylglucuronide and 20 mg/L internal standard and a real sample (determined ethylglucuronide concentration 4.72 mg/L, internal standard 20 mg/L). Analysis performed using a linear polyacrylamide coated capillary (inner diameter: 75 µm, length: 60 cm) with 10 mM nicotinic acid, EACA and 10% acetonitrile as BGE at -30 kV (with permission from [164]).

1.3.7. VERY SMALL SAMPLE VOLUMES

The use of capillary electrophoresis as separation technique leads to the lowest absolute limits of detection of all routine separation techniques using the same detection strategy. Amounts of only a few fg on-column can still be detected and analyzed. The achievable limits of detection in terms of concentration are comparable to HPLC methods, if detection techniques like MS or LIF are used instead of classical UV-detection, achieving only relatively high limits of detection due to the small optical path length of mostly 50 or 75 µm (Lambert-Beer's law). The work of Rittgen et al. [175] is a very good example of a highly repeatable analysis at extremely low analyte concentration in small sample volumes: they developed an NACE-MS method for the trace analysis of fentanyl derivates in seizures, in heroin (where it was used as substitute or adulterant to heroin to enhance the potency) and from extracts of clothes contaminated with nebulized carfentanil. Although the size of the seizures were only 21 mg, 6.14 mg or 3.21 mg of heroin, partially containing only 0.3 ng fentanyl per mg (0.3 ppm), unambiguous identification and quantification was possible by NACE-MS, corroborated by isotope dilution analysis. Also, cis– and trans–methylfentanyl could be separated and identified. The applied buffer system was a mixture of 200 mM ammonium acetate in glacial acetic acid with acetonitrile (1:9) in a bare fused silica capillary. RSD values for migration times were below 0.07% after correction with two internal standards and linearity was demonstrated in the range from 2.53 nM to 6.33 µM for carfentanil.

Another remarkable example for the analysis of small amounts of sample is the work of Tagliaro et al. in 1998 [176], who needed only 50-100 mg (about 4 cm) of hair for a precise analysis of morphine, cocaine and MDMA, achieving detection limits as low as 2-8 ng/mL (S/N = 5), representing 0.01 ng/mg sample (10 ppb). The high sensitivity was based on a head-column field-amplified sample stacking procedure [177,178], where analytes are preconcentrated via electrokinetic injection through a plug of 0.1 mM solution of diluted acid (here: phosphoric acid). Separation was accomplished in a 100 mM phosphate buffer (pH 2.5). The method was also characterized by a good linearity (0.025-0.5 ng/mg) and repeatability when internal standards and internal calibration were used. In 2007, the hair analysis using field amplified sample stacking

was transferred to a CE-MS hyphenation [179], thus adding a verification step by MS/MS experiments, while achieving similar values in terms of LOD and LOQ. The high versatility of CE combined with head-column field amplified sample stacking can also be seen in the work of Wey et al. [180] in the analysis of opioids in bodyfluids (human plasma, serum and urine) using only 20-100 µL sample volume in total. The article also presents a readily understandable introduction to head-column field amplified sample stacking and sums up the key factors and conditions for high repeatability and highly efficient online-preconcentration as well as sample preparation. Standards could be separated within 9 min in a 41 cm (effective length: 22 cm, i.d.: 50 µm) capillary with phosphate (75 mM Na_2HPO_4 and 25 mM NaH_2PO_4) in an ethylene glycol-water mixture (6:4 v/v) (pH 7.9). It was possible to detect opioids at a few ng/mL, however, the obtained results were strongly dependent on the pH value in the extraction procedure, limiting the method to the analysis of closely related analytes and only closely related opioids could be extracted at once.

1.3.8. IMMUNOASSAYS AND IMMUNOAFFINITY CAPILLARY ELECTROPHORESIS

Immunoassays are usually very easy to use and fast, but also prone to cross-reactivity and high limits of detection. By transferring the assay to a capillary electrophoresis system, these weaknesses can be overcome via the introduction of a separation step to gain additional selectivity and detect the enzyme and the product separately. CE provides the additional advantage of the absence of a stationary phase, possibly interfering with ligand binding. Furthermore, the implementation of different detection techniques enables low limits of detection and additional selectivity. Immunoaffinity capillary electrophoresis is currently regarded as an almost ideal enrichment tool for very low concentrated analytes in complex biomatrices, like blood, serum or urine [181].

An early, thoroughly executed investigation of a CE-based immunoassay from 1998 can be found in the work of Thormann et al. [182]. They investigated and compared the feasibility of a micellar electrokinetic capillary chromatography based immunoassay

(buffer: 6 mM sodium tetraborate,10 mM disodium hydrogenphosphate and 75 mM SDS, pH ~ 4.6) and a CE-based immunoassay (buffer: 50 mM sodium tetraborate) to two previously developed CE-UV methods and a CE-MS method [42,183] for the unambiguous identification of methadone and its metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine in urine samples. Both novel electrokinetic capillary immunoassay approaches [42,183] were indeed capable of lowering the limit of detection to 10 ng/mL without exhaustive sample pretreatment or enrichment and were judged as being suitable for screening purposes. The capillary zone electrophoresis immunoassay approach [31] added selectivity as it separated the free tracer from the tracer-antibody complex, which comigrated in case of the micellar electrokinetic capillary chromatography immunoassay. For the confirmation of their findings the authors developed a CE-QqQ-MS method using a simple ammonium acetate buffer (20 mM ammonium acetate, 20 mM acetic acid, pH 4.6) and cross-validated it with an LC-MS approach. Even earlier than Thormann, in 1992, Bao & Regnier [184] transferred a classical immunoassay into a CE format. They combined electrophoretic mixing with the separation of the substrate, the enzyme and the product. With the high resolution achieved, they obtained limits of detection as low as 46 attomol of glucose-6-phosphate dehydrogenase, which was used as model analyte. Later, in 2000, Wey et al. [185] introduced an MEKC- and a CE-based immunoassay for the analysis of opiates and metabolites thereof in urine, both being capable of recognizing opiates in urine (10 ng/mL), but not able to discriminate between different opiates. This was only achieved by a third CE method with MS detection. The importance of separation techniques for immunoassays was shown during the MEKC-based analysis, as the authors found out, that the commercial fluorescence tracer was actually composed of two different tracers, which reacted competitively. Their final method was as sensitive as the immunoassay, for most compounds, but an increase in selectivity by a factor of four was found. Nevertheless, the method could only prove the presence of opioids or codenoids, no quantitative data were obtained. A pure CE-MS method for the analysis of the analytes was developed later, superseding the need for the fluorescence reaction, on the cost of an LOD raised by a factor of four [186].

1.3.9. ORTHOGONALITY

"The identification of a substance has to be realized with several analytical techniques, independent from each other" (translation from [187]). This sentence is written in the guidelines for quality assurance in forensic-chemical analysis of medical drugs and controlled substances from the German Society of Toxicological and Forensic Chemistry that was prepared by a working group of chemists from forensic science institutes of different police offices. Of course it is logical and most reasonable to employ a secondary analytical technique with an ideally orthogonal separation or detection principle. Electromigrative separation techniques are excellently suited as a complement to chromatographic techniques, as the sample matrix and solvent are usually compatible with both analytical methods, but the separation principle is entirely different. Indeed, several publications performing cross validation of an electromigrative separation technique with chromatographic methods are available, demonstrating at least equal performance of the electromigrative compared to the chromatographic techniques. This was already demonstrated by Penalvo et al. in 1996 [188] for the analysis of cefotaxime and impurities. Their MEKC analysis yielded comparable results to the reference HPLC analysis consequently they rendered MEKC analysis as a valuable orthogonal separation technique. Shortly after, Sadeghipour et al. [189] compared HPLC and CE for the analysis of amphetamines in drug seizures and evaluated the results using a Student's paired *t*-test. They found no significant difference between both methods in terms of linearity (0.5-20 µg/mL), precision, accuracy, limits of detection and quantification (between 130-300 ng/mL) and speed of analysis (8 min for the compounds of interest). Another example is a CE method with UV-LIF detection for the analysis of tryptamines which was compared to an HPLC method with DAD detection [76]. Both methods were found to be suitable for the analysis of psychotropic tryptamines, but HPLC proved to be more robust in terms of peak areas, whereas CE, probably somewhat surprising, was more stable in migration times. The CE method used a simple 20 mM NaH_2PO_4, 15 mM α-cyclodextrin buffer (pH 3) in a 66 cm (46.5 cm effective length) 75 µm bare fused silica capillary.

Theobald et al. [190] used CE-MS in addition to GC-MS for studies on the metabolism of a new designer drug (4-iodo-2,5-dimethoxy-β-phenethylamine (2C-I)). They could

identify most of the metabolites by GC-MS/MS experiments and proposed a scheme for the metabolism of 2C-I in rats. However, GC-MS failed to verify, why the metabolite (2-hydroxy-4-iodo-5-methoxyphenyl)acetic acid was not derivatized in the sample preparation, in contrast to the other isomer. Additionally, it was not clear, weather the detected lactone was an artifact of the harsh conditions in the GC-interface or present already in the sample. To solve these particular questions, CE-MS was employed for its ability to separate and detect unstable free carboxylic acid metabolites and indeed an O-demethyl-deamino-carboxy metabolite was separated from the two isomeric N-acetyl-O-demethyl metabolites of 2C-I and identified via MS. A simple 20 mM ammonium formate buffer (pH 10) containing 25% isopropanol was used in a 75 µm bare fused silica capillary.

Another very interesting fact is that electromigrative separation methods themselves exhibit orthogonal separation principles, if e.g. MEKC and CE are employed for the analysis of the same sample. Then, even the same analytical instrumentation can be used: already in 1996 Tagliaro et al. [191] studied the complementary use of two CE and one MEKC method for the mutual confirmation of results in forensic drug analysis using Spearman's test and principle component analysis to determine their degree of orthogonality. They found out that a CE method and an MEKC method were of high orthogonality and therefore well suited for comparative analysis providing a more comprehensive analysis.

1.3.10. MOBILITY AXIS

Drifts or instable migration times may have to be encountered in CE due to several reasons: I) changes in the BGE composition (evaporation of organic solvents, pH value changes due to electrolysis), II) matrix effects with matrix or analyte compounds adhering to the capillary surface.

In case I) both effective electrophoretic mobility of analytes and electroosmotic mobility change, so a more stable BGE (e.g. with better buffering capacity) has to be chosen or a better sealing of the BGE vials against evaporation has to be achieved. Likewise, buffer exchange or replenishment procedures may be used. In Case II) only the EOF

velocity may be affected. Rinsing procedures may be implemented or the use of high ionic strength buffers, addition of modifiers (organic solvents, detergents) or the use of capillary coatings may be helpful. Coatings may also aid for more stable migration times in case of BGEs of intermediate pH values (4-7), which are critical as they lie in the range of the pK_a values of the silanol groups of the capillary surface. This reveals the steepest dependence of the electroosmotic mobility on pH in this range, which can be circumvented by changing the capillary surface. In cases where only the EOF is affected, repeatability problems can be minimized by avoiding compounds adsorbing onto the capillary surface, e.g. by increased ionic strength or addition of organic solvents, detergents or shielding the silanol surface by capillary coatings. However, coatings cannot be used in all approaches, e.g. they are mostly not resistant to high contents of organic solvents in the BGE. Nevertheless, high repeatability can be achieved, since the analyte effective electrophoretic mobility is a physicochemical parameter of the analyte in the given BGE and is therefore constant, when the BGE composition does not change. A transformation of the time axis into an effective electrophoretic mobility axis effectively eliminates detected migration time differences due to EOF changes between runs. In some cases also improved repeatability for quantitative results was observed as demonstrated by Schmitt-Kopplin [192,193]. The transformation can be realized according to Equation 2 or Equation 3, either using the EOF or an internal standard as reference point.

$$\mu_{eff} = \frac{L_d L_t (t_{EOF} - t_m)}{U t_m t_{EOF}}$$

Equation 2

$$\mu_{eff} = \mu_{\text{int}} + \frac{L_d L_t (t_m - t_{\text{int}})}{U t_m t_{\text{int}}}$$

Equation 3

Equations 2 & 3 for the transformation of a time scale into a mobility scale, with μ_{eff} being the effective electrophoretic mobility, L_d being the capillary length to the detection system, L_t being the total capillary length, t_{EOF} being the migration time of the electroosmotic flow, t_m being the measured migration time, U being the applied voltage and t_{int} being the migration time of the internal standard.

This transformation is also very useful for the alignment of electropherograms, when signals from different detection systems have to be evaluated, like UV, LIF and MS

signals [127], that are necessarily acquired in different segments of the capillary (e.g. UV-detection on-column at ca. 8 cm distance from the capillary outlet and MS-detection post-column due to the ESI sprayer geometry). Unfortunately, this technique has attracted little attention in general and even less in the field of forensic science, even though the transformation is often implemented into the data handling software of the commercial instruments. To the best of our knowledge, only one article with a forensic background using a mobility axis is available [127]. The alignment of the signal of an LIF detection system and a UV-detection system allowed for the identification and quantification of minor and major sassafras oil constituents within one run. For the sake of completeness, the work of Nevedomskaya et al. [8] is worth mentioning here, as it features a freely available software package, which can be used for the alignment of an unlimited number of CE-MS electropherograms to each other, even if they suffer from non-linear shifts in the migration times. This approach can also correct for differences in μ_{eff}, however, mass spectrometric detection is required.

1.3.11. POTENTIAL FOR ON-SITE SCREENING INSTRUMENTS

In today's globalized world, it is often financially inefficient to transport samples to a lab for routine analysis for example from a logistic high throughput location like an airport or a port. Also, if the sample is perishable it would be beneficial to have on-site analytics or in the case of a crime scene, on-site analytics could provide the investigator with "useful intelligence at an early stage in a criminal investigation" [40], and can also be very important for self-protection e.g. during the scene of crime work in a clandestine laboratory for illicit drugs or even improvised explosives. At the moment, there are only few handheld or easily transportable analytical instruments available, usually coming with a narrow range of applications or being optimized for a specific compound. However, within the general trend to lab-on-a-chip analytics and portable devices, electrophoresis shows outstanding properties within all the techniques of separation science. This is due to the ease of its miniaturization, especially when using C4D and is only outmatched by ion-mobility-spectrometry or thin-layer chromatography. A more detailed discussion about portable CE instruments can be found in the very vivid review by Lewis et al. [194].

Wallenborg et al. [195] developed a method, employing 4-fluoro-7-nitrobenzofurazane for a very fast on-chip derivatization (3-5 min) to enable the chiral separation of amphetamine and related compounds by chip-electrophoresis with LIF detection with the potential to be used in a portable system. A 50 mM phosphate buffer (pH 7.35) with 10 mmol/L highly sulfated γ-cyclodextrins and an SDS concentration of 1.5 mmol/L (probably evoking MEKC-based separation as the CMC is very low at this high ionic strength). 6 of the 7 investigated chiral compounds were baseline resolved within 7 min in a 160 mm separation channel.

In summary, one can conclude that the possibilities of electromigrative separation techniques in mobile devices are currently being more deeply investigated and the technical implementation is on-going for drug analysis. In different fields, e.g. in the analysis of explosives and small organic compounds, mobile [196] and even remotely controlled small analytical platforms are already available [197]. The presented methods demonstrate the high potential of portable devices in the field of forensics and we are sure that within the ongoing miniaturization, small, portable (chip)-CE instruments will be available.

As an outlook, several recent publications can be mentioned here, with methods that can easily be transferred to a mobile platform or handheld instrument. Blanco et al. [198] for example developed a very fast (run time: 55s) and reliable (RSD of the migration time for ten analytes in 240 injections: 0.61-2.07%) method and instrumental setup for the analysis of 10 inorganic anions in e.g. soil, which are relevant for pre- and postblast analysis. A short capillary (35 cm long, 25 µm inner diameter), coated with hexadimethrine bromide was used with a buffer consisting of 50 mM Tris, 50 mM CHES and 0.05% poly(ethyleneimine) to ensure the high repeatability. A C4D-system was employed. Sarazin et al. [199,200] developed methods for the analysis of ten inorganic anions well separated from seven interfering anions and carbohydrates with the intention to transfer these methods to portable devices for postblast analysis.

A very well validated and optimized approach can be found in the work of Kobrin et al. [201] who used an purpose-built, portable CE instrument for the postblast analysis of

residues from improvised explosives and commercial organic explosives from various matrices within 4 min. By injecting the sample from both ends of the capillary, they were capable of analyzing anions and cations in one run using a C4D as universal detection system. The instrument was equipped with a bare fused silica capillary (length: 50 cm, i.d.: 50 µm) and a mixture of 20 mM MES, 20 mM HIS, 30 µM CTAB and 2 mM 18-crown-6 was used as BGE. Low limits of detection (lowest 3.4 µM for K^+ up to 35.7 µM for Cl^-) were found. A principle component analysis allowed the identification of the specific explosive.

1.3.12. LIMITATIONS

Some limitations of CE methods may be summarized here: first of all, the loadability in CE is limited compared to liquid chromatography. Only a few nL can be injected, which is only partially counteracted by the clearly higher separation efficiency. Detection limits are thus usually higher compared to standard chromatography. Usually the loadability can easily be enhanced by various preconcentration techniques, first of all simple stacking when the sample has a lower conductivity than the buffer or transient ITP, where a transient leader (e.g. sodium or ammonium and chloride are added to the sample. For well defined sample matrices, e.g. in organic extracts, electrokinetic injection may be applied giving rise to impressive detection limits [202]. Other preconcentration techniques may be used by more experienced users.

Intermediate precision can be affected by matrix components, which adsorb onto the capillary surface. As this will influence the EOF, changes in migration times, often with drift phenomena will occur. Rinsing protocols, the use of a mobility axis or coatings may be used or a more sophisticated sample cleanup.

When transferring CE-UV to CE-MS methods, one has to consider that the outlet vial is replaced by the sheath-liquid interface. The sheath-liquid composition is not the same as the BGE, consequently ions from the sheath-liquid may travel into the capillary and influence the separation [203].

With MS detection, the choice of buffer additives is limited, which is especially critical for MEKC. APCI as an alternative ionization method or partial filling techniques may be used.

1.4. QUICK STARTING GUIDE FOR METHOD DEVELOPMENT IN CAPILLARY ELECTROPHORESIS

As the intention of this review is to introduce the reader to the wide applicability of electromigrative separation techniques, the authors want to provide some useful standard buffer compositions as starting points for further method development, suitable for a wide range of analytes (Table 1). For an easily comprehensible access, we try to structure the choice for a specific method and buffer composition in a table, including simple remarks for a decision-making based on our own experience and publications in the field. A relatively complex flow chart derived from the principles of MEKC can be found in the work of Terabe [113] for decision making in MEKC separation optimization.

Of course, our table does not include all possible combinations or approaches, nor does it guarantee that the chosen conditions will be applicable in all cases, however, we hope that this guideline will help inexperienced operators to quickly reach a sound working base.

For **neutral compounds**, we recommend the use of MEKC, if MS detection is not required. Otherwise CEC may be applied, though additional equipment is necessary. For relatively polar compounds, buffer System 1 is a good starting point. For SDS as the most common micellar phase as well as all detergents, a change of the phase ratio between stationary and mobile phase for tuning of the retention factors is possible, just by changing the concentration of the detergent in the BGE. This may also serve as a good tool for the optimization of the overall analysis time. If the analytes are detected too close to or even together with the micelle marker, the addition of acetonitrile or methanol (up to 30% v/v) can decrease the polarity of the mobile phase. For compounds with an octanol water partition coefficient of about 4-5, the combination of acetonitrile and urea is a good compromise (see also: [204]). Even more hydrophobic compounds can be well separated with MEEKC with the “traditional” buffer System 2. Selectivity tuning can easily be achieved using different detergents, the choice of which can occur via the analyte characteristics as described in Section 1.3.2.2.

With MEKC, also **mixtures of neutral and charged compounds** (both anions, and cations) can be separated; the chromatographic distribution of the charged compounds between the phases is then overlaid by their migration in the electric field. Care has to be taken with the choice of the detergent, which may have like or dislike charge and thus induce coulombic attraction or repulsion; and likewise very high or low retention factors may be obtained. To the author's opinion, MEKC will achieve the separation of any pair of analytes as the number of possible optimization parameters is impressive. Mostly however, changing one parameter will affect many entities relevant for the separation including resolution, migration time window, overall analysis time, selectivity. But due to its impressive separation efficiency, normally a fast method development can be achieved. MEKC is ideal for the separation of structural isomers. Chiral separation may also be achieved using micelles with chiral head groups or via the inclusion of secondary complexation equilibria using cyclodextrins.

For the CE separations of **charged compounds**, a large number of buffers have been employed with borate and phosphate being most often applied and also among those with highest robustness (System 3). Other frequently encountered buffering compounds with their corresponding pH value ranges are: phosphate (pH 1.2-3.2 and pH 6.2-8.2), ammonium acetate (pH 3.8-5.8), borate (pH 8.1-10.1), HCO_3^-/CO_3^{2-} (pH 5.4-7.4), TRIS, glutamic acid (pH 7.3-9.3), MES (pH 5.2-7.2), HEPES (pH 6.6-8.6), Tricin (pH 7.2-9.2), see also publication by Reijenga et al. [205] for further reading. In general, a good separation is achieved, when the pH of the BGE is close to the pK_a or pI-value of the analytes (to maintain a sufficient effective electrophoretic mobility, we recommend a pH not more than 0.8 pH units away from the pI or pK_a). Beside via pH variation, which is usually the first tuning parameter, selectivity changes may be introduced using a large variety of additives, predominantly organic solvents (water soluble ones such as acetonitrile, simple alcohols and formamide), complexing agents (trifluoro acetic acid, EDTA, etc.), chiral selectors (mostly α-, β- or γ-cyclodextrins or modified versions of them, including charged (mostly highly sulfated) cyclodextrins). We recommend 10 mmol/L solutions of the BGE as a compromise on ionic strength for good separations and reduced adsorption to the capillary wall vs. problems with Joule heating. With these buffers, normally high voltages (+/-30 kV) can be employed for fast

analysis. The use of coatings is only recommended when: 1) required against adsorption, 2) for the analysis of fast anions 3) for BGE systems with a pH in the range of ~ 5 (the range of the pK_a of the silanol groups of the capillary surface) or 4) in rare cases with very complex separation problems for resolution optimization via the electroosmotic velocity (see also: [206]).

In many cases, for **basic analytes,** NACE may give rise to reduced adsorption phenomena and also very high separation selectivity in case of structural isomers and diastereomers. Two BGE systems are well known and often applied (System 4). Optimization can be reached changing the solvent ratios and the concentration of the electrolyte [102,103] and the choice of strong ion-pairing counterions

Strong bases or permanent cations (quaternary ammonium compounds) normally give rise to very fast separations. Nearly any BGE may be used here, though a higher selectivity may be obtained using a high pH. Optimization via pH is not possible, but many additives may provide selectivity tuning. The EOF may be reduced by increasing the ionic strength or decreasing pH for higher resolution.

For the separation of **weak bases**, the pH-value of the BGE has to be adjusted to the pK_a or pI of the analytes with good selectivity often obtained close to these values. However, care has to be taken in an intermediate pH range, where coatings may help to increase migration time repeatability. In many cases, a good selectivity may also be obtained with analytes being (almost) fully charged (thus at very low pH values) with size-based separation. Here, selectivity tuning using organic solvents or complexing agents is possible.

Strong acids and inorganic anions have to be classified upon their effective electrophoretic mobility as different EOF requirements prevail. Very fast anions overcome the EOF and separation may be carried out at reversed polarity (in CE-MS, coatings are required as the EOF has to be towards the MS inlet). Problems occur, when the analytes effective electrophoretic mobility is similar to the electroosmotic mobility. Very high resolution may be obtained, though likewise too high analysis times. Coatings are recommended, e.g. via a sulfonation of the capillary surface to obtain a fast, pH-independent EOF.

Weak acids (normally with a low effective electrophoretic mobility) can be characterized in a similar manner as weak bases with all optimization strategies as discussed above. Normal polarity can be used as these analytes are effectively transported to the detector by the EOF, which is high at the present pH-value, necessary for effective ionization. Due to counterelectroosmotic migration, resolution is usually also relatively high.

For **ITP**, we recommend to start with a fast leading combined with a slow termination ion. Usually chloride/borate or hydroxide ions(dilute base) (LE/TE) and potassium or ammonium/TRIS or H^+-ions (dilute acid) (LE/TE) are good choices to provide a very wide mobility window (see Systems 5 and 6). During optimization, this window may be reduced in order to speed up the analysis or to use leaders slower than common salts to cut off the sample matrix with high loads of chloride and sodium ions. For transient ITP as a preconcentration technique, it is simplest to add sodium or ammonium ions to the sample for cation analysis or chloride for anion analysis. These compounds then migrate as transient leaders. Often, some preconcentration automatically is obtained due to sample-induced transient ITP as most samples contain significant amounts of these ions anyhow. The optimization of selectivity proceeds as for CE for an increase of differences in the effective electrophoretic mobilities. In literature a focus is set on complexing agents.

Special buffer system with a focus on detection methods

MS detection requires volatile BGEs. Suitable buffer components include acetic acid (pK_a 4.76), formic acid (pK_a 3.77), carbonic acid (pK_a 6.35, 10.33), citric acid (pK_a 3.09, 4.75, 5.41) and ammonia (pK_a 9.21) and sometimes ε-aminocaproic acid (EACA) (pK_a 4.43, 10.75) for anion analysis. Addition of organic solvents to the BGE normally facilitates the electrospray due to reduced surface tension. Also additives should be volatile, though many publications also apply semi-volatile separation enhancing substances such as ion pairing reagents (e.g. trifluoroacetic acid) or cyclodextrins (preferably hydroxylated ones). Partial filling techniques may be applied, though its use should be restricted to more experienced users. Many coating agents (predominantly

physically adsorbed coatings or covalently attached coatings) are amenable to adjust the EOF. Details on the choice of coatings for CE-MS can be found elsewhere [206].

When laser-induced fluorescence (LIF) detection is applied, especially in case of employing a UV-Laser (e. g. frequency-quadrupled Nd:YAG-Laser emitting at 266 nm) for the analysis of native fluorophors, special care has to be taken regarding the quality and the chemical purity of the buffer compounds, especially concerning additives as surfactants, EOF modifiers, chiral selectors and coating polymers which sometimes contain impurities that are natively fluorescent at UV excitation resulting in disturbed base-lines and artifact peaks. MEKC buffers should be thoroughly and frequently filtered to avoid generation of excessive stray light.

C4D measures the differences between sample and BGE conductivity and thus relies on the differences in the effective electrophoretic mobilities of the analyte ion and its coion. In theory, the use of either high or low conductivity buffers is possible, though in order to reduce Joule heating effects, low conductivity buffers are usually recommended. Mostly, so called Good buffers can well be employed as they have a high buffering capacity but a low specific conductivity, e.g. MES, HEPES, Tricin, histidine among others. For small inorganic and organic anions and cations, buffer Systems 7 and 8 may be suitable as a starting point.

Indirect UV detection of non-UV-absorbing analytes requires the addition of probe ions at a concentration of typically 5-10 mmol/L to the BGE, which should show an overall low conductivity to achieve an effective displacement of the analyte. A compromise has to be found on low ionic strength vs. high buffering capacity and detection limits. The probe ions' effective electrophoretic mobility should match well with the analytes' effective electrophoretic mobilities to avoid band broadening with typical saw-like peak shapes due to electrodispersion. Probe ions often applied are: a) anion separation: chromate (-0.80 cm^2/Vs, UV range 250-280 nm), sorbic acid (-0.24 cm^2/Vs, UV range < 290 nm), dinitrobenzoic acid (-0.22 cm^2/Vs, UV range < 260 nm), naphthylsulfonic acid (-0.16 cm^2/Vs, UV range 200-225 nm); b) cation separation: histamine (+0.53 cm^2/Vs, UV range < 220 nm), imidazole (+0.46 cm^2/Vs, UV range < 220 nm), 9-aminoacridin (0.42 cm^2/Vs, UV range 250-265 nm), 1-aminonaphthalin (0.17 cm^2/Vs, UV range ~

300 nm). Buffer Systems 9 and 10 as used in the German Federal Criminal Police Office for the analysis of small inorganic and organic anions and cation may serve as a starting point (see Figure 10). Optimization may include changes of the pH, addition of organic solvents and the type and concentration of the probe ion. Indirect fluorescence detection is hardly used and despite more expensive equipment is not advantageous with regard to the high noise at higher fluorescence signals.

Table 1: Common standard conditions as starting points for method development in electromigrative separation techniques.

BGE systems and analytes	Separation technique	Buffer for the first experiments	Possible modifications	Detection modes
System 1 (neutral and charged analytes of low to medium hydrophobicity)	MEKC	80 mM SDS, 8.5 mM borate, 8.5 mM phosphate buffer (pH value 8.5) with 15% acetonitrile	type and concentration of detergent; organic solvents, urea, cyclodextrins; pH in case of charged analytes	UV, LIF
System 2 (neutral compounds of high hydrophobicity)	MEEKC	3.3% SDS, 1.5% n-hexane, 6.6% 1-butanol in a 8.85 mM sodium tetraborate solution (pH 9.5)	SDS concentration, concentration of 1.butanol, pH	UV, LIF
System 3 (cations and anions)	CE	see buffering ions in text	pH, organic solvents, complexing agents, cyclodextrins	UV, LIF, MS
System 4 (cations, especially alkaloids)	NACE	35 mM ammonium acetate in acetonitrile methanol (8:2) or 60 mM ammonium formate and 1M acetic acid in acetonitrile	Variation of solvent ratios, of ammonium acetate concentration, pH	UV, LIF, MS
System 5 (cationic analytes)	ITP	LE: 5-10 mM ammonium or potassium, pH 3 for analytes to be well charged (using e.g. acetic acid)	pH via counterion, addition of organic solvents, complexing agents	C4D, UV

		TE: Tris or H+ (diluted counterion, e.g. 20 mM acetic acid) counterion: 10 mM β-alanine		
System 6 (anionic analytes)	ITP	LE: 5-10 mM chloride salt TE: borate or OH- (diluted counterion solution (basic) counterion: e.g. Tris	pH via counterion, addition of organic solvents, complexing agents	C4D, UV
System 7 (absorbing and non UV-absorbing anions)	CE	7 mmol/L sorbic acid/15 mmol/L arginine; dynamic coating with 0.001% hexadimethrine hydroxide (Polybrene), pH 9.0 at + 30 kV	type and concentration of buffer; pH, organic solvents, complexing agents	C4D
System 8 (absorbing and non UV-absorbing cations)	CE	25 mmol/L MES/25 mmol/L histidine with 1 mmol/L 18-crown-6-tetracarboxlic acid (for the separation of NH4+ and K+), pH 6.1	type and concentration of buffer; pH, organic solvents, complexing agents	C4D
System 9 (non UV-absorbing anions)	CE	anions (bromide, chloride, nitrate, sulfate, phosphate, internal standard azide): 2.25 mmol/L sodium hydroxide, 6.5 mmol/L benzene-1,2,4,5-tetracarboxylic acid (pyromellitic	type and concentration of probe ion; pH, organic solvents, complexing agents	indirect UV

		acid), 1.6 mmol/L triethanolamine, 0.75 mmol/L hexamethonium hydroxide solution (as a dynamic coating for EOF reversal), pH 7.7 (with NaOH); -28 kV, UV detection at 254 nm		
System 10 (non UV-absorbing cations)	CE	cations (ammonia, potassium, sodium, calcium, magnesium, lithium, methyl amine): 10 mmol/L imidazole, 2 mmol/L crown ether 18-crown-6-tetracarboxylic acid (for the separation of NH4+ and K+), adjusted to pH 4.0 with sulfuric acid; +30 kV; UV detection at 214 nm		indirect UV

Table 2: Experimental details to selected literature covered in this article, sorted alphabetically with regard to the analyte (class). (LLE = liquid-liquid extraction, SPE = solid phase extraction, AM =amphetamine, MA = methamphetamine)

Analyte	Matrix	Sample preparation	CE Method	BGE	Capillary	Linear range	Detection limit	Reference
(2-hydroxy-4-iodo-5-methodxyphenyl) acetic acid	Rat urine	SPE	CE-MS	20 mM NH_4Form +25% isopropanol (pH 10.0)	90 cm, 75 µm	Not given	Not given	[190]
17 drugs of different classes	Standards, urine, plasma	Extraction with chloroform-isopropanol (9:1)	CE-UV	50 mM NaH_2PO_4 (pH 2.35)	60(52.5) cm, 75 µm	Not given	Not given	[99]
19 Cathinone derivatives	Standards*	Dissolution	CE-UV	50 mM NH_4Ac, 20 mg/mL sulfated-ß-CD +10% ACN (pH 4.5)	78.5(70) cm, 50 µm	Not given	Not given	[159]
34 drugs, heroin + impurities, cocaine + impurities	Seizure	Dissolution	MEKC	85 mM SDS, 8.5 mM phosphate, 8.5 mM borate + 15% ACN (pH 8.5)	122(100) cm, 50 µm, 72(50) cm, 50 µm, 72(50) cm, 25 µm, 47(25) cm, 50 µm	Not given	Impurities 0.2% rel. to heroin	[114]
AM, MA	Urine	LLE, 0.45 µm filtration	CE-UV	75 mM Tris +10 mM DM-ß-CD, 5mM ß-CD, (pH 2.5, phosphoric acid)	64.5(56) cm 50 µm, diol-modified	Not given	Not given	[154]
AM, MA	Urine	LLE, SPE	CE-MS	1 M formic acid, 3 mM ß-CD, 10 mM DM-ß-CD, (pH 2.2)	100 cm, 50 µm	0.2-10 µg/mL	0.03-0.05 µg/mL	[155]
AM, MA	Urine	LLE	CE-MS	1 M formic acid, 0.85 mM heptakis(2,6-diacetyl-6-sulfato)-ß-CD, (pH 1.7)	100 cm, 50 µm	0.05-10 µg/mL	0.02 µg/mL	[156]
AM, MA, amphetamine type stimulants	Standards, seizures	Dissolution, filtration	CE-MS	2.5 mM highly sulfated-γ-CDs (pH 3.1)	56 cm, 50 µm	Not given	2 pg on column	[160]
AM,MA	Urine	filtration	CE-MS	1 M formic acid/ 1 M NH_4Ac (10:0.2	100cm, 50 µm	0.05-10 µg/mL	0.02 µg/mL	[157]

				mixture), 1.5 mM heptakis(2,6-diacetyl-6-sulfato)-ß-CD, (pH 2)				
Caffeine	Beverages, smart drugs	Dilution, methanolic extraction	MEEKC	3.3 % SDS, 1.5% n-hexane, 6.6% 1-butanol in a 8.85 mM sodium tetraborate solution (pH 9.5)	50(40) cm, 50 µm	5-100 µg/mL	LOD 2 µg/mL, LOQ 5 µg/mL	[62]
Cannabinoids, hashish, marijuana	Seizure	Extraction in MeOH: chloroform (9:1)	CEC-UV	25 mM phosphate buffer (pH 2.57) in 75% acetonitrile	49 cm (40 cm) 100 µm, 40 cm, 3 µm, Hypersil C18	Not given	0.5 µg/mL for d9-THC	[67]
Codeine, MA, morphine, ketamine, benzodiazepines	Urine	LLE with EtOAc	Large volume sample stacking sweeping MEKC	50 mM NaH_2PO_4, 150 mM SDS + 10% MeOH (pH 2.3)	50(40) cm, 50 µm	0.025-1.5 µg/mL, 0.05-3.0 µg/mL	0.0075-0.030 µg/mL	[167]
Fentanyl derivatives	Standards, Seizures, heroin, garment	Dissolution, MeOH extraction	NACE-MS	200 mM NH_4Ac in glacial acetic acid + 90% ACN	80 cm, 50 µm	0.002-6.33 µmol/L	0.001 µmol/L	[175]
Food colorants	Seizures, tablets	Extraction with BGE	CE-MS	200 mM formic acid (pH 2.2)	82 cm, 75 µm	Not given	Not given	[10]
GHB, GBL	Standards, Gatorade	-	MEKC	50 mM phosphate + 3% SDS (pH 6.5)	32(23.5) cm, 50 µm, dynamic cationic coating	300-9 700 µg/mL (GHB), 600-9 700 µg/mL (GBL)	Not given	[73]
Heroin	Standards, Seizures	-	CE-UV	CElixir B, 13.3% DM-ß-CD (pH 2.5)	64(55.5) cm, 50 µm, dynamic anionic coating	250-800 µg/mL	Not given	[115,73]
Heroin, amphetamines, impurities	Seizures	Not given	MEEKC	3.3% SDS, 6.0 % 1-butanol, 0.9% octane, 89.8% in a 5 mM sodium tetraborate solution (pH 9.5)	40(30) cm, 75 µm	1-600 µg/mL	LOD 1.0 µg/mL, LOQ 3.0 µg/mL	[116]
Heroin, basic impurities, basic adulterants	Standards, seizures	Dissolution	CE-UV	100 mM HP/DM-ß-CD in CElixir Reagent B (pH 2.5)	64(55.5) cm, 50µm, dynamic, polyanionic	10-800 µg/mL; 0.6-80 µg/mL	Not given	[115]

					double layer			
Indole alkaloids, Kratom alkaloids	Standards, Plant material	Extraction with MeOH, centrifugation	NACE-MS	60 mM NH_4Form in a mixture of ACN/glacial acetic acid (100:6)	67.5 cm, 50 µm	1 -500 000 µmol/L	0.025-0.075 µmol/L	[102]
LSD, opium	Standards, Seizures	-	CE-UV	CElixir B, 3.94% HP-ß-CD, 9.98% DM-ß-CD (pH 2.5)	32(23.5) cm, 50 µm, dynamic anionic coating	0.8-26 µg/mL (LSD),	Not given	[207,73]
MA, MDMA, heroin, ketamine	Banknotes, plastic bag, kraft paper, silver paper	Dispersive LL microextraction	CE-UV	100 mM KH_2PO_4 + 20 mM β-CD	37(30) cm, 50 µm	0.15-6000 µg/L	0.05-0.2 µg/L	[158]
Neutral, acidic, and weakly basic adulterants	Standards	-	MEKC	50 mM phosphate-borate + 3% SDS (pH 6.5)	32(23.5) cm, dynamic cationic coating	8-900 µg/mL (caffeine)	Not given	[115,73]
Neutral, acidic, weakly basic heroin impurities	Seizures	Dissolution	MEKC	103.2 mM SDS, 50 mM phosphate-borate (pH 6.5)	32(23.5) cm, 50 µm, polycationic coating (CElixir A)	7-900 µg/mL (caffeine), 3-50 µg/mL (phenacetine)	Not given	[115]
Opiates	Standards	Dissolution in MeOH	CEC-UV	10 mM Tris, 50 mM SDS buffer (pH 8.3) in 20% acetonitrile	28 cm (unknown), 75 µm, 15 cm 1.5 µm C18 packing	Not given	Not given	[68]
Opiates amphetamines	Standards, urine	SPE	CEC-MS	25 mM NH_4Form (pH 3) in 30% acetonitrile	36 cm (27.5 cm), 100 µm, 26 cm, 3 µm CN-modified packing	0.0025-0.1 ng/mL	LOD 0.0008 µg/mL, LOQ 0.00025 µg/mL	[119]
Opiates, amphetamines	Standards, urine	SPE	CEC-UV	20 mM sodium phosphate (pH 2.5) in 20% acetonitrile	33 cm (24.5 cm), 75 µm, 23 cm, 3 µm CN-modified packing	0.1- 1.2 µg/mL	LOD 0.005 µg/mL, LOQ 0.01 µg/mL	[118]
Opiates, amphetamines, cocaine + metabolites	Urine	Centrifugation, filtration	CE-MS	1 M formic acid (pH 1.8)	80 cm, 50 µm, double layer, dynamic polyanionic coating	0.01-1.0 µg/mL (cocaine), 0.021-1.0 µg/mL	0.002 µg/mL	[108]

						(methadone)		
Opioids	Serum, plasma, urine	SPE, LLE	Head-column FASS CE-UV	75 mM Na_2HPO4 and 25 mM NaH_2PO_4 in ethylene glycol-water mixture (6:4 v/v) (pH 7.9)	41(22) cm, 50 µm	0.02-0.5 µg/mL (dihydrocodeine) in plasma	0.001 µg/mL (dihydrocodeine) in plasma	[180]
Phenethylamines, cocaine, oxycodone	Standards, Seizures	-	CE-UV	CElixir B (pH 2.5)	33(24.5) cm, 50 µm, dynamic, anionic coating	3-100 µg/mL	Not given	[208,73]
Piperazine derivatives	Seizures, tablets	Extraction with H_2O/isopropanol (1:1)	CE-MS	100 mM formic acid, 10 mM 2-HP-ß-CD + 10% MeOH (pH 2.4)	82 cm, 75 µm		Not given	[10]
Plant alkaloids	Standards	-	CE-UV	100 mM NH_4Ac (pH 3.1) +50% ACN	55(50) cm, 50 µm	Not given	0.25 µg/mL (for gramine)	[100]
Plant alkaloids	Standards	-	CE-UV-MS	100 mM NH_4Ac (pH 3.1) +50% CAN	75(20) cm, 50 µm	Not given	Not given	[100]
Psychoactive mushrooms	Standards	-	CE-UV	CElixir B (pH 1.8)	32(23.5) cm, 50 µm, dynamic anionic coating	Not given	Not given	[73]
Psychoactive plant materials, preparations	Plant material, aq. Solutions	Extraction with MeOH, centrifugation	NACE-MS	58 mM NH_4Form in a mixture of ACN/glacial acetic acid (975:60)	67.5 cm, 50 µm	1 -500 000 µmol/L	0.025-0.075 µmol/L	[103]
Weakly-strongly acidic/basic drugs of forensic interest	Standards	Dissolution in mobile phase	CEC-UV	25 mM phosphate buffer (pH 2.5) in 75% acetonitrile	34 cm (25 cm), 100 µm, 25 cm, 3 µm Hypersil C8	Not given	Not given	[117]

1.5. CONCLUSION AND OUTLOOK

From the previous subchapters it should be obvious, that electromigrative separation techniques are capable of playing a larger role in forensic science and forensic toxicology than they currently do, especially with regard to the potential use of CE for forensics in developing countries (see Section 1.3.6) and for on-site screening. All points of criticism which are still sticking to this technique can be regarded as outdated as many publications demonstrated robustness and well validated methods with high accuracy, repeatability and separation efficiency. The ease of method development, adjustment to new questions and the orthogonality are other aspects to be considered. We hope that this review can aid separation scientists who want to gain a first glimpse into electromigrative separation techniques in forensic science, as well as allow for an easy startup into the application of CE techniques, by providing appropriate literature examples and a simplified starting guide.

At the moment, there is an ongoing discussion in Germany if doping, especially the distribution of doping agents will be considered as a crime in the near future, thus further extending the large variety of analytes to deal with, starting from steroid solutions to complex glycoproteins like erythropoietin (EPO). Doping is already considered as a crime in several European countries. As electromigrative separation techniques have attracted a lot of attention in peptide and protein analysis lately and can be seen among the top analytical platforms in biopharma, we are sure that they will also find their way into more and more forensic laboratories in the future.

2. CHAPTER II: STUDY ON THE INFLUENCE OF THE BACKGROUND ELECTROLYTE ON SEPARATION AND DETECTION IN NON-AQUEOUS CAPILLARY ELECTROPHORESIS-MASS SPECTROMETRY

Based on: T.N. Posch, A. Müller, W. Schulz, M. Pütz, C. Huhn. Electrophoresis 2012, 33, 583–598

2.1. CHAPTER SUMMARY

Non-aqueous capillary electrophoresis background electrolytes are most often composed of a mixture of methanol and acetonitrile with soluble ammonium salts added as electrolyte. In this chapter, a study on NACE-MS is presented using a mixture of glacial acetic acid and acetonitrile, providing an acidic background electrolyte with a very low dielectric constant. Impressive changes in selectivity and resolution were observed for structurally closely related indole-alkaloids including diastereomers upon addition of ammonium formate as electrolyte and upon variation of the solvent ratio (acetonitrile and glacial acetic acid). In order to obtain best separation and MS-detection conditions and to reveal the influence of the parameters of the BGE on separation and detection and vice versa of the MS parameters on separation, an optimization strategy was employed using a design of experiments in a central composite design with response surface methodology. It was proven that at the present high EOF conditions capillary electrophoretic separations and thus optimization can be realized without interference from the coupling to an MS-system. Several significantly interacting parameters were revealed, which are not accessible with classical univariate optimization approaches. With this optimization, alkaloid mixtures from a plant extract of *Mitragyna speciosa*, containing a large number of diastereomeric compounds were successfully separated.

2.2. INTRODUCTION

The aim of this study was to develop a new capillary electrophoretic method for the analysis of the indole-based alkaloids from methanolic plant extracts of Kratom, a biogenic recreational drug made from the leaves of the tropical plant *Mitragyna speciosa*. The effects of this drug are said to be induced by its main alkaloids mitragynine, paynantheine and 7-hydroxy-mitragynine [3] (for structures see Figure 13). The alkaloid composition of Kratom is determined by a high number of diastereomers of each individual active component which have to be well separated to be discriminated by mass spectrometric detection, necessitating a careful method optimization. In recent years, NACE [209-211] has increasingly been applied to determine alkaloids in plant samples with good results regarding the separation of structurally closely related compounds [212-214].

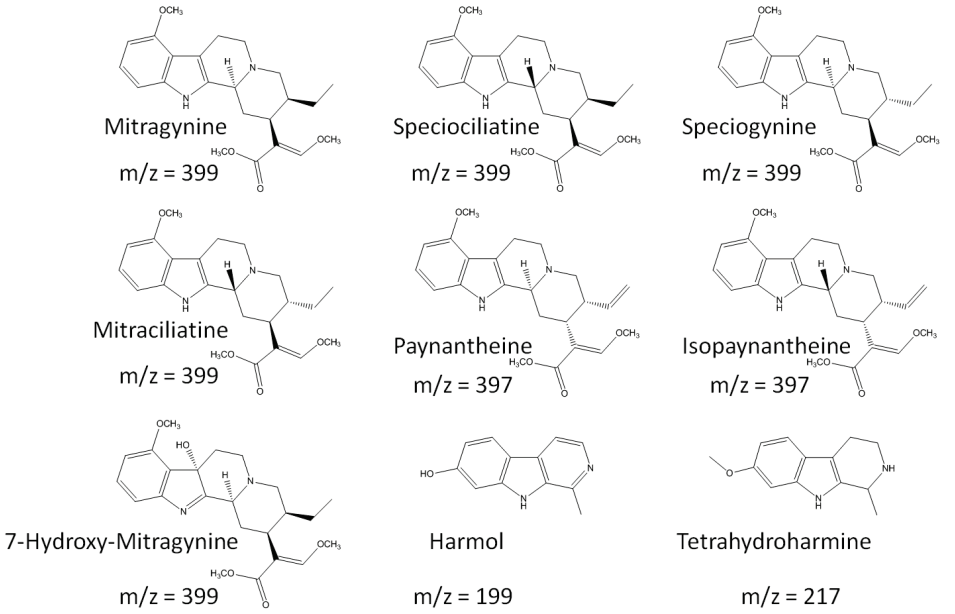


Figure 13: The main alkaloids of Kratom together with harmol and tetrahydroharmine as examples for harmala alkaloids.

The application of organic solvents in buffer systems in NACE has been investigated since the early years of capillary electrophoresis [97,215] and even earlier in capillary isotachophoresis [216]. A number of advantages of NACE can be stated: Organic solvents enable the analysis of hydrophobic and even water insoluble compounds with CE. They have a very low viscosity and especially those organic solvents with a low dielectric constant show only very low electric currents in CE, thus they allow the use of high electric field strengths without producing excessive Joule heating [132]. If the EOF is increased in the non-aqueous buffer, very fast separations are possible [106,217]. The predominant reason to use NACE is the introduction of selectivity changes in comparison to aqueous systems. NACE was found to be of special value for the separation of structurally closely related compounds, including structural isomers and diastereomers. Several authors stated, that baseline separation of complex mixtures of pharmaceutical or forensic drugs was possible in NACE [217], but could not be achieved in aqueous solution, except when modifiers such as cyclodextrins [52] were used or MEKC [218] was applied.

Naylor and coworkers [219,220] introduced non-aqueous buffer systems for CE-MS in 1994 for the analysis of drug metabolites. Since then, NACE-MS has been investigated in more detail [221,222] and is increasingly used for alkaloid separation [52,223]. The application of organic solvents as buffer system exhibits several advantages for MS-detection: The low volatility of the solvents improves ionization efficiency. In addition, the scope of NACE-MS can be broadened as water-insoluble compounds can be analyzed without the introduction of MS-incompatible dissolving agents (e.g. detergents). However, the general knowledge about the underlying processes can still be regarded as insufficient in comparison to aqueous capillary electrophoresis [97]. Most of the published papers use the same buffer system as in the very first application on NACE i.e. mixtures of ACN/methanol/2-propanol containing ammonium acetate and acetic acid which are excellently suited for UV-detection [97].

However, some authors also investigated other solvent systems [224-227] or ionic liquids as additives [228], most of them suitable for both UV and MS detection. The separation mechanism in organic solvents has been described to be different from those in organic solvents with ion-pairing and heteroconjugation [46,136,226,227,229-236] as the most prominent mechanisms present due to the decreased dielectric constant. Separations based on differences in pk_a values can also be present [237,238]. In our NACE setup we employed a BGE of very low dielectric constant using varying ratios of glacial acetic acid (ε_r = 6.2) and acetonitrile (ε_r = 36.6) ([239] and unpublished results). Strong effects on resolution, selectivity and speed of analysis were observed upon addition of ammonium formate and variation of the solvent ratio. With this low dielectric constant, separation mechanisms based on ion-pairing and homo- or heteroconjugation can be expected to be stronger than in BGEs of moderate ε_r. We here aim at the determination of the extent of these mechanisms present in the NACE separation and thus varied the composition of the BGE over a wide parameter range (electrolyte concentration and solvent ratio). The effects of these parameters are expected to be strongly correlated, thus, a univariate approach studying one parameter at a time after the other is not suitable. In addition, the influence of the BGE on MS detection is studied as complexation and ion-pairing are known to influence mass spectrometric detection, mostly leading to a severe decrease in sensitivity. Some of these BGE effects may be counteracted by a suitable sheath-liquid composition and flow rate significantly diluting the interfering

BGE components and thus leading to a better sensitivity. In return we also have to study the influence of the MS parameters on the NACE separation, which may be significant as the viscosity of the BGE (η = 1.056 (AcOH), η = 0.369 (ACN) [240]) will be very low and separation impairing effects such as the nebulizer pressure leading to a suction effect in the capillary may be detrimental. For this reason and for in-depth method optimization, we decided to more closely investigate the effects of experimental variables of both BGE composition and MS parameters in NACE-MS on separation and detection by applying statistical tools. The NACE-MS system evaluation and method optimization is thus based on two sets of variables: the process variables describing the experimental conditions set by the user (e.g. pH-value of the BGE, capillary length, applied voltage) and the response variables describing the effects on the separation or detection parameters (e.g. migration time, plate number, resolution, efficiency, signal intensity). The process variables are controlled by the user within the experimental setup, while the response variables depend on the choice of the process variables and are determined from the electropherograms. The few available publications on multivariate method optimization in NACE or NACE-MS usually employ low numbers of experiments (16-32 single experiments [52,231,241,242]), the optimization is realized in very narrow parameter ranges and always conducted with standard substances. Nevertheless, a DOE is always justified by revelation of interacting parameters and a superior method optimization. However, in addition to be able to study interacting variables, the judgment of the results of a multivariate approach achieved by statistical tools also allows to visualize the effects of interaction parameters more clearly than it would be possible with univariate approaches.

A central composite design was applied as it combines a relatively low number of experiments needed with a high statistical significance [243,244]. It was set up in the following way: In pre-studies the experimental limits of the process variables were defined in a way that a higher or lower value would lead to run failures. These limits were identified as the alpha points (2n star point design) of the CCD setup. A centerpoint of this multidimensional parameter space is calculated from the alpha points as well as so-called cube points (cube levels) (2^n factorial design) marking the frame of the more robust process variable settings. The number of necessary experiments is obtained as $2^n + 2n + m$, with n = number of process variables and m = number of replications of the centerpoint. In this study, we used m = 15 and n = 6,

with each alpha point and cube level measurement conducted in duplicate to minimize the influence from random effects. In total, 167 experiments were conducted in a random order within twelve days. The CCD was evaluated using response surface methodology. This design has already been approved as validation and optimization tool for established LC-MS methods by Müller et al. [243,244].

2.3. EXPERIMENTAL

2.3.1. CE-MS

All experiments were performed on an Agilent 7100 Capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent 6520 Accurate-Mass qToF-MS (Agilent Technologies, Santa Clara, CA, USA) via an Agilent CE-MS-adapter-kit. Sheath-liquid was added by an Agilent isocratic pump 1260 (Agilent Technologies, Waldbronn, Germany). The separation voltage was set to +30 kV in all cases. The capillary (50 µm i.d. length: 67.5 cm) was flushed for 4 min with running buffer prior to injection and was kept at an operational temperature of 25 °C during analysis. Injection was performed hydrodynamically for 5 s at 50 mbar. Acetophenone was spiked to the sample as neutral marker for the EOF determination. The nebulizer had to be set to 2 psi during injection and vial handling to prevent the introduction of air into the capillary and was subsequently increased to the desired value at the beginning of voltage application. 5 L/min drying gas were supplied at 325 °C, the fragmentor voltage was set to 175 V and capillary voltage was set to -4000 V. The skimmer was set to 65 V and the octopole to 750 V. The mass range was 100-1700 m/z, acquiring 2 full spectra per second. The calibration was performed with an Agilent standard calibration mixture for QTOF-MS systems. All other parameters were varied according to Table 3.

*Table 3: Boundaries of the process variables for the DOE. The variable **Buffer** determines the amount of ACN in 1 mL buffer, the remaining volume is filled with glacial acetic acid; **SL-Acid** determines the amount of glacial acetic acid in the sheath-liquid; **SL-Comp** determines the amount of 2-propanol in the sheath-liquid in 50 mL total volume. The remaining volume is filled with Milli-Q water (resistance(R)> 18 MΩ).*

	+alpha	Cube +1	Centerpoint	Cube -1	-alpha
Buffer [µL]	1000	902	846	790	687
*Formate [mmol/L]	60	44	35	26	10
SL-Acid [%]	5.0	3.4	2.6	1.8	0.1
SL-Comp [mL]	50	42	35.5	29.5	17
SL-Flow [µL/min]	10	7	6	5	1
Nebulizer [psi]	9	7	6	5	3

**A stock solution of 1 mol/L ammonium formate in glacial acetic acid was prepared and added to the BGE to achieve the desired ammonium formate concentration.*

2.3.2. STANDARDS AND CHEMICALS

Harmaline (purum), harmane (purum), norharmane (purum), harmalol hydrochloride dihydrate (>98%), ammoniumhydroxide (25%), 2-propanol (LC-MS-grade) were purchased from Sigma-Aldrich (Steinheim, Germany). Ammonium formate was purchased from BDH Prolabo (Darmstadt, Germany) and acetonitrile was purchased from Honeywell (Morristown, NJ, USA). Harmine (98+ %) and tryptamine (98+ %) were purchased from Alfa Aesar (Karlsruhe, Germany). Harmol hydrochloride dihydrate was bought from ABCR GmbH & Co.KG (Karlsruhe, Germany). Methanol, ethanol (LC-MS grade), glacial acetic acid (100%) and formic acid (98-100%) were purchased from Merck (Darmstadt, Germany). The calibration solution was from Agilent Technologies (Waldbronn, Germany) and capillaries were from Polymicro Technologies (Phoenix, AZ, USA). All biogenic drug samples were provided by the Federal Criminal Police Office of Germany, KT-34 (Wiesbaden, Germany). Tetrahydroharmine was synthesized according to Pütz et al. 2003 [245]. A stock solution of 1 mol/L ammonium formate in glacial acetic acid was prepared and added to the BGE to achieve the desired ammonium formate concentration (see Table 1). As enantiomerically pure standards are not commercially available Stereoisomers were not discriminated and are represented via the name of the main alkaloid or the corresponding m/z value (e.g. speciociliatine, speciogynine are depicted as mitragynine or m/z = 399) in the figures.

2.3.3. SOFTWARE

The capillary electrophoresis unit was controlled by ChemStation® B.04.02 (Agilent Technologies, Santa Clara, CA, USA), the QTOF was controlled by the Mass Hunter® software B.03.01 (Agilent Technologies, Santa Clara, CA, USA). Extracted Ion Electropherograms were extracted with ±20 ppm accuracy. Simple statistics were performed with Microsoft Office Excel® (Microsoft Corporation, Redmond, USA), Origin® (Northampton, USA) and MatLab® (The MathWorks, Massachusetts, USA). The DOE was designed and evaluated with Unscrambler 9.5 (CAMO Software AS, Oslo, Norway).

2.4. RESULTS & DISCUSSION

2.4.1. AQUEOUS VS. NON-AQUEOUS BGE

Pre-studies were conducted to find a promising buffer system suitable for the optimization process and to estimate the boundaries for the DOE in order to circumvent problems arising from instable analysis conditions. Since commercial standards for Kratom alkaloids are not available, the development was performed with harmala alkaloids as model substances and a methanolic extract of dried, commercial Kratom samples. Thus, we include two different classes of indole alkaloids.

Separations in classical aqueous buffer systems (10 mmol/L ammonium acetate in 10 mmol/L acetic acid, pH = 4.7 or a 3:1 mixture of 1 mol/L acetic acid and 1 mol/L formic acid, pH = 2.1) suffered from strong adsorption effects and poor overall performance in combination with a long analysis time Figure 14A). However, in aqueous media, the expected clear separation of the two analyte classes (here harmala and the larger Kratom alkaloids) is observed, originating from the separation mechanism related to the hydrodynamic radius of each molecule. The change to a non-aqueous buffer system composed of ACN and glacial acetic acid [239] clearly changes the separation mechanism: The separation of the two analyte classes is no longer present in the non-aqueous buffer system and the separation of more diastereomers is possible in NACE (Figure 14B). We used ammonium formate as buffer electrolyte, which is soluble in the solvent mixture applied and is expected to induce selectivity changes due to a separation mechanism based on ion-pairing and heteroconjugation.

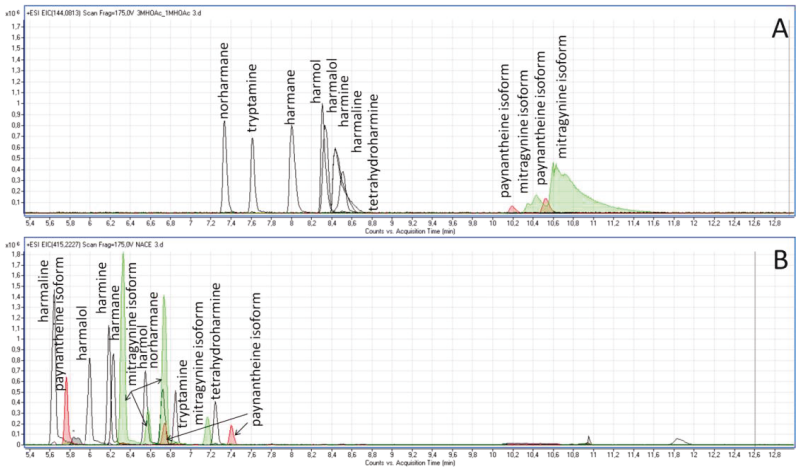


Figure 14: A) Base peak electropherogram of a mixture of harmala alkaloid standards and a methanolic Kratom extract (shaded) in an aqueous buffer system (3:1 mixture of 1 mol/L acetic acid and 1 mol/L formic acid, pH = 2.1); B) Base peak electropherogram of a mixture of harmala alkaloid standards (black) and a methanolic Kratom extract (shaded) in a non-aqueous buffer system (35 mmol/L ammonium formate in a mixture of acetonitrile/acetic acid (875:125))

2.4.2. DOE-DEVELOPMENT: PROCESS VARIABLES

The choice of the process variables was based on the importance of the parameter for separation and detection of the coupled CE-MS system and the need for its multivariate optimization. Thus, all BGE parameters were taken into account. Regarding mass spectrometry, parameters such as the fragmentor voltage were not included in the DOE as it was regarded to be largely independent of others and thus justifies a simple univariate approach. Other parameters such as drying gas temperature and drying gas flow rate were observed to have a negligible influence on the separation and detection during pre-studies (data not shown) and were thus not included in the DOE as the number of necessary experiments for the DOE would otherwise largely increase (with the 6 parameters chosen finally, 167 experiments had to be conducted, one additional parameter would have increased the number of experiments to 299. Please refer to the description of the DOE in the introduction for the calculation of the number of experiments). The following parameters were considered for the DOE:

The composition of the buffer is the most important parameter for separation in capillary electrophoresis. pH-value, ionic strength, conductivity, viscosity, heat capacity are only some of the many properties that change with the composition of

the buffer. Since it is impractical to use different types of solvents as buffer within the DOE (they represent categorical data), pre-studies were conducted to find a suitable composition and concentration range of buffer components, as described above. As a result, a mixture of ACN and glacial acetic acid was used in this DOE, which we observed previously to yield good separations with high resolution for basic analytes (for harmala alkaloids [239] and unpublished results for basic drugs of abuse). Starting from this suitable basic buffer composition, we varied the ratio of ACN to AcOH content as design **Variable 1: Buffer**. The addition of small amounts of ammonium formate to increase pH and ionic strength and the type of co- and counter-ions for the analytes (in addition to acetic acid) showed strong effects on the selectivity in pre-studies. Therefore, the concentration of ammonium formate in the buffer was chosen as a second BGE-related design **Variable 2: Formate.**

Previous CE-MS investigations [246-249] showed that for each analyte the sheath–liquid composition regarding the solvents and their ratios (here 2-propanol and water) must be considered when optimizing a CE-MS method. The composition of the sheath-liquid was thus introduced as design **Variable 3: SL-Comp**. Obviously, the influence of the acid content is an interesting parameter with ambiguous influence depending on the characteristics of the analytes of interest [250,251]. Therefore the amount of glacial acetic acid added to the sheath–liquid was chosen as design **Variable 4: SL-Acid**.

Besides the ambivalent role of the sheath-liquid composition on ionization efficiency and thus signal intensity of analytes, it is often reported that a high SL-flow rate and high nebulizer pressure influence the separation by a suction effect and thus the introduction of a parabolic bulk flow. This may lead to a reduction of the residence time in the electrical field and thus a reduced resolution, but also lowered separation efficiency due to band broadening [249-251]. The presence of a suction effect is likely with our BGE as it has a very low viscosity (see values above). We investigated this effect by varying the sheath- liquid flow rate and the nebulizer pressure as design **Variable 5: SL-Flow** and **Variable 6: Nebulizer**.

The parameter range of all process variables was intentionally set as broad as possible and is given in Table 3.

2.4.3. DOE-DEVELOPMENT: RESPONSE VARIABLES

2.4.3.1. SEPARATION

The choice of a response variable reflecting the quality of the separation is not trivial. The separation efficiency quantified with plate numbers was observed not to be a robust parameter as its determination showed a high RSD of 20% (using 15 repetitions of the center point) mainly due to differences in the peak width. In general, plate numbers were high in nearly all measurements of the DOE with N ~ 100 000 on average for the center point measurements. In order to establish a response variable for the separation power of an analysis, a variable based on effective electrophoretic mobility is defined, which is independent of the peak width, reflecting the electrophoretic resolution:

$$R_{\mu_i} = \frac{\Delta\mu_{eff}}{\overline{\mu}_{eff}}$$

Equation 4: With $\Delta\mu_{eff}$ *= the difference in effective electrophoretic mobilities of a pair of neighboring peaks and* $\overline{\mu}_{eff}$ *as the mean effective electrophoretic mobility of the peak pair.*

This equation was already employed to characterize the electrophoretic resolution by Balaguer and Neusüss [252]. EOF determination for the calculation of effective electrophoretic mobilities was performed with regard to the neutral acetophenone peak and the electrical current profile. The resulting numerical value R_μ, which characterizes the separation power of a single run is obtained by addition of the single $R_{\mu i}$ values of all neighboring signals in the run (Eq. 5).

$$R_\mu = \sum_1^i (R_{\mu 1} + R_{\mu 2} + \ldots + R_{\mu i})$$

Equation 5: Separation power R_μ

An advantage of the use of effective electrophoretic mobilities is that differences in electroosmotic mobilities between runs can be eliminated mathematically. Some authors have used the term of $R_{\mu i}$ to describe selectivity and selectivity changes [253]. This does not apply here as with $R_{\mu i}$ we do not follow one specific analyte pair, because of the frequent changes in migration order (see e.g. Figure 15). Instead, for

each electropherogram we calculated $R_{\mu i}$ for every neighboring peak pair present, regardless of the identity of the signal itself. The separation power R_μ thus reflects the overall separability of the analytes of interest rather than the separability of one or several selected pairs of specific analytes. The validity of the response variable was tested and is impressively shown in Figure 15: The superior separation is well reflected in R_μ values of 0.67 and 0.96 and R_μ can be regarded as appropriate for the description of the separation quality. It is interesting to note, that the separation capacity reflected in the increase of the migration window between the first and the last analyte [254], is also comprised by R_μ. However, the use of the peak capacity directly as a response variable cannot be recommended especially for structurally closely related analytes and the investigation of different analyte classes: Here a large peak capacity does not tell about the separation between analytes as a large peak capacity could also be obtained, if only two groups with non-separated analyte classes are present in the electropherogram (similar to e.g. the electropherogram in Figure 14A). In contrast, a high R_μ value is obtained in case of a high peak capacity in combination with ideally evenly spread analytes. As visible from Figure 15, also minor changes in the separation of analytes can be comprised with R_μ, thus avoiding subjective judgment of separations. The use of R_μ facilitates the evaluation of the separation power as a visual inspection of both electropherograms would be difficult, since overlapping peaks exist in both electropherograms and differences in the peak shape might influence the judgment as well.

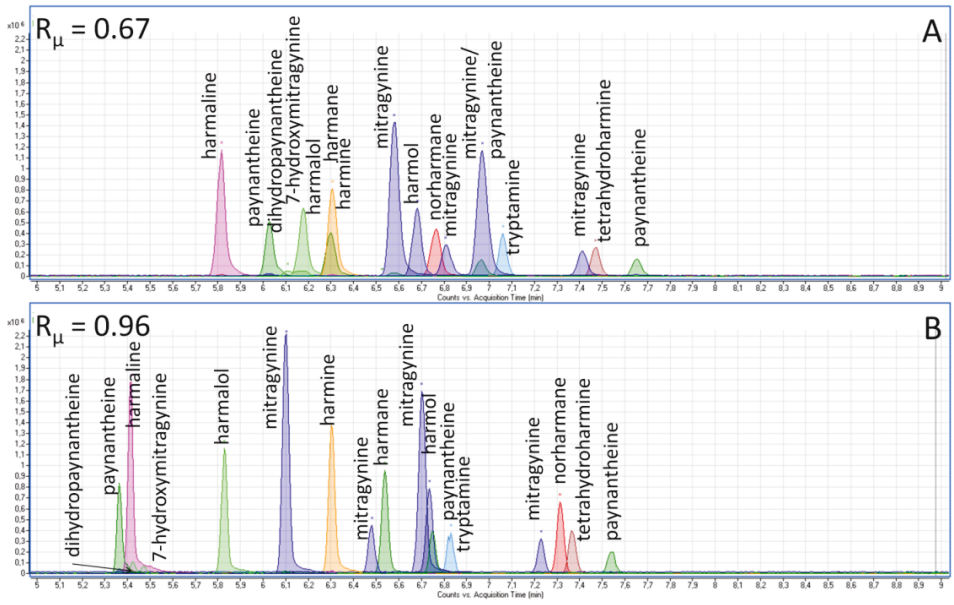


Figure 15: Extracted ion electropherograms of the same sample under different experimental conditions as validation for R_μ: A) Buffer: 26 mmol/L ammonium formate in 80:20 (ACN/AcOH); sheath-liquid: 59% 2-propanol in water (R> 18 MΩ) with 1.8% AcOH B) 45 mmol/L ammonium formate in 90:10 (ACN/AcOH); sheath-liquid: 84% 2-propanol in water (R> 18 MΩ) with 3.4% AcOH, sheath-liquid flow rate 4 µL/min, and nebulizer pressure 6 psi.

2.4.3.2. DETECTION

To characterize the overall signal intensity, the signal height of all integrated peaks was summed up, as sum parameter SH.

$$SH = \sum_{1}^{j} (SH_1 + SH_2 + ... + SH_j)$$

Equation 6: Sum parameter SH

By the combination of all peak heights to a sum parameter, the individual optima of every analyte might be missed, but it is assumed that this approach leads to a global optimum within the boundaries of the DOE. The signal to noise ratio was discarded as a parameter, since different spray conditions influenced the S/N of the analytes to an unpredictable extend leading to unreliable results.

2.4.3.3. OUTLIER TREATMENT

To ensure that the DOE is undisturbed by instable conditions, only intensive analyte mass traces were used, as some of the very low abundant analytes from the Kratom extract were undetectable for a few process parameter settings. Despite the careful choice of parameter ranges during the pre-studies, the parameter combination of a low **SL-Flow** with a low **Nebulizer** pressure was not sufficient to create a stable spray and it was not possible to determine peak parameters from the extracted ion electropherograms. Thus, this run had to be provided with an artificial numerical value marking the coding parameters as worst case, while matching the general population of R_μ to circumvent contortions of the results. The value was therefore set to 90% of the minimum value of the population of R_μ or SH.

2.4.4. DOE-RESULTS

Significant ANOVA results for R_μ and SH from the DOE are presented in Table 4. All results are graphically summarized in Figure 16, which shows the significant separation process variables in the upper, the detection process variables in the lower section. Interaction of variables is indicated with double arrows. The effects on R_μ and SH are visualized with solid arrows for linear and broken lines for quadratic dependencies. It can easily be seen, that signal height is influenced by nearly all process variables including detection and BGE-related process variables, whereas the response variable R_μ is predominantly influenced by the BGE-related variables, but not by MS-related experimental settings.

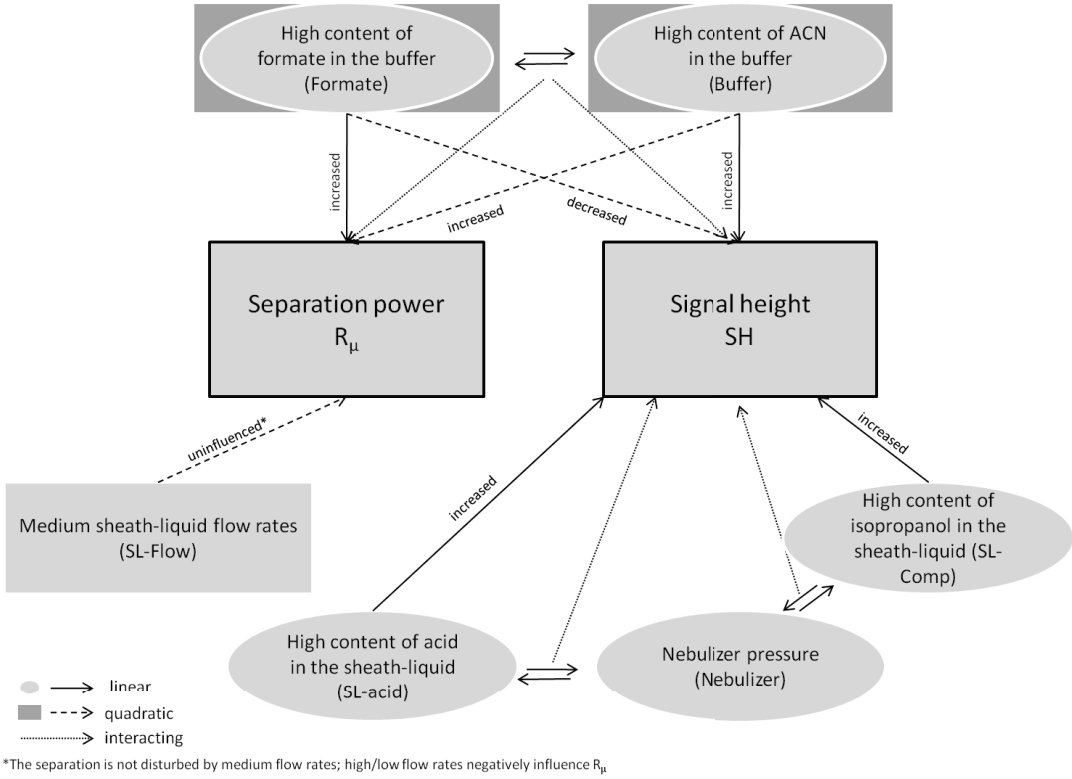


Figure 16: Visualization of the interactions and influences of the process parameters on the response variables. Capillary electrophoretic parameters are shown on top, mass spectrometric parameters are shown below the response variables.

Table 4: Significant (p < 0.05) results of the ANOVA for the separation power Rμ and the signal height SH

Process variable	p-value Rμ	p-value SH
Nebulizer	0.8158	0.5386
Buffer	0	0
Formate	0	0
SL-Flow	0.2842	0.1282
SL-Acid	0.1703	0
SL-Comp	0.5252	0
Nebulizer & Buffer	0.2832	0.607
Nebulizer & Formate	0.5577	0.4896
Nebulizer & SL-Flow	0.2511	0.9188
Nebulizer & SL-Acid	0.9446	0.0119
Nebulizer & SL-Comp	0.1739	0.0211
Buffer & Formate	0.0028	0.0007
Buffer & SL-Flow	0.3113	0.9925
Buffer & SL-Acid	0.3099	0.2465
Buffer & SL-comp	0.6253	0.7929
Formate & SL-Flow	0.1231	0.2448
Formate & SL-Acid	0.9733	0.1011
Formate & SL-Comp	0.6635	0.2789
SL-Flow & SL-Acid	0.8411	0.804
SL-Flow & SL-Comp	0.6034	0.082
SL-Acid & SL-Comp	0.9742	0.9001
Nebulizer & Nebulizer	0.555	0.9808
Buffer & Buffer	0	0.6178
Formate & Formate	0.5754	0.0042
SL-Flow & SL-Flow	0.0002	0.1036
SL-Acid & SL-Acid	0.9597	0.0848
SL-Comp & SL-Comp	0.0919	0.4513

For the separation power R_μ, the acetonitrile content (**Buffer**) and the ammonium formate concentration in the buffer (**Formate**) as well as interacting effects of the variables **Buffer** and **Formate** are significant. A significant quadratic dependence on the separation profile from the **SL-Flow** and the **Buffer** is present (Table 4). The highest obtained value for R_μ (best separation) was 1.8 and the lowest value was

0.56. The effects of **Formate** and **Buffer** are demonstrated in the electropherograms in Figure 17 and their dependency is visualized in Figure 18A.

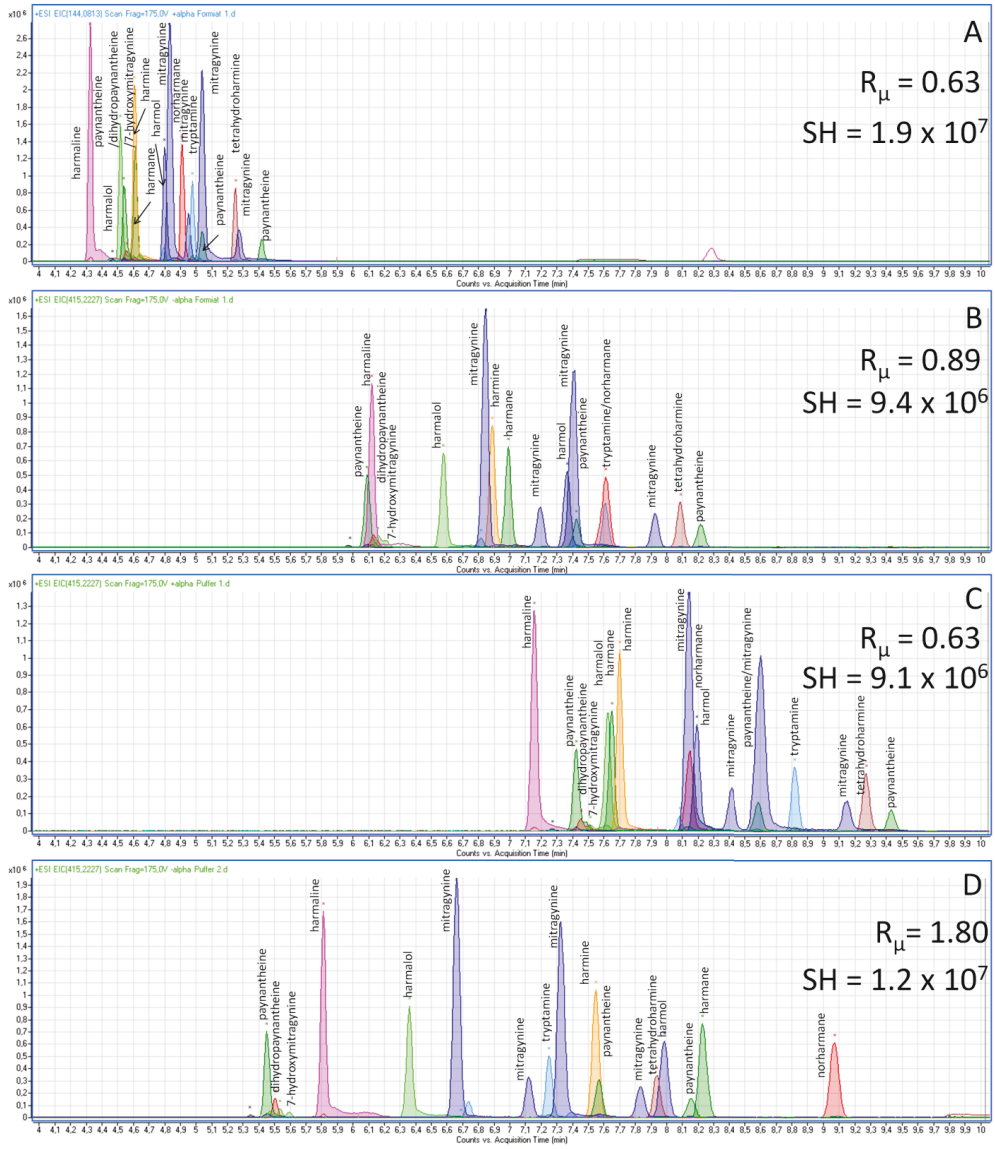


*Figure 17: Electropherograms of the alpha-points (extreme values) from the DOE. Experimental conditions as in Table 1 (centerpoint) except: A) content ammonium formate: 10 mmol/L (-alpha **Formate**); B) content ammonium formate: 60 mmol/L (+alpha **Formate**); C) buffer composition: 687:313 (ACN:AcOH) (-alpha **Buffer**); D) buffer composition: 1000:35 (ACN:AcOH) (+alpha **Buffer**)*

The ANOVA results for the dependence of the response variable SH (Table 4) on the DOE process variables show significant linear influences by **Buffer**, **Formate**, **SL-**

Acid and **SL-Comp** (see Figure 18B, C & D). Significant interacting parameters are the **Nebulizer** with **SL-Acid** and the **Nebulizer** with **SL-Comp**. Combined effects are also present for **Buffer** and **Formate**, similar to what has already been observed for the separation power. The highest obtained value for SH was 2.8 x 10^7 the lowest value was 0.36 x 10^7.

2.4.4.1. SEPARATION POWER R_μ

It can be concluded from the ANOVA that the MS-variables **Nebulizer**, **SL-Acid** and **SL-comp** ($p > 0.05$) are robust parameters for the separation conditions within the boundaries of the design and their effect on the separation power and thus resolution is negligible in this experimental setup. Optimization for signal intensity can thus be achieved independently of the separation conditions. Although these results are pleasant, they were rather unexpected, since Chen et al. [255], Peri-Okonny et al. [256] and others [249-251] described significant effects of the nebulizer pressure on the separation conditions by induction of a parabolic flow profile leading to band broadening. Therefore, the peak width was determined in a separate set of experiments for 5 different stages of the nebulizer pressure (2, 5, 6, 7, 9 psi) but no significant effect was observed (mean peak width = 0.13), but one has to keep in mind that an increase of a flow by a suction effect also leads to a faster analyte transport through the detector, artificially creating narrower signals. The effects of a parabolic flow are therefore difficult to predict and conversely discussed in the literature, as e.g. Soga et al. [257] found these effects negligible, while Ruiz-Calero et al. [258] found these effects significant. However, the robustness of the separation regarding **Nebulizer** pressure in our study stems from the overall high EOF present and it can deduced that the effect of the **Nebulizer** is pronounced only during injection. The suction effect of the nebulizer was quantified in a simple experiment (injection of a sample plug without application of voltage, while recording the time the plug needs to travel to the detector) to exhibit a flow rate of << 1 cm/min. This flow rate is not sufficient to influence the separation, since the EOF velocity is much higher, however it is sufficient to create the problems during injection as described in Section CE-MS. The experimental setup chosen here, circumvents these arising problems by reduction of the nebulizer pressure during injection (2 psi). Another explanation is that the usage of effective electrophoretic mobilities instead of migration times eliminates the influence of the suction mathematically.

The effect of the interacting parameters **Buffer** and **Formate** on R_μ are shown in Figure 18A. Separation power increases with increasing amount of ACN in the buffer on the one hand, and with increasing formate concentration on the other. The effects of these two process variables on R_μ have to be discussed concertedly as they are significantly correlated: It is anticipated, that all analytes are fully protonated in every buffer composition within the DOE with AcOH as strong protogenic solvent. ACN and AcOH have low dielectric constants relative to water (ε_r = 78.39) [97]. Thus ion stabilization is relatively low (especially compared to water) and ion-pair formation is strongly enhanced [230]. In the BGE composition chosen, ion-pairing between the analytes and acetate will be present. In addition, heteroconjugation is expected in ACN (see also discussion on the separation mechanism 2.4.6).

The separation power increases with increasing ammonium formate concentration in correlation with a high ACN content. On the one hand, this is a simple effect of the ionic strength, reducing the EOF, pronouncing the electrophoretic separation and leading to a superior resolution with extended analysis time (Figure 17A & B). As will be discussed below, ammonium is involved in heteroconjugation, strongly effecting the separation. A third aspect of the addition of ammonium formate might be the increase in the apparent pH* of the BGE [238,256], which might introduce some separation selectivity due to differences in the apparent pK_a* values of the analytes. It is interesting to note, that ACN broadens the separation window towards a lower migration time range without increasing the analysis time (Figure 17C & D).

Ideal separation conditions according to the model are consequently a combination of a high concentration of ACN and formate. In order to find a true optimum, the borders of the DOE would have to be extended. Within the frame of this DOE, the slope of the R_μ dependence on the process variables **Buffer** and **Formate** near the highest value of R_μ is at its maximum, pointing to a lowered robustness. However, the repeatability of separation and detection parameters at conditions reaching highest R_μ with this BGE composition were acceptable with 2.3% RSD for migration time and 12.5% RSD for the relative peak area. Linearity was given for the harmala alkaloids ranging from 0.1 µmol/L to 0.5 mmol/L on average (calculations were performed according to the guidelines of the GTFCh [259]). The results are acceptable with regard to the fact that all these experiments were conducted in the complex matrix of a Kratom plant extract.

A quadratic dependency for R_μ on the parameter **SL-Flow** was found (Figure 19). An optimum close to the centerpoint is visible with sloping at higher and lower flow rates. Although this effect is significant, its overall impact is comparatively low (R_μ range ≈ 0.58-0.72). The influence is expected to be a suction effect [260] leading to shortened residence times of the analytes in the electric field, thus less time is available for separation. Optimizing the **SL-Flow** is therefore not an improvement of the method, but rather the elimination of a disturbing effect.

The results of the detailed investigation of the separation power and its influencing factors are within the expectations and prove that the separation at high EOF is largely undisturbed by potential influences of the interface coupled to the system, except the influence from **SL-Flow**. The separation can thus be regarded to be independent from the detection or MS process variables and can be optimized with respect to the maximum separation power (Figure 16, top section). At least for systems with a high EOF, we can thus conclude, that separate DOEs can be setup regarding separation vs. detection conditions. This significantly reduces the number of experiments needed.

2.4.4.2. SUM OF SIGNAL INTENSITIES SH

The ANOVA results from Table 4 (see also Figure 16) clearly demonstrate the necessity of a DOE: The MS-parameters regarding the electrospray cannot be optimized independently from each other as a number of interacting process variables is present. Therefore, two general effects have to be taken into account affecting SH: (i) finding optimal conditions for the formation of a stable electrospray as well as (ii) finding conditions to provide ideal global ionization conditions for the analytes. Only a critical evaluation of both effects leads to a robust method with low overall detection limits.

The dependency of SH on the MS-parameters **SL-Comp**, **SL-Acid** and **Nebulizer** is shown in Figure 18B & C. Comparison of both figures shows a similar behavior for the response value with regard to the process variables **SL-Acid** and **SL-Comp**: A maximum for SH is reached for highest **SL-comp** or **SL-Acid** values together with the lowest **Nebulizer** adjustments used in the DOE. High **SL-Acid** values aid the analyte ionization processes due to the lower pH-value. **SL-Comp** determines the volatility, viscosity and surface tension. Thus, on the one hand they determine the

conditions and the robustness of the electrospray and influence the ionization efficiency of the analytes for detection on the other. A high content of 2-propanol leads to a highly volatile sheath-liquid with a low surface tension (coefficients of surface tension are: 2-propanol: 2.124 10^{-2} Nm^{-1}, water 7.181 10^{-2} Nm^{-1}, ACN 2.760 10^{-2} Nm^{-1}. The surface tension coefficients for water : 2-propanol mixtures range from 2.228 10^{-2} Nm^{-1} for a 50 : 50 mixture down to 2.165 10^{-2} Nm^{-1} for a 10 : 90 mixture [50,261]); this favors ionization by facilitating coulombic explosion in the electrospray at an early stage of the ionization process. However, in this NACE setup the additional effect of **SL-Comp** on the ion-pair formation present in the BGE of low dielectric constant or disruption has to be taken into account. Sensitivity will be reduced in SL solutions enhancing ion-pair formation as ion-pairs are more difficult to be ionized than solvated free ions. The ion-pairs may be disrupted using more aqueous or more protic SL conditions evoking better signal intensities.

Changes in the dielectric constant of the electrospray solution can be induced by changes of the composition of the SL or the BGE. For a high content of ACN in the BGE (low values for the variable **Buffer**) with its relatively low dielectric constant (ε_r = 37.5) and its extremely low tendency to stabilize ions by hydrogen bonding, ion-pair formation in the electrospray is enhanced leading to a reduced ionization efficiency. In contrast, the dielectric constant of mixtures of 2-propanol and water are significantly higher (ε_r = 19.92 (2-propanol), 78.39 (water)) [50]. Hydrogen bonding is strong in these polar solvents, thus ion-pair formation is reduced and higher signal intensities can be expected. The sheath-liquid composition with a higher polarity (**SL-Comp**) and higher salt content (**SL-Acid**) is also suitable to close the electric contact of the CE as the SL conductivity is higher. In addition, the higher conductivity in conjunction with a higher viscosity compared to the BGE will most likely induce a stacking effect at the BGE:SL interface with higher plate numbers and peak heights accordingly.

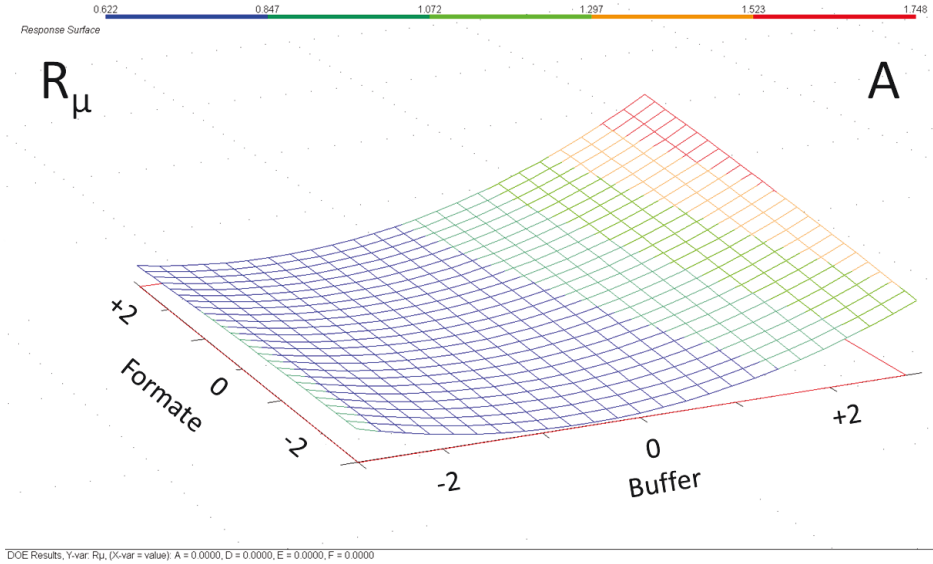
Figure 18D shows the effects of buffer composition (**Buffer**) and ammonium formate concentration (**Formate**) on the signal height. A high ammonium formate concentration leads to low ionization efficiencies for the analytes when accompanied by a high ACN content in the BGE. Contrary, ammonium formate facilitates ionization when accompanied by a high AcOH content in the BGE, although with less overall effect. As a consequence, the best choice for high overall signal strength is a low

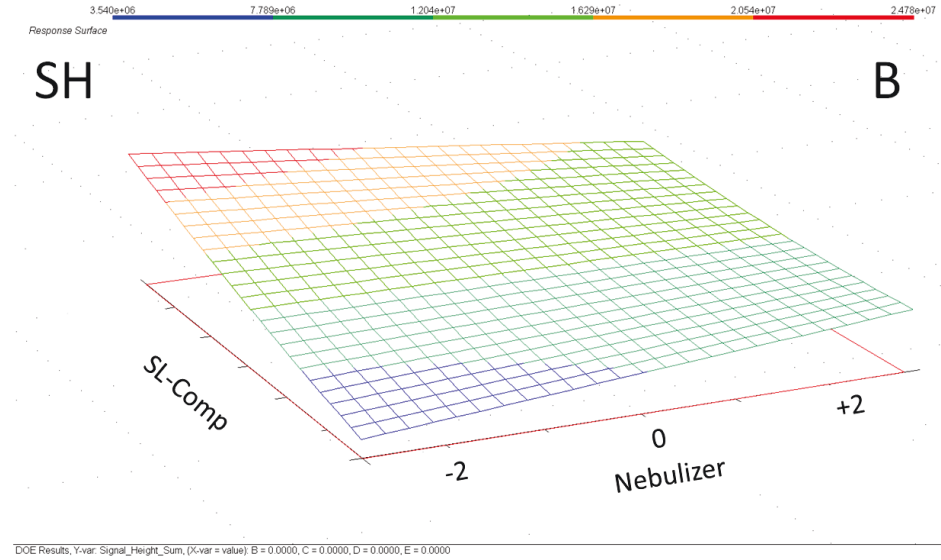
concentration of ammonium formate and a high content of ACN. This finding can be explained by two independent processes: The first process is the quenching process, originating from the ion-pairing as described above. In addition, a high ammonium formate concentration increases the apparent pH* and thus less protons are available for ionization in the electrospray, leading to severe quenching effects [210]. Another possible explanation is a simple diffusion process: Since increased formate concentration leads to longer analysis time, the effect of longitudinal diffusion is more pronounced, producing broader peaks with reduced signal height [262]. This counteracts a possible stacking effect, which may be present due to conductivity differences at the interface. However, due to the separation mechanism most likely involving ion-pairing and heteroconjugation, this effect is difficult to judge without a further experimental basis.

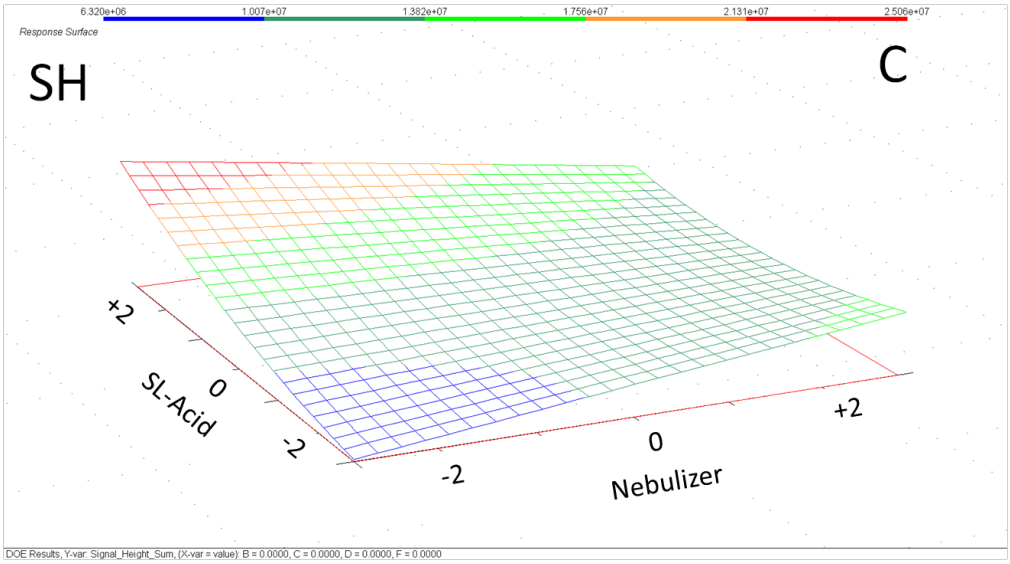
Interestingly **SL-Flow** has no significant influence on the signal height. Consequently the effect of the sheath-liquid flow rate can be neglected within the boundaries of the model and adjusted to generate a robust, low-noise spray without interfering with the detection. This is in contrast to most work published in the literature [246] and a result of two counteracting processes balancing each other: As observed in the pre-studies, where the BGE was shown not to be suitable as sheath-liquid (data not shown), the applied buffer system is unfavorable for the ionization efficiency of the analytes. This is due to both the concentration of formate and ACN in the BGE leading to increased pH* values and increased ion-pair formation as described above. The introduction of the SL with its composition suitable for efficient analyte ionization in this special case leads to a dilution of the quenching buffer solution [263] in the electrospray and therefore improves ionization efficiency and thus detection limits by breaking up the ion pairs. This is especially important as the EOF velocity is generally high and thus is the flow rate of buffer into the electrospray. This positive effect of diluting the unfavorable BGE effluent is balanced by the simple dilution effect. Most probably the combination of these two effects leads to the low overall impact of the sheath-liquid flow rate on the signal height in this NACE-MS setup.

In summary, signal height depends on all process variables (except the SL-flow rate) (Figure 16) as electrospray properties are strongly influenced by all parameters. The strongest effect was observed for **Formate**. In every case where a significant interaction or dependency was observed, the assumed ideal value was beyond the

borders of the DOE (and sometimes outside the experimental parameter range to ensure a stable electrospray or stable separation process), but signal intensity was always sufficient for the complete analysis of all analytes included in the study.







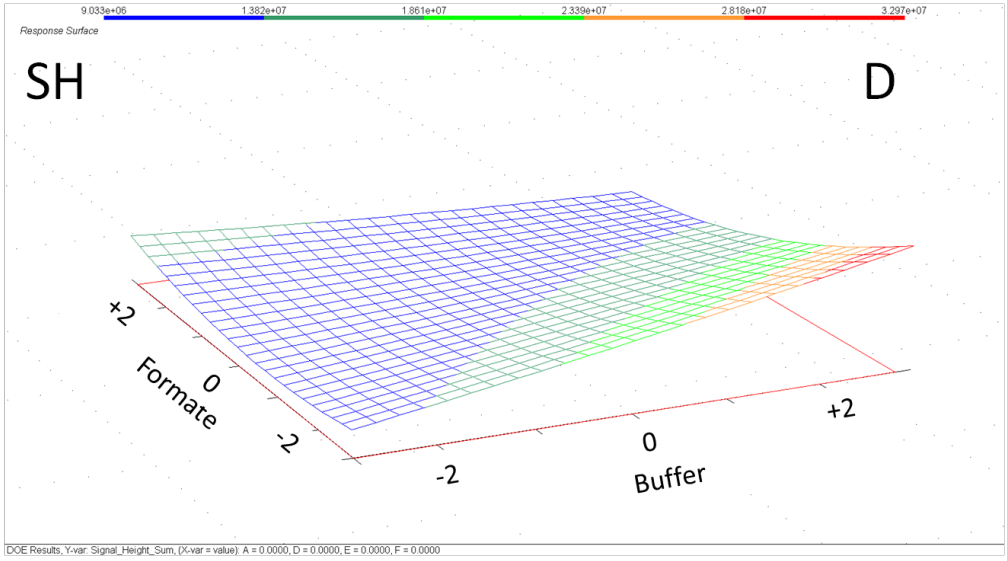


*Figure 18: A) Response surface landscape plot for the response variable R_μ depending on the **Buffer** concentration versus the **Formate** concentration; B) Landscape plot of the dependence of the response variable SH on **SL-Comp** and **Nebulizer**; C) Landscape plot of the dependence of the response variable SH on **SL-Acid** and **Nebulizer**; D) Landscape plot of the dependence of the response variable SH on the process variables **Formate** and **Buffer***

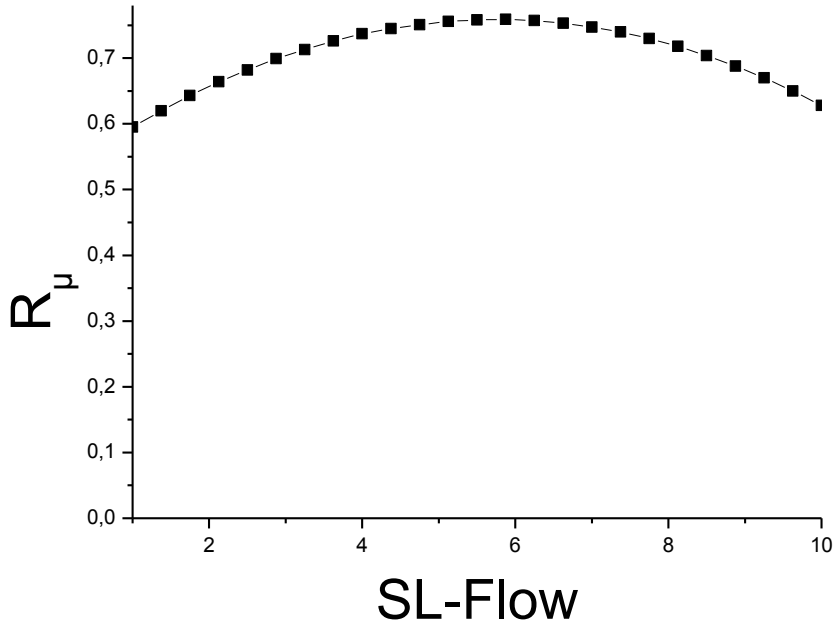


Figure 19: DOE-Results of Rμ plotted versus SL-Flow.

2.4.4.3. OPTIMIZED PROCESS PARAMETERS

The optimal choice of process parameters is a compromise of high separation power and strong signal intensity. Since MS-detection requires separation of diastereomers to differentiate between them, the parameters **Buffer** (1000 µL)**, Formate** (60 mmol/L) and **SL-Flow** (7 µL/min) were identified as optimal values for R_{μ}, while **SL-Acid** was set to the optimal value for SH (5%). **SL-Comp** (66%) and **Nebulizer** (6 psi) were set slightly below/above the optimal value for SH as a compromise to take the robustness of the electrospray into account. The validity of these optimal parameters was confirmed with the separation of the alkaloid ingredients of a Kratom extract: Figure 20 shows that a very good separation for major and minor abundant alkaloids was obtained. The optimized method is applied in a provenience study on Kratom (see Chapter III & V).

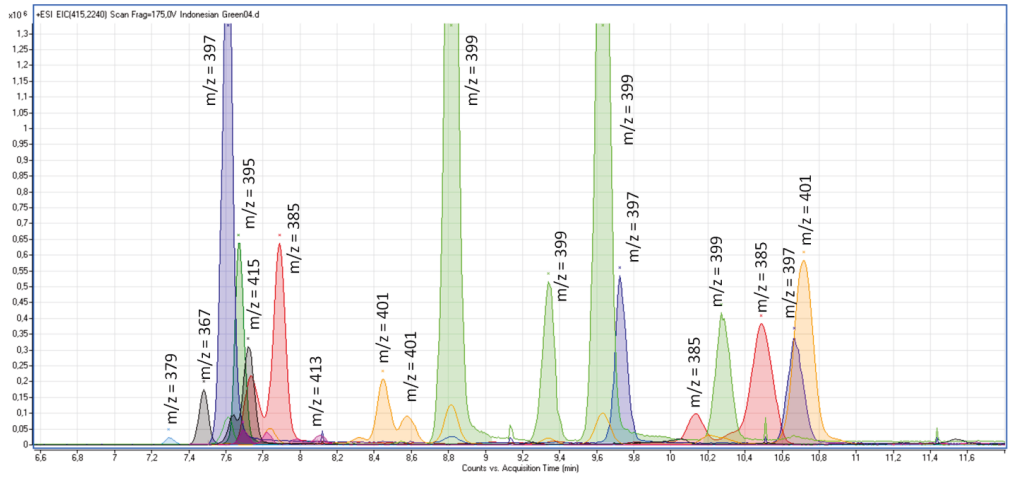


Figure 20: Extracted ion electropherograms of the Kratom sample "Indonesian Green". See Figure 13 for m/z-values.

2.5. DISCUSSION OF THE INFLUENCE OF THE BACKGROUND ELECTROLYTE

First of all, our results show that a BGE composed of acetonitrile, glacial acetic acid and ammonium formate is principally suitable for the separation and detection of indole alkaloids in NACE-MS over a broad range of acetonitrile content and ammonium formate concentration. With all BGE systems employed during the DOE, stable electropherograms could be obtained and no interday shift could be observed according to the centerpoint analysis. Compared to aqueous BGEs, adsorption of the analytes onto the capillary wall is clearly suppressed, most probably due to the destabilization of the anionic charge of the silanol groups on the capillary wall in the non-aqueous BGE with its low dielectric constant as well as ion-pairing and heteroconjugation of the analytes and BGE constituents. Figure 14 clearly shows that the NACE method is based on a different separation mechanism compared to aqueous CE with the two analyte classes not being separated any more. In addition, the migration order changes significantly, e.g. harmaline is the prelast harmala alkaloid in aqueous CE (Figure 14A), but the first to be detected in NACE (Figure 14B). Harmine and harmaline almost co-migrate in the very acidic aqueous BGE (pK_a values in water: harmine 7.45, harmaline 9.55 [264]), whereas a strong resolution is visible in NACE. The migration order also changes significantly among the other harmala alkaloids in NACE, clearly visible in Figure 17C vs. D.

The evaluation of the detailed separation mechanism present in the BGE of very low dielectric constant and with the protogenic solvent acetic acid is beyond the scope of this article but will be studied in further detail in future. However, from the results presented here and from literature data, several assumptions can be made: Using organic solvents instead of water for the BGE results in a large number of changes in physicochemical constants. The most important are the changes in the viscosity and dielectric constant or better, the solvents ratio of dielectric constant vs. viscosity (ε_r/η) [97], directly related to electrophoretic and electroosmotic mobility. Large changes in the pK_a values of acids and smaller changes of cation acids in the form HB^+ as present in our work have been described to evoke significant changes in the separation selectivity used for the optimization of the separation [237,238]. In our work we use the dipolar aprotic solvent ACN and the protogenic solvent acetic acid. A similar combination has not often been applied in NACE, mostly an amphiprotic solvent like methanol is used in conjunction with acetonitrile instead of AcOH. A few publications used protophilic solvents such as N-methyl formamide [225]. With the protogenic solvent AcOH in ACN, we expect the analytes to be fully charged (see [64]) because of AcOH's high proton-donating tendency. Even bases being very weak in water will at least be partially protonated. The separation and more importantly the selectivity changes present in this study are thus not dominated simply by differences in the pK_a values.

For organic solvents, different analyte-solvent as well as analyte-electrolyte interactions have to be taken into account to understand the separation mechanisms in NACE. These involve ion-ion, and ion-dipole (either permanent or induced) interactions or dipole-dipole interactions as well as dispersion forces and more importantly hydrogen-bonding [265,266]. The fraction of charged analyte species of the total amount of B (HB^+ or charged conjugates) relevant for the electrophoretic separation depends on all these factors. For the mixtures of the two solvents employed here, we have to take strong ion-pairing and heteroconjugation and to a minor extend homoconjugation into account. Ion-pairing in NACE has been discussed in great detail in the literature in various solvents as separation mechanism [46,227,230-232,267] and has been used for chiral discrimination using chiral ion-pairing reagents [136,226,233]. Likewise some authors studied heteroconjugation as a separation mechanism in NACE [234-236].

Acetic acid is highly associated into dimers $(AcOH)_2$ [265] in equilibrium with ions present as ion pairs $[AcOH_2^{+\cdots}AcO^-]$. The autoprotolysis constant is referred to as 14.45 [265]. It differs to water with a similar autoprotolysis constant by its high proton-donating tendency. Due to its low dielectric constant protonated anions will form (neutral) ion-pairs in the form $[AcOH_2^{+\cdots}A^-]$ or higher aggregates and only slight dissociation into free ions will be present. Likewise, bases will be involved in ion-pairing in the form $[BH^{+\cdots}AcO^-]$. Dissociation into free ions is not likely to be present. A similar situation is present for acetonitrile, where the dissociation of ion-pairs involving the protonated base BH^+ is likewise not present to a large extent. The predominant equilibrium involved here is the formation of homoconjugates of the form $BH^{+\cdots}B$ [265].

For our BGE this would indicate, that complete ion-pairing is present with the analytes and acetate or formate (with acetate having a higher ion-pair formation constant [230]). A disturbance or reduction of this ion-pairing with formate as envisioned at the beginning of the study does not take place as all BGEs tested, composed of mixtures of ACN, acetic acid and different amounts of formic acid (no ammonium salt added), revealed neutral analytes, transported with the EOF together with the neutral marker. However, the addition of ammonium formate obviously led to the heteroconjugation of either BH or the ion pair $[BH^{+\cdots}OAc^-]$ with NH_4^+, thus giving rise to an electrophoretic mobility of the analyte species different from zero. The separation mechanism in this study thus involves several equilibria such as ion-pairing, the formation of hetero- and to a minor extent homoconjugates and eventually the dissociation of the ion-pairs into free ions. For the separation, the mobilities of all charged analyte species (heteroconjugates and free analyte anions) also have to be taken into account to describe the separation. Lämmerhofer et al. [233] made similar observations with neutral analytes with and without the addition of a suitable co-ion. Also there, the co-ion was described to influence the separation. The explanation, however, was based on the presence of competing ion-pairing equilibria with constituents of the BGE composed of ACN with triethylamine and acetic acid or octanoic acid. A more detailed study on the separation mechanism has to be accomplished.

The separation power is shown in this DOE to strongly depend on both ACN content and ammonium formate concentration with the two variables being strongly

correlated (see Figure 18A and Table 4), clearly showing the need for a multivariate approach for method optimization and the understanding of the underlying effects of the separation in the case of Kratom and harmala alkaloids. Interestingly, the effect of the ammonium formate concentration is only pronounced at a high content of acetonitrile (and a low content of glacial acetic acid). With the separation mechanism discussed here, the vast selectivity changes (see Figure 17) we observed during our study can be understood from the different roles of acetonitrile/acetic acid and ammonium formate in influencing the equilibria of ion-pairing and heteroconjugation as well as the electrophoretic mobilities of the charged analyte species involved.

A further aspect of NACE has to be stated: In aqueous CE, several diastereomers of Kratom alkaloids could not be separated (see Figure 14A). The NACE method presented here thus provides a promising alternative to the use of complexing agents like cyclodextrins with only limited compatibility with MS detection. The impressive resolution observed between diastereomers (e.g. mitragynine diastereomers Δt = 1 min) might be a result of the combined effects of the absence of a large solvate-shell around the analytes in aprotic solvents, like ACN, exhibiting low analyte-solvent interactions and a different ion-pairing constant of the diastereomers originating from steric hindrance. For mitragynine and its stereoisomer speciociliatine large structural differences and thus a large difference in the analyte radii were described [3]. Interestingly, these large differences evoke a very high resolution only in NACE but not in aqueous CE.

High ammonium formate concentration leads to a lowered EOF velocity (see Figure 17A and B). The opposite holds true for high acetonitrile content. This leads to the unique opportunity to optimize the selectivity and the speed of analysis (due to the EOF velocity) by adjustment of the ammonium formate concentration vs. ACN content (compare Figure 15 and Figure 17B vs. C) to achieve a fast separation with a large separation window. This is visible in Figure 15A & B, where the analysis time is the same, but different selectivity and a higher peak capacity is visible in B. The separation window here is nicely widened towards lower migration times.

With the BGE systems involved here, it can clearly be seen that at the high EOF conditions present, the NACE separation is not influenced by the coupling of an electrospray interface. Thus, the detection parameters can be optimized

independently after the optimization of the separation conditions without interfering with the CE.

In literature, the SL interface is very often regarded to negatively influence the signal intensities by a dilution effect. In contrast, with the DOE design chosen, we clearly show that in our case, a dilution of the capillary effluent has a positive effect, as a reduction of quenching from the BGE at the high EOF present, mainly due to ion-pairing or conjugation can be achieved by the addition of sheath-liquid. This points to the fact that the separation can be optimized first and quenching effects by the BGE can afterwards be reduced by a proper selection of the composition and flow rate of the sheath-liquid. A similar effect was observed for intact proteins [263], where the signal intensities were clearly enhanced when using an optimized sheath-liquid instead of a sheath-liquid made of BGE. In a third step, the negative influence from the **SL-Flow** and the **Nebulizer** could be minimized to provide optimal analytical conditions. However, one should be aware that the response surface methodology used for the DOE evaluation points to theoretical optima outside the investigated parameter range, which are, however, beyond robust or even stable CE-MS conditions e.g. for the lowest investigated **Nebulizer** pressure adjustment (see Section DOE-development: Process variables).

2.6. CONCLUSIONS

The application of a DOE as a tool for method development starting with a very broad parameter range was successful and revealed the interactions of parameters, which are not sizeable in classical univariate method development. The introduction of the separation power R_μ as an interesting parameter was proven to be very useful to judge separation quality as it comprises resolution as well as peak capacity within one parameter. The validity of this response variable is obvious from our results. A further optimization may include the overall analysis time. Another option would be to introduce a limiting upper value for $\Delta\mu_{eff}$ in this CCD approach in order to avoid an overestimation of strongly resolved peak pairs and focusing on poorly resolved signals. However, with $\Delta\mu_{eff}$ limited to 0.01 cm² V^{-1} min^{-1} in this resolution regime, nearly every process variable became significant.

The results of the DOE for separation show, that this multivariate approach helps in the judgment of the separation mechanisms and principles in NACE and NACE-MS

coupling. Clearly, the strong effects of ammonium formate at high acetonitrile content become visible with the response surface. Further in-depth analysis on the effects of ammonium formate on the separation has to be conducted including the influence of other co- and counter-ions on the separation.

The obtained results (Figure 16) simplify further work with the presented technique, since the effects and interactions of the parameters are now known and can be changed on purpose. This is especially useful for a possible further optimization of the method and a robustness assessment by the application of a second DOE with narrow ranges for process parameters close to the optimum [243]. As the important parameters are determined, considerably fewer experiments are required. A second DOE with narrow ranges could also verify if local maxima or minima have been overlooked in the very broad setup of the DOE. This is possible, if other than linear or quadratic dependencies are present.

The results here clearly reveal the impressive selectivity changes and probably even more importantly the possibilities of tuning selectivity, resolution and speed of separation in NACE. We were able to show that the combination of less protic conditions (leading to differences in the solvation and thus in the size of the diastereomeric analytes) and ion-pairing or heteroconjugation is very interesting for the separation optimization of structurally closely related compounds including diastereomers.

3. CHAPTER III: NON-AQUEOUS CAPILLARY ELECTROPHORESIS-MASS SPECTROMETRY AS A VERSATILE, STRAIGHTFORWARD TOOL FOR THE ANALYSIS OF PLANT ALKALOIDS

Based on: T.N. Posch, N. Martin, M. Pütz, C. Huhn, Electrophoresis 2012, 33, 1557-1566

3.1. CHAPTER SUMMARY

This chapter serves as a powerful demonstration that non-aqueous capillary electrophoresis mass spectrometry, carefully optimized by a design of experiment, can be applied to a very large number of alkaloids from different plant extracts. It is possible to characterize the component pattern of the psychoactive alkaloids in several plant samples and preparations made thereof, each presenting different challenges in their analysis: The method is shown to be able to separate structurally closely related substances, diastereomers and further isobaric compounds, to separate members of different alkaloid classes within one run and to tolerate significant matrix load. A comparison with methods presented in the literature reveals, that a near-generic NACE-MS method for the fast profiling of alkaloids in plant samples has been developed and can be used for screening purposes of analytes, relevant to forensic investigations.

3.2. INTRODUCTION

The use of organic solvents in non-aqueous capillary electrophoresis (NACE) was investigated nearly parallel to the advance of aqueous capillary electrophoresis and isotachophoresis [97,215,216]. The most cited reasons to use organic solvents as BGE are the possibility to analyse hydrophobic compounds or to increase the window of solubility [131]. Very fast separations are possible when using solvents with a low dielectric constant by applying high field strengths [132]. Additionally, the lower vapor pressure of most organic solvents (compared to water) make NACE perfectly suited for its coupling to mass spectrometric detection [131].

Forensic analysis of recreational drugs is markedly different from routine analysis e.g. in pharmaceutical quality control [40]. Methods in forensic science have to handle samples with very different properties, concentrations and difficult matrices (tablets, liquids, powders, synthesis raw materials, body fluids, etc.), combined with a high demand on precision and the need to identify even completely unknown substances (new designer drugs, impurities in synthesis mixtures, etc.). But also trace analysis (e.g. in wipe samples from contaminated surfaces) has to be conducted [268]. This necessitates instrumental setups, which are characterized by high flexibility, wide linear dynamic range, high matrix tolerance, speed of analysis and short and easy optimization procedures and of course high selectivity and resolution [269]. These requirements are perfectly matched by capillary electrophoresis coupled to mass spectrometry [106]. In this study, we present the versatility, high separation power and matrix tolerance of a previously developed and optimized non-aqueous capillary electrophoresis-mass spectrometry method for the analysis of Kratom (*Mitragyna speciosa*) [102], now for the analysis of different biogenic drug samples. These drugs samples were chosen according to different properties of their alkaloid composition: *Banisteriopsis caapi* and *Peganum harmala* as different sources for harmala alkaloids which are structurally closely related substances; *Mitragyna speciosa* and Kratom preparations as samples with a high number of diastereomeric compounds; *Voacanga africana* as a representative sample containing iboga-alkaloids with an overall high number of different alkaloids; *Datura stramonium* as source for tropane alkaloids and *Mimosa tenuiflora* as tryptamine source. Finally, the analysis of Ayahuasca as an aqueous sample is presented showing a complex mixture of alkaloids. The method is capable to deal with this wide variety of indole based

alkaloids, like ß-carbolines, tryptamines, and iboga alkaloids as well as tropane alkaloids and opioids. Each analysis is compared with methods from the literature, where possible.

The most prominent psychoactive tryptamines are dimethyltryptamine (plants), bufotenine (cane toad) and psilocybin (mushrooms) [270]. The oral uptake of N,N-DMT is effectless, as the monoaminooxidase A (MAO) degrades the tryptamine before passing the blood brain barrier. But they have a very high psychoactivity known when smoked, injected, taken snuffed or combined with MAO inhibitors. N,N-DMT, N,N-DET and 5-MeO-N,N-DMT are regulated by the German Narcotic Act. Harmala alkaloids are a subgroup of the indole alkaloid class, which are found in a wide variety of plants, with *Banisteriopsis caapi* and *Peganum harmala* as the most prominent ones [270]. They are strong reversible MAO inhibitors and have some antibacterial functions [271,272], but they lack strong psychoactive properties on their own. Harmala alkaloids are used in herbal preparations like the beverage Ayahuasca for their MAO inhibiting properties and their analysis is therefore of interest in forensic science, although they are not regulated in the German Narcotic Act [239,245]. Ayahuasca, a Shamanic hallucinogenic drink from the Amazon region as a complex mixture of plants, contains tryptamines as psychoactive compounds and harmala alkaloids as inhibitors for MAO.

Kratom alkaloids are found in the stem, leaf and fruits of *Mitragyna speciosa*, a tree endemic to Southeast Asia with a traditional and medicinal use by the natives in the countries of origin for a long time. They were used as cure for tapeworm infestation and diarrhea. Kratom alkaloids are large, indole based alkaloids and are characterized by a very high number of diastereomers und structural isomers of the main active compounds [3,273]. The effects of the main alkaloids (mitragynine, paynantheine and 7-OH-mitragynine) are a paradox mixture with a cocaine-like euphoria for the first 2 hours after consumption, followed by an opiate-like dizziness for several hours. The plant is of interest for pharmaceutical investigations as it was also traditionally used as a substitute to cure opiate addiction.

Tropane alkaloids are closely related to cocaine and found ubiquitous in a wide variety of Solanaceae (e.g. *Atropa Belladonna* or *Datura stramonium*). The most important tropane alkaloids are tropane, scopolamine and hyoscopolamine. Their uptake readily occurs over the mucosa or the intact skin. Tropane alkaloids have a

direct impact on the central nervous system, leading to shortness of breath and death, but they are widely used as medicine under controlled dosage [274-276]. We will show that the method developed previously can be used for the analysis of all those different alkaloids without any modification [102].

3.3. EXPERIMENTAL

3.3.1. CE-MS

Experiments were performed on an Agilent 7100 Capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent 6520 Accurate-Mass qToF-MS (Agilent Technologies, Santa Clara, CA, USA) or an Agilent 3D-CE (Agilent Technologies, Waldbronn, Germany) coupled to a 6340 Ion Trap (Agilent Technologies, Santa Clara, CA, USA), both coupled via an Agilent CE-MS-adapter-kit. Sheath-liquid was added by an Agilent isocratic pump 1260 (Agilent Technologies, Waldbronn, Germany) in both cases. The capillary (50 µm i.d. length: 67.5 cm) was flushed for 30 s with methanol, then for 3.5 min with running buffer prior to injection and was kept at an operational temperature of 25 °C during analysis. Injection was performed hydrodynamically for 10 s at 50 mbar. The nebulizer was set to 2 psi (138 mbar) during injection and vial handling to prevent the introduction of air into the capillary and was subsequently increased to the desired value of 6 psi (414 mbar) at the beginning of voltage application. Instrumental settings for the qToF-Instrument were: 5 L/min drying gas were supplied at 325 °C, the fragmentor voltage was set to 175 V and capillary voltage was set to -4000 V. The skimmer was set to 65 V and the octopole to 750 V. The mass range was 100-1700 m/z, acquiring 2 full spectra per second. The calibration was performed with an Agilent standard calibration mixture for qToF-MS systems. Ion Trap Settings were: 5 L/min drying gas were supplied at 300 °C, SPS-settings were optimized for a mean m/z-value of 300 (Capillary +3500 V, Skimmer -40 V, Cap Exit -109.0 V, Trap drive 70.6). Max. Accumulation time was 200 ms and the ICC was set to 500 000. MS^3-experiments were performed using the Auto-MS function with a fragmentation amplitude of 1.0 V using a ramp from 30-300%. The two precursors were selected automatically, when exceeding the threshold limit of 400 000 cps. Tentative identification of the analytes has been performed with their accurate mass from the qToF-MS and the MS^3-spectra from the Ion Trap.

3.3.2. STANDARDS AND CHEMICALS

Harmaline (purum), harmane (purum), norharmane (purum), harmalol hydrochloride dihydrate (>98%), ammonium hydroxide (25%), 2-propanol (LC-MS-grade) were purchased from Sigma-Aldrich (Steinheim, Germany). Ammonium formate was purchased from BDH Prolabo (Darmstadt, Germany) and acetonitrile was purchased from Honeywell (Morristown, NJ, USA). Harmine (98+ %) and tryptamine (98+ %) were purchased from Alfa Aesar (Karlsruhe, Germany). Harmol hydrochloride dihydrate was bought from ABCR GmbH & Co.KG (Karlsruhe, Germany). Methanol, ethanol (LC-MS grade), glacial acetic acid (100%) and formic acid (98-100%) were purchased from Merck (Darmstadt, Germany). Capillaries were purchased from Polymicro Technologies (Phoenix, AZ, USA). All biogenic drug samples were provided by the Federal Criminal Police Office of Germany, KT-34 (Wiesbaden, Germany). Tetrahydroharmine was synthesized according to Pütz et al. 2003 [245]. A stock solution of 1 mol/L ammonium formate in glacial acetic acid was prepared and added to the BGE to yield an ammonium formate concentration of 60 mmol/L.

3.3.3. SOFTWARE

The CE7100 capillary electrophoresis unit was controlled by ChemStation® B.04.02 (Agilent Technologies, Santa Clara, CA, USA), the qToF was controlled by the Mass Hunter® software B.03.01 (Agilent Technologies, Santa Clara, CA, USA). Extracted Ion Electropherograms were extracted with ±20 ppm accuracy. The 3D-CE was controlled by ChemStation B.01.03-SR2 (Agilent Technologies, Santa Clara, CA, USA), the Ion Trap was operated with 6300 Series TrapControl Version 6.2 (Bruker Daltonik GmbH, Bremen, Germany). Data evaluation was performed with Agilent DataAnalysis 6.2 (Bruker Daltonik GmbH, Bremen, Germany).

3.3.4. SAMPLE PREPARATION

Samples were cut into small pieces and ground with mortar and pestle to yield a fine powder for the methanolic extraction. The ground sample was mixed with methanol (10 µL methanol per mg sample powder) in a 2 mL reaction tube. Extraction was performed by sonication for 15 min and subsequent storage in the fridge at 8° C over night. The samples were centrifuged at 13 000 rpm for 10 min and the clear supernatant was transferred into a new microtube. This extraction procedure was designed for efficient extraction of 7-hydroxymitragynine from Kratom leaves by

Kikura-Hanajiri et al. [277], but also proved to be effective for the extraction of several other plant materials. Samples were usually measured in a 1:100 dilution in methanol, if not mentioned otherwise.

3.3.5. BUFFER COMPOSITION

A mixture of 975 µL acetonitrile with 60 µL of a 1 mol/L solution of ammonium formate in glacial acetic acid was used as BGE. This leads to a buffer of 58 mmol/L ammonium formate and 1.01 mol/L acetic acid in acetonitrile.

3.4. RESULTS & DISCUSSION

3.4.1. METHOD VALIDATION

The method applied for the analysis of indole alkaloids was optimized as described in Chapter I [102] by a design of experiment focusing on best global peak intensities and a very good overall resolution for a large number of alkaloids including harmala alkaloids as commercially available standards as well as the alkaloids of an extract of *Mitragyna speciosa*. For the study presented here, this method was further optimized by the introduction of a rinsing step with pure methanol for 30 s at 1 bar before each analysis, followed by flushing with BGE for 3.5 min to eliminate memory effects and reduce peak tailing, which were observed in the unmodified method after the injection of highly concentrated samples. This also improved the repeatability of the migration times and the peak areas determined with commercially available standards of harmala alkaloids and tryptamine. The average relative standard deviation of migration times was < 0.75%. The relative standard deviation of peak areas was below 10% for all analytes at the measured concentrations, except for tryptamine and harmol. The reason for the higher deviation of the peak area of tryptamine is the occurrence of in-source-decay, resulting in a fragment at m/z = 144 as the most intense peak instead of the pseudo molecular ion with m/z = 161. This could have been circumvented by lowering the cap exit voltage (or fragmentor voltage at the qToF instrument) but at the cost of sensitivity for the harmala alkaloids. Harmol is migrating close to harmane, probably leading to some quenching in the small overlapping area. Limits of detection (on basis of an S/N ratio of > 3) were found to be 25 nmol/L for harmaline, harmine, harmalol and norharmane, 50 nmol/L for tetrahydroharmine and harmol and 75 nmol/L for tryptamine and harmane. Limits of quantification (on basis of a S/N ratio of > 10) were found to be 50 nmol/L for

harmaline and harmine, 75 nmol/L for harmalol and norharmane, 100 nmol/L for harmane, harmol and tetrahydroharmine and 150 nmol/L for tryptamine.

3.4.2. STRUCTURALLY CLOSELY RELATED ANALYTES: HARMALA ALKALOIDS IN *BANISTERIOPSIS CAAPI*

Banisteriopsis caapi is a plant from the Amazon region, which is used for the production of Ayahuasca (see Section 3.4.6). The very large liana is woody and highly branched. Its leaves are 8-18 cm long and up to 8 cm wide. The harmala alkaloids harmine, harmaline and tetrahydroharmine are the active compounds and the highest content of those is found in the bark. Bark and wood of a short piece of liana were analyzed separately in this study.

The comparison of their electropherograms (Figure 21) shows baseline separation of all analytes. The good separation of major and minor compounds allows the detection of very small peaks with still surprisingly good S/N-ratios (e.g. harmalol). This is important for MS-detection as the detection of minor compounds in presence of quenching co-eluting main compounds is difficult and prone to errors. However, in terms of relative quantitation, the reduced content of harmalol in the wood is surprising, as the previously obtained bark to wood ratio (3:1 [245]) is not corroborated here, possibly as a result of the long storage time of the sample.

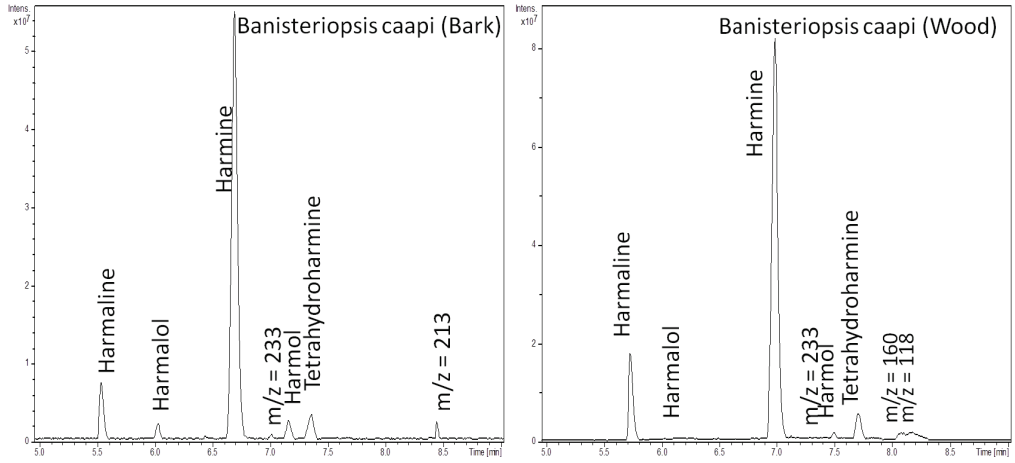


Figure 21: Corrected base peak electropherograms of the analysis of Banisteriopsis caapi. For a better comparison of the qualitative analysis, twice the amount of sample was injected from the wood-sample. Separating conditions: 30 kV separation voltage, 67.5 cm capillary length, BGE: 60 µL of a 1 mol/L solution of ammonium formate in glacial acetic acid in 975 µL acetonitrile.

3.4.3. STRUCTURALLY CLOSELY RELATED ANALYTES: HARMALA ALKALOIDS IN *PEGANUM HARMALA*

Peganum harmala, also known as Syrian rue, is a wild growing herb, endemic to the semi-dry regions of Southeast Europe to Central Asia that has spread over Africa, the Middle East, and South America [270]. Its application in folk medicine as abortifacient agent and central nervous system stimulating agent is well known in Turkey (as "yüzerlik" or "üzerli") and Iran (dried capsules, known as "espænd" or "esfænddāneh"). It was also used in ritual context originating from pre-Islamic times, where the smoke of the burnt seeds was said to protect from "the evil eye" [272,278]. Due to its slightly psychoactive properties its use as a drug in ancient preparations is likely [272]. The main compounds of *Peganum harmala* are the ß-carboline alkaloids harmine, harmaline and tetrahydroharmine, along with vasicine and compounds related to it [270]. The use of *Peganum harmala* in Ayahuasca-analogues is reported, as it is an easily available substitute for *Banisteriopsis caapi* as harmala source (see also: *Ayahuasca*).

Two different samples of *Peganum harmala* from different head-shops were compared in this study (Sample A and B). The results of the analyses are presented in Figure 22. Baseline separation has been achieved for all analytes, even for vasicine (also known as peganine) and its related analytes, representing another alkaloid class here. We were able to identify two doubly glycosylated vasicine peaks (Figure 22 mass spectrum), which have not been observed in any study of *P. harmala*. As far as the authors know, there is only one literature source mentioning one doubly glycosylated vasicine peak, observed in an HPLC method in a Spanish patent (Spanish patent: ES 2 360 547 A1). Separation of the two peaks is suggested to be a result of a different interglycosidic linkage, resulting in a different conformation although the observed fragments indicate a 1→6 interglycosidic linkage in both cases [279]. It might also be possible that more than two glucoses are attached to the vasicine during separation, but are lost by in-source decay. The similarity of the observed peak areas between the two samples, gives a strong indication that both samples represent *Peganum harmala*, as their relative alkaloid contents are in agreement with proportion values from the literature [270]. The small difference of the peak areas between the two samples is explainable by different times of harvest or places of origin, as the alkaloid content in plants is related to the climate conditions

[270]. The depicted MS³-experiments (Figure 22) show that the NACE-MS method might be useful for the analysis of Phase I or II metabolites from plants.

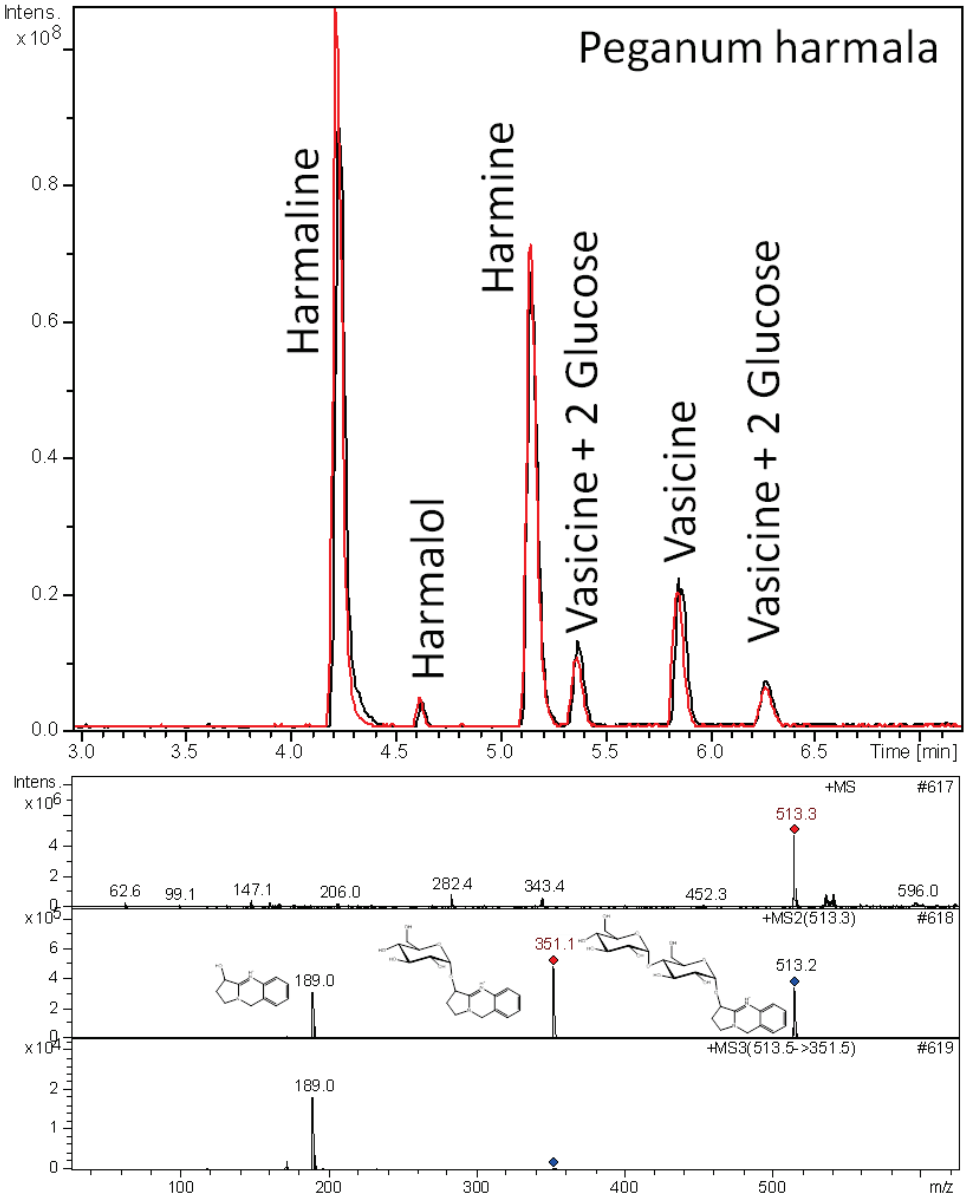


Figure 22: Spectral corrected base peak electropherogram of the analyses of 2 samples Peganum harmala from different sources (above) and MS[1]-MS³-spectrum of the signal m/z = 513.3 (below). Further experimental separation conditions, see Figure 21.

3.4.4. SEPARATION OF DIASTEREOMERS: *MITRAGYNA SPECIOSA*

The dried leaves of *Mitragyna speciosa* are sold via the internet as biogenic recreational drug ("Kratom") and appear in a large variety of quality and preparations (raw, extracts or resins) on the German and European market [280]. Additionally, products with further added active compounds (e.g. O-desmethyltramadol) were identified [2,281]. It is obvious that the addition of O-desmethyltramadol in high amounts represents a high health risk for the unaware consumer, as the common dose used for brewing is in the range of 3-5 grams of Kratom. A commercial Kratom product called "Indonesian Premium" was analyzed and compared with another Kratom product from a seizure ("Krypton"). The listed extracted ion electropherograms (Figure 23) shows the very good separation of diastereomers and further isobaric substances in both analyses. The separation of the diastereomers is regarded to be a result of ion-pairing or heteroconjugation as separation mechanism in the non-aqueous buffer system (see [102] or Chapter 2 for a more detailed discussion). The diastereomers of the main alkaloids mitragynine (m/z = 399 trace) are separated by up to 1.5 minutes on a total migration time of about 8 minutes. This is an effective electrophoretic mobility difference of $\Delta\mu_{eff}$ = $7.53 \cdot 10^{-5}$ $cm^2\ V^{-1}\ s^{-1}$ between the first m/z = 399 peak (μ_{eff} = $1.94 \cdot 10^{-4}$ $cm^2\ V^{-1}\ s^{-1}$) and the last one (μ_{eff} = $1.19 \cdot 10^{-4}$ $cm^2\ V^{-1}\ s^{-1}$) [192].

The very large m/z = 250 peak in the analysis of "Krypton" is identified as O-desmethyltramadol which is present in very large excess compared to the Kratom alkaloids. O-desmethyltramadol is an opioid which was suggestively added to the sample by the distributor to fake enhanced potency. The inspection of the mass trace m/z = 413 shows the separation of at least four analytes with the same mass, separated by a maximum of 2.5 min ($\Delta\mu_{eff}$ = $2.51 \cdot 10^{-4}$ $cm^2\ V^{-1}\ s^{-1}$) between analytes. They are suggested to be diastereomeric dehydro-derivates of hydroxy-mitragynine where the position of the hydroxy-group is different. However, a further investigation of these analytes by MS^3-experiments showed no differences in their fragmentation pattern. But it demonstrates the possibilities of the method to resolve unknown compounds in complex mixtures. The main alkaloids in the mass trace m/z = 399 were tentatively identified to be (from the left to the right) mitragynine, speciogynine, speciociliatine and mitraciliatine by comparison of the magnitude of the peak areas of the obtained electropherogram with peak areas obtained from an LC analysis where

the elution sequence is known from Arndt et al. [2]. The trace for m/z = 401 shows 10 signals, four of which are isotope signals from m/z = 399. The resulting six substances exhibiting a separation window of 4 min have not been described in literature, but might be artifacts, as the occurrence of ±2 u analytes increases with age of the sample. We suggest that they are the result of a hydrogenation of the aromatic ring system [282] and are consequently separated as double-bond isomers and diastereomers. It is obvious from these electropherograms that separation methods with high resolving power are necessary as diastereomers could only be differentiated by their relative migration time, as exact mass and fragmentation patterns of these analytes are the same. The achieved separation is suggested to be a result of differences in the Stokes-radii, as they are pronounced in the absence of the large hydroshell (Chapter 2 and [102]).

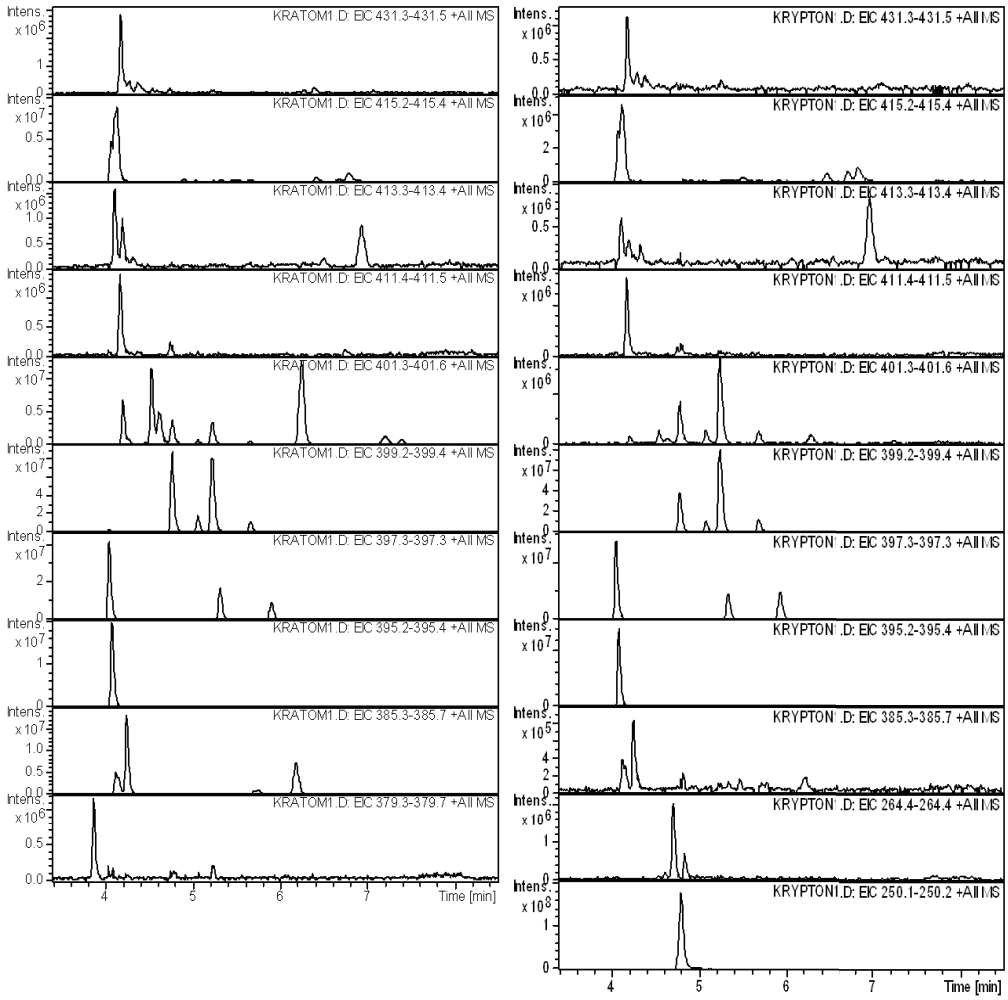


Figure 23: Extracted ion electropherograms of the analyses of Kratom (left) and "Krypton" (right). Further experimental separation conditions, see Figure 21.

3.4.5. TRYPTAMINES: *MIMOSA TENUIFLORA*

The bark of the tree *Mimosa tenuiflora (*synonym: *Mimosa hostilis)* are used in Yurema preparations, as they are a potent and easily available source for the psychoactive N,N-dimethyltryptamine. The root bark of the Mexican form of this tree contains up to 1% N,N-DMT [270]. As Yurema does not contain an MAO inhibitor, it is suggested that the Yurema mamin acts as an adjuvant for tryptamine [283]. Figure 24 shows the analysis of a *Mimosa tenuiflora* extract. The tryptamines 5-MeO-tryptamine and N,N-dimethyltryptamine are present in high concentration,

accompanied by unidentified analytes. It is suggested that the peaks at m/z =203 and m/z = 187 are dehydro-varieties of 5-MeO-tryptamine (m/z = 205) and N,N-dimethyltryptamine (m/z = 199), as they exhibit comparable fragmentation patterns. This reduction of analytes has been observed in several methanolic extracts over time.

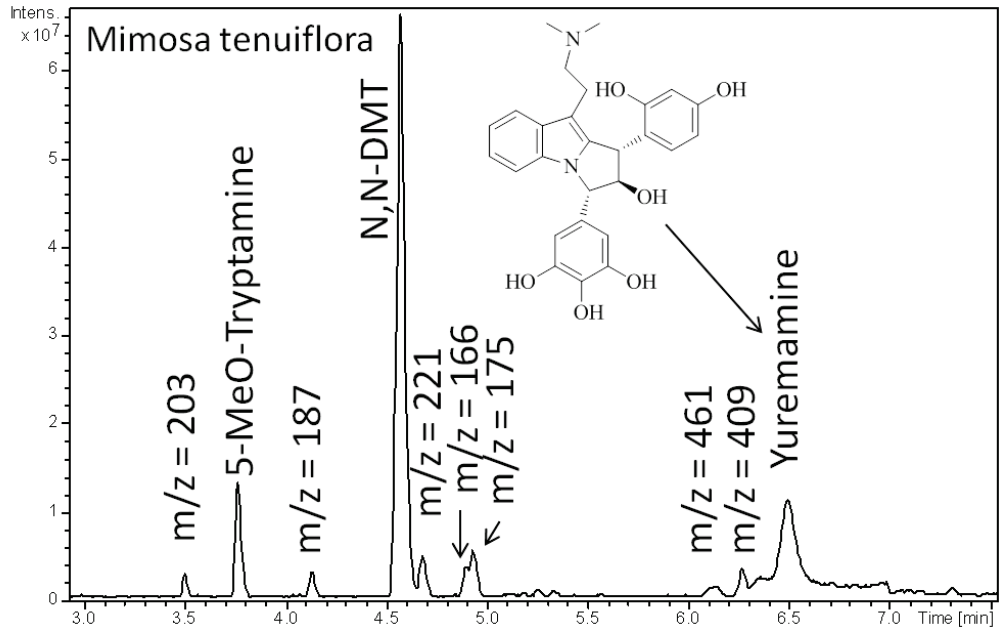


Figure 24: Spectral corrected base peak electropherogram of the analysis of Mimosa tenuiflora. Further experimental separation conditions, see Figure 21.

3.4.6. VERY COMPLEX ALKALOID COMPOSITIONS: *VOACANGA AFRICANA*

The seeds and the bark of *Voacanga africana* contain up to 10% iboga alkaloids and were traditionally used in West Africa as cure for diarrhea with anti-ulcer properties [284], as hunting drug, stimulant and as an aphrodisiac agent. It is also used by African “wizards” for creating visions [270]. The analysis of the alkaloids voacamine, ibogaine, voacangine, 3-oxovoacangin and voacristine is of interest, as they are partially known to decrease withdrawal symptoms from morphine, nicotine and alcohol [285]. Unfortunately, no analysis of extracts from *Voacanga africana* could be found in the literature. The spectral corrected base peak electropherogram (Figure 25) shows a very complex mixture of unknown analytes in the methanolic extract

over a wide range of masses. The alkaloid composition is characterized by many isobaric substances, by analytes with only slight differences in mass (± 2 u) and comparatively large analytes (m/z > 600) exhibiting a good resolution. A detailed identification of each peak goes beyond the scope of this study, especially as there is no comparable literature data available, but the electropherogram demonstrates the high resolving power of the method for iboga alkaloids. Nevertheless, it also shows the limits of very fast separations as a detailed identification is impossible using only the exact mass and an MS/MS spectrum from the qToF instrument. The precision of the Ion Trap instrument is not capable to isolate only one analyte when e.g. the analytes with m/z = 367 and m/z = 365 have very similar migration times and the isolation window is typically ± 4 u. Additionally, the cycle time of the Ion Trap is not fast enough to perform several MS^n-measurements within this short time frame. This hinders further identification as structural information is not obtainable by MS^n-experiments. However, this example shows the distinct difference of the NACE separation mechanism compared to classical aqueous CE, as e.g. analytes with m/z = 720 are migrating between analytes with m/z = 325 and 355, clearly showing that charge and size are not the only relevant separation parameters. Separation occurs due to differences in the ion-pairing constant (Chapter II [102]).

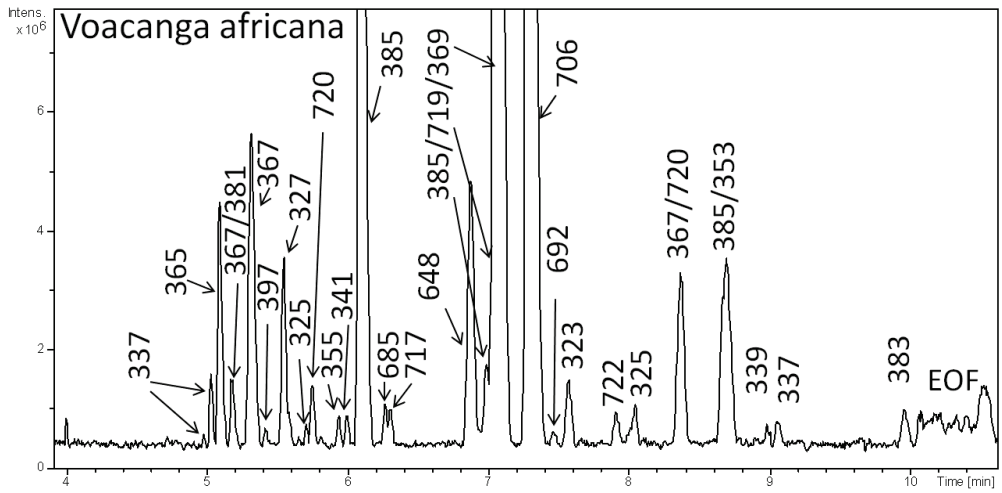


Figure 25: Spectral corrected base peak electropherogram of the analysis of Voacanga africana. The numbers describe the m/z-value of the indicated peaks. Further experimental separation conditions, see Figure 21.

3.4.7. HIGH MATRIX TOLERANCE: *DATURA STRAMONIUM*

The analysis of tropane alkaloids from plant extracts is performed by GC-MS [286] reverse phase-HPLC [276,287] LC-MS [288], MEKC [274], CE-MS [275] and also NACE [131,289]. While for the GC and HPLC analysis sample cleanup before injection was necessary, pure extracts could be injected in MEKC, CE and NACE. The analysis time for the different approaches varies from more than 30 min for LC and GC to 10-20 minutes for MEKC and LC to < 15 min for NACE and CE. In this study, three different parts of a plant were investigated: Seeds, leaves and the fruit-shell. The electropherogram of the analysis of *Datura stramonium* leaves (Figure 26) shows the two main tropane alkaloids of the plant, which are well resolved. The electropherogram shows that the analysis of tropane alkaloids is generally possible with the restriction that enantiomeric separation is not possible, as no chiral selector was added. However, NACE-MS is valuable for the analysis even with the very high matrix load, visible in the very large signal of the EOF and a corresponding drop in the current profile (data not shown). In comparison with the existing methods, only GC [286] showed a better separation and qualitatively more trace analytes, but requires a labor intensive sample preparation. LC-methods suffer from broad peaks, reducing plate height and therefore resolution. MEKC and NACE [131,274] methods showed better separation properties for standard mixtures, but proved to be less matrix-tolerant. All reference methods were able to identify further tropane alkaloids in other species and in *D. stramonium*. Clearly our extraction procedure requires further optimization, which was, however beyond the scope of this study. Nevertheless, the results from the comparison of the different parts of a plant, reveals differences in relative analyte concentration, but not with regard to the presence of other types of analytes. Unsurprisingly, the seeds have the highest concentration of atropine and scopolamine, while no analytes had a concentration above the LOD in the fruit shell. Summarizing, this NACE-MS method is a valuable tool for the analysis of the tropane alkaloids with very similar mass to charge ratios, as already presumed by Cherkaoui et al. [131].

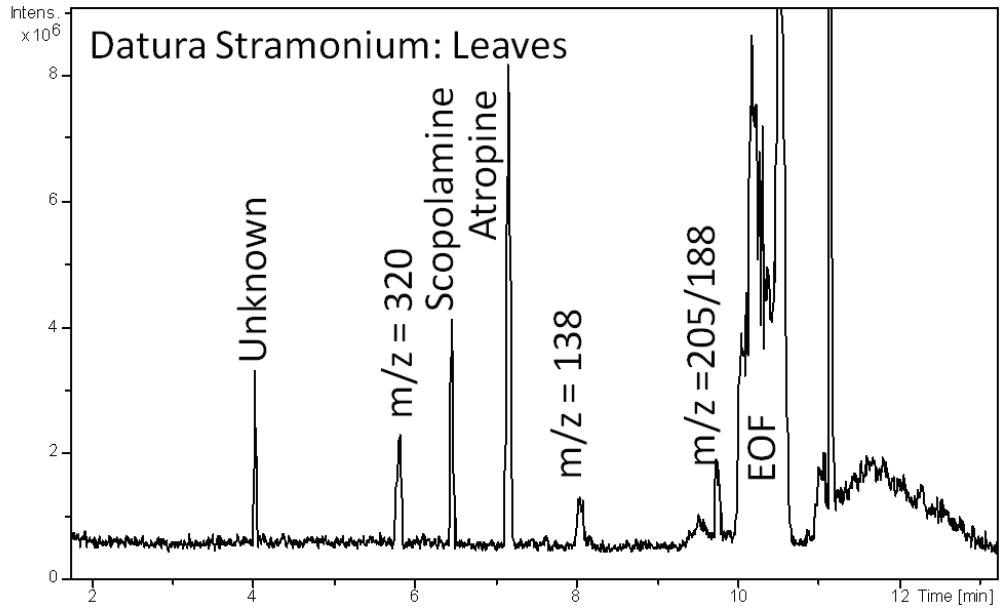


Figure 26: Spectral corrected base peak electropherogram of the analysis of Datura stramonium. Further experimental separation conditions, see Figure 21.

3.4.8. EXTRACTS OF AQUEOUS SAMPLES: AYAHUASCA

Ayahuasca (Quechua for "vine of the souls") is a shamanic brew from the Amazon region, traditionally made from the liana *Banisteriopsis caapi* and the plant *Psychotria viridis* (Chacruna) or *Diplopterys cabrerana* (Chaliponga, syn. *Banisteriopsis rusbyana*). Ayahuasca has a traditional, ritual and sacramental use in the endemic regions, as a diagnostic tool for the examination of ill people used by the shaman [270] or within the ritual context of the syncretistic churches Santo Daime and the União do Vegetal.

The matrix of Ayahuasca is difficult as it is a combined extract from two plants with a high amount of sugar. The existing methods for the analysis of Ayahuasca prefer to detect the *N,N*-dimethyltryptamine with a GC using a nitrogen-phosphorus detector and the ß-carbolines with an HPLC method in two individual approaches [290,291] or only with GC-NPD after sample cleanup [292]. A previously published study used capillary electrophoresis-laser induced fluorescence-mass spectrometry for the analysis of tryptamines and ß-carbolines and reached detection limits < 40 nmol/L [239]. In our study precipitation occurred when diluting the samples with pure methanol, making a liquid-liquid extraction necessary. 300 µL of the drink basified

with ammonia (10%) were extracted with 100 µL methylene chloride and neutralized with acetic acid afterwards. 10 µL of the methylene chloride solution were then diluted with 990 µL MeOH and hydrodynamically injected for 10 s at 50 mbar. The separation voltage was 30 kV, capillary length: 58 cm. The spectral corrected base peak electropherogram (Figure 27) shows the well separated main alkaloids of *Banisteriopsis caapi* harmaline, harmine and tetrahydroharmine, as well as a small peak of Harmalol. Several additional small peaks were recognized (visible in EIEs but not in the BPE), but not further identified. The presence of *N,N*-DMT without occurrence of other tryptamines is a good indicator that *Psychotria viridis* was used as source [239]. The relative concentration of harmine in comparison to harmaline indicates the usage of *Banisteriopsis caapi* as harmala source (see Figure 21) and verifies the Ayahuasca sample as an original brew. The separation performance of this method is superior to all published LC [291,293] and CE-MS methods [239] while requiring less analysis time. A better separation was only achieved with prolonged analysis time in GC-MS analysis (40 min) [294] and MEKC (30 min) [245].

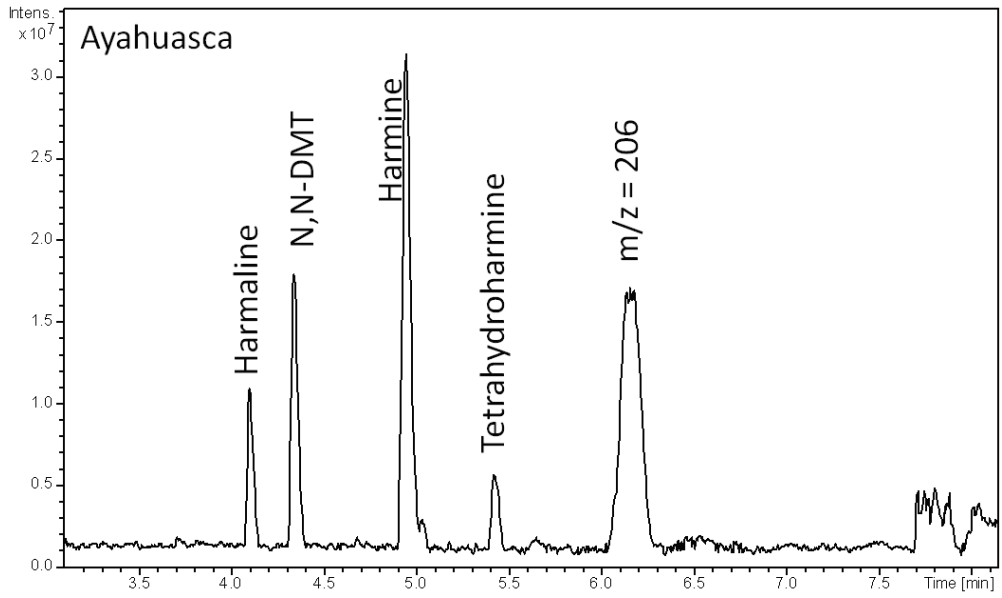


Figure 27: Spectral corrected base peak electropherogram of an Ayahuasca sample. See text for further experimental parameters.

3.5. DISCUSSION

3.5.1. LIMITATIONS OF THE METHOD

The method has proven valuable for the analysis of a wide variety of alkaloids and samples, e.g. different plant parts, aqueous samples after liquid-liquid extraction or complex mixtures of samples. However, it was not able to deal with very oily samples (e.g. *Griffonia simplicifolia*) or with alkaloids, exhibiting a methylated nitrogen in the ring system (e.g. ergot alkaloids). When analyzing samples of the latter kind, no separation took place, all analytes were detected as stable ion pairs that is electrokinetically neutral together with the EOF. Additionally, the system was unstable when aqueous samples were injected or a sample exhibited a too low conductivity. The latter problem can easily be circumvented by the addition of 10% BGE to the sample. Some samples (see *Voacanga africana*) exhibit a very complex alkaloid pattern, where a full identification or relative quantification is difficult even with the high resolving power of our NACE-MS method.

3.5.2. METHOD PERFORMANCE

The methods unique properties to separate diastereomers with retention time differences of e.g. 1.5 min in an 8 min ($\Delta\mu_{eff}$ = $7.53 \cdot 10^{-5}$ cm^2 V^{-1} s^{-1}) analysis run makes the method very attractive for the analysis of structurally closely related analytes. The ease of application and high matrix tolerance makes it suitable as a fast screening method, especially since no pretreatment of the capillary and only little sample preparation is necessary. The analysis of different indole alkaloid types within one analytical method has already been realized by Unger et al. (1997) for capillary electrophoresis [100] and Stöckigt et al. (2002) for LC-MS [295]. However, the comparisons of the presented method with existing methods allows the conclusion, that a better separation in shorter analysis time has been achieved [100,296] although a comparison is not always proportionate. Good comparison is given to the work of Stöckigt *et al.* [295] as here, too, harmala alkaloids were used as model substances, showing that the aqueous CE system is generally capable of separating them, but only with prolonged analysis. We have proven that NACE-MS is a nearly generic method for the analysis of indole alkaloids and its application is easy and fast and should be considered as an alternative to the established HPLC and GC methods when analysis of plant material for alkaloids is necessary.

4. CHAPTER IV: ANALYSIS OF PASHS IN DESULFURIZED FUELS FROM ATHABASCA BITUMEN

Based on: T. Nolte, T.N. Posch, C. Huhn, J.T. Andersson. Energy & Fuels 2013, 27, 97-107

The work in this chapter describes the transfer of a CE-UV method developed by Thies Nolte in the group of Prof. Andersson in Münster, to a CE-MS system. Sample preparation and preliminary CE experiments were performed by Thies Nolte, first NACE-MS experiments were performed by Tjorben Posch. The final series of measurements was performed in cooperation of Nolte and Posch. [297].

4.1. INTRODUCTION

One of the indisputable strengths of NACE is the very easy adaptation of the buffer system to new analytical challenges. As the developed method has shown its large potential and broad applicability (see Chapter 4), it was also applied for the analysis of methylated polycyclic aromatic sulfur heterocycles (PASHs) from desulfurized fuels. PASHs are of high importance in oil and the fuel production as residues of these compounds are undesirable in the end products, being harmful for engines. Desulfurization strategies are therefore employed, however residues still remain and a reliable analysis for these compounds is needed, for subsequent further treatment. As petroleum represents one of the most challenging and complex samples, there are already several strategies for the analysis, mostly based on GC or LC methods, coupled to different detection systems. Both separation techniques have already achieved outstanding successes, however both techniques also have its drawbacks, as GC is limited by the volatility of the samples, while LC analysis suffers from an overall low resolution in comparison and long analysis times. Capillary electrophoresis can therefore potentially close this gap and deliver the high resolution it is known for, while not being limited by volatility. This chapter describes the adaption of NACE-MS methods for the analysis of PASHs from desulfurized fuels from Athabasca Bitumen. These very complex samples are characterized by an exponentially increasing number of isomers with increasing molecular size. It is therefore of interest if the achieved separation is sufficient for such a complex sample.

4.2. MATERIAL & METHODS

4.2.1. CE-MS

Experiments were performed on an Agilent 7100 Capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent 6520 Accurate-Mass qToF-MS (Agilent Technologies, Santa Clara, CA, USA) coupled via an Agilent CE-MS-adapter-kit. Sheath-liquid was added by an Agilent isocratic pump 1260 (Agilent Technologies, Waldbronn, Germany). The capillary (50 µm i.d. length: 74.5 cm) was kept at an operational temperature of 25 °C during analysis. Injection was performed hydrodynamically for 10 s at 50 mbar. The nebulizer was set to 2 psi (138 mbar) during injection and vial handling to prevent the introduction of air into the capillary and was subsequently increased to the desired value of 6 psi (414 mbar) at the beginning of voltage application. Instrumental settings for the qToF-Instrument were: 5 L/min drying gas were supplied at 325 °C, the fragmentor voltage was set to 175 V and capillary voltage was set to -4000 V. The skimmer was set to 65 V and the octopole to 750 V. The mass range was 100-1700 m/z, acquiring 2 full spectra per second. The calibration was performed with an Agilent standard calibration mixture for qToF-MS systems.

4.2.2. SOFTWARE

The CE7100 capillary electrophoresis unit was controlled by ChemStation® B.04.02 (Agilent Technologies, Santa Clara, CA, USA), the qToF was controlled by the Mass Hunter® software B.03.01 (Agilent Technologies, Santa Clara, CA, USA). Extracted Ion Electropherograms were extracted with ±20 ppm accuracy.

4.2.3. SAMPLE PREPARATION

The samples preparation was performed by the cooperation partner. The interested reader is referred to the publication of Nolte et al. [297] for details. In short: The methylation of the PASHs was performed at the sulfur using methyl iodide and silver tetrafluoroborate as methylation reagent. This induces a permanent positive charge in the molecules, making them accessible for CE separation and ESI-MS analysis.

4.2.4. BUFFER AND SHEATH LIQUID

The buffer system was a solution of 4% glacial acetic acid in methanol with 70 mM ammonium acetate. A mixture of 2-propanol and water (50:50) with 1% acetic acid was used as sheath liquid at a flow rate of 4.5 µL/min.

4.3. RESULTS & DISCUSSION

The first experiments showed that the NACE-MS method, presented in Chapter 2 and 4, is generally also suitable for the analysis of PASHs, however an exchange of the aprotic organic solvent acetonitrile by the protic methanol was chosen for the analysis, as its separation characteristics are more comparable to classical, aqueous capillary electrophoresis (migration of the analytes according to their charge vs. the hydrodynamic radius). This allows for a simplified data evaluation of electropherograms, as the sequence of elution is predetermined and in agreement with the rising masses of analytes. Due to this direct correlation of migration times and mass, a possible misinterpretation of isobaric substances is reduced. The analytes we focused on in this analysis were the homologous series of the compounds dibenzothiophenes, tetrahydrodibenzothiophenes, tetrahydrobenzonaphthothiophenes, and dihydrophenanthrothiophenes (DHPTs). The homologous series for each analyte started with the mass over charge ratio of single methylated ion (due to sulfur methylation), increasing by 14 mass units for each additional methyl group attached to the aromatic ring or present as a prolonged alkyl substituent. Figure 28 shows the results of an analysis of hydrotreated heavy gas oil. As can be seen from Figure 28a, the NACE-MS method delivers a very good separation of the complex mixture. The single isoforms of the DBTs are well separated up to the C4-DBT in this sample. Also the separation of the THDBTs (Figure 28b) shows individual peaks to a certain amount, although the analysis of these analytes suffers from its low relative concentration in the sample. The separation of the THBNTs and DHPTs (Figure 28c) is of additional difficulty, as the mass over charge ratios of the two analyte groups nearly overlap with the corresponding species of a higher degree of methylation. Despite the circumstances, the high resolution of the qToF instruments allows for the discrimination of the individual groups. The full evaluation of the analysis of these samples goes beyond the scope of this subchapter, as its purpose is to demonstrate the impressive range

of application of simple NACE-MS methods and their suitability for the analysis of very complex samples.

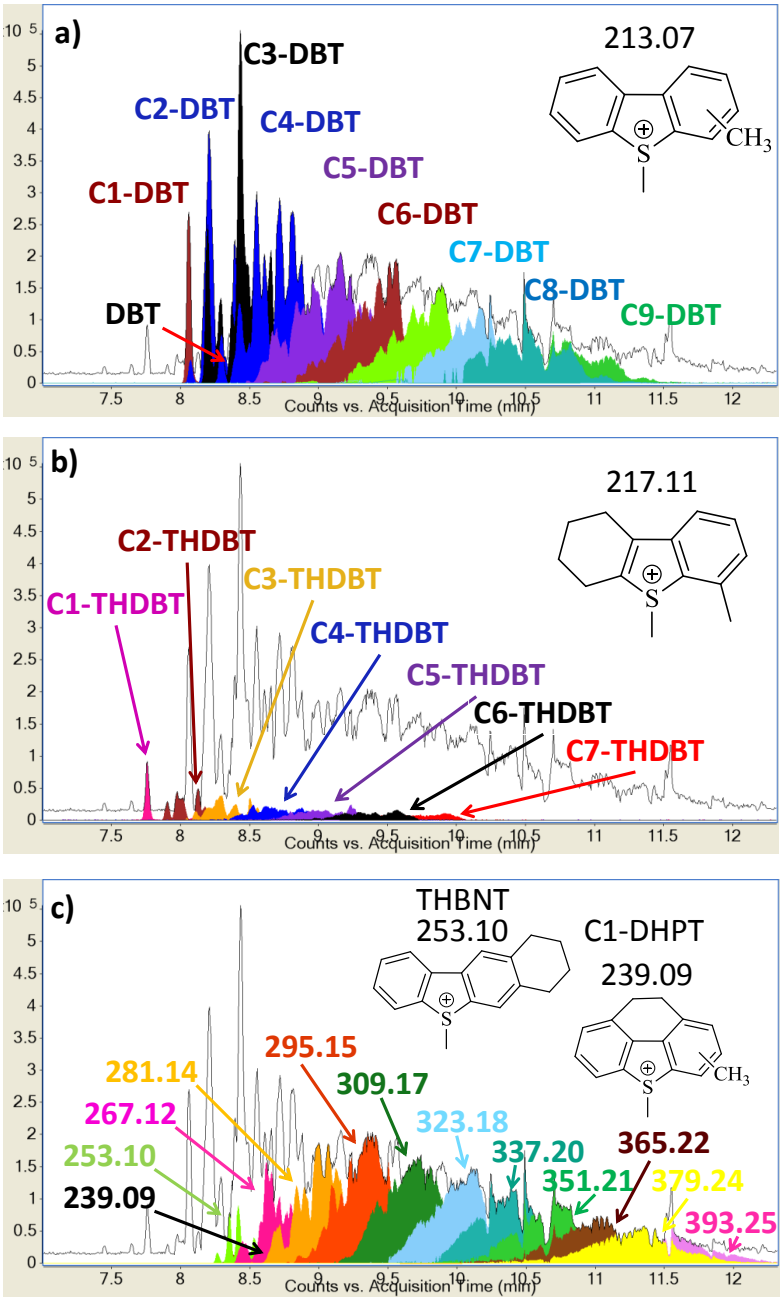


Figure 28: Overlay of methylated a) DBT to C_9-DBT; b) C_1- to C_7-THDBT; c) THBNTs/DHPTs. The figure shows extracted ion electropherograms of non aqueous NACE-MS measurements of a heavy gas oil PASHs (from Nolte et al. [297], with permission from ACS publications).

5. CHAPTER V: CHEMOMETRIC DATA EVALUATION FOR THE PROFILING OF ANALYTES OF FORENSIC INTEREST BY NON-AQUEOUS CAPILLARY ELECTROPHORESIS-MASS SPECTROMETRY

Based on: T.N. Posch, M.N. Palmblad, P.A. Kler, H. Mahler, L. Welge, M. Pütz , C. Huhn, in preparation

5.1. INTRODUCTION

The profiling of drugs is the analysis of a drug's chemical or physical profile which can further be used to prevent their illegal trade [298]. Seizures of illegal drugs can be fully profiled to gain comprehensive information on different levels, strategic and tactical intelligence, according to Lurie et al. [1]: "Strategic intelligence gives information on the processing and/or geographic origin of an exhibit; whereas, tactical intelligence tells whether two or more samples came from the same source." This information reflects the similarities and differences of the drug batches and can further be used to establish links between samples, drug distribution patterns, origin of precursor substances, assessment of impurities and synthesis pathways [299]. Especially since the beginning of fighting against the drug-financed international terrorism [300], in-depth profiling becomes more than just an analytical tool as it offers the possibility to trace origin, production processes or distribution channels of illegal drugs or precursors, if based on robust analytical methods and chemometric models [301]. A prerequisite for such a comprehensive investigation is an analytical platform that can characterize a high number of analytes of interest in a short time and preferably with a high degree of automation. Additionally the analytical technique has to be reliable and robust against matrix effects; a short and simple sample preparation is desired.

Despite its long history of use and abuse, opium and heroin are still subject to ongoing analysis, observation and profiling programs. Methods for the analysis of heroin and opium are well established [302], mainly based on liquid-chromatography [1,303-305] and gas chromatography [306-313]. Also well characterized and validated capillary electrophoretic methods are available, exhibiting an orthogonal separation principle to chromatographic methods, most of them based on MEKC [107,114,115] MEEKC [116] or NACE [104,105]. Also pure CE-MS methods are available [108]. Unfortunately, MEKC is hardly compatible with mass spectrometry, except when low concentration of surfactants [56,314] or volatile micelles are used, like the perflouorooctanic acid [315]. Therefore, it is desirable to develop other electromigrative separation methods with a high separation capability, based on MS-compatible buffer systems to be combined with the benefits of mass spectrometric identification. Non aqueous capillary electrophoresis is an ideal tool for this purpose,

especially with its unique features to separate structurally closely related analytes according to an ion-pairing principle [102].

In this chapter, we will investigate the feasibility to apply non aqueous electromigrative separation techniques for the discrimination of (il)legal drugs as basis for a new comprehensive profiling. The overall goal of this chapter is to present fast, but reliable procedures with a minimal manual workload, thus reducing potential influence from the operator while maintaining high quality of analysis.

For this purpose we introduce a non aqueous capillary electrophoretic method for the non-targeted analysis of raw opium and heroin. The method was used to create a chemometric model for discriminating opium and heroin samples from different seizures and for the identification of the most important qualifiers. Additionally, we here apply the previously developed NACE method (see Chapter 2) to analyze a large number of samples of the biogenic drug “Kratom” (*Mitragyna speciosa*) from a large, recent seizure.

We will also present a comprehensive workflow, starting from the sample extraction over method development and analysis, data pretreatment and extraction up to the chemometric evaluation by means of principle component analysis. This workflow is independent from commercial software packages (like e.g. Marker View™ from AB SCIEX) which are used for similar purposes in biomarker analysis. For this, the raw data files from CE-MS are converted into an open access mass spectrometric file format to be subject of an automated alignment of the files and data extraction using an open software package.

The sample preparation is kept as simple as possible, using a simple methanolic extraction followed by centrifugation and direct injection after dilution. Method development, optimization and robustness assessment is conducted using the design of experiments principle. The information gathered from our analysis will be further subject to a principle component analysis, a valuable chemometric tool, used here for two specific purposes: 1) profiling of the drugs and classification into distinct groups according to characteristic analytes, characteristic concentration ranges or analyte compositions/combinations; 2) identification of the analytes delivering the highest degree of information for the differentiation of the groups for a subsequent

data reduction and reduction of the work load, from a full analysis of nearly all analytes to the targeted analysis of a few ingredients, the so called qualifiers.

The latter has already been applied for opium and heroin, but with more sophisticated analytical and chemometric tools [301,316]. Therefore it is of interest weather our approach can deliver comparable information for these samples though in a straight-forward manner and thus be an orthogonal tool and secondary procedure.

The situation is different for the analysis of Kratom, where detailed studies are still lacking, as most of the available literature focuses on the analysis of Phase I and Phase II metabolites, mostly from rat urine [317,318], human body fluids [2,5] or on method development without application [277], to the best of our knowledge. The only available systematic forensically-oriented investigation of Kratom samples was performed by Arndt et al. [4] and impressively shows the problem in the analysis of Kratom samples: There are no obvious systematic differences that can be used for the classification of these samples, an experience we also made in a previous investigation (unpublished results). Additionally, the concentration range of the main alkaloids is very large in the investigated samples and cannot be compared or tracked to any of the distributions which are described by Takayama et al. [3] in the investigations of fresh plant samples in the countries of origin.

5.2. MATERIAL & METHODS

5.2.1. INSTRUMENTATION

Experiments were performed on an Agilent 7100 capillary electrophoresis system (Agilent Technologies, Waldbronn, Germany) coupled to an Agilent 6520 Accurate-Mass qToF-MS (Agilent Technologies, Santa Clara, CA, USA) via an Agilent CE-MS-Adapter-kit. Sheath-liquid was added by an isocratic pump 1260 (Agilent Technologies, Waldbronn, Germany). The capillary (50 µm i.d., length: 60 cm) was kept at an operational temperature of 25 °C during analysis. Injection was performed hydrodynamically for 3 s at 50 mbar. The nebulizer was set to 2 psi (138 mbar) during injection and vial handling to prevent the introduction of air into the capillary (Venturi effect [319]) and was subsequently increased to the desired value of 6 psi (414 mbar) at the beginning of voltage application. Instrumental settings for the qToF-Instrument were: 5 L/min drying gas were supplied at 325 °C, the fragmentor voltage

was set to 175 V and capillary voltage was set to -4000 V. The skimmer was set to 65 V and the octopole to 750 V. The mass range was 100-1700 m/z, acquiring 2 full spectra per second. The calibration was performed with an Agilent standard calibration mixture for qToF-MS systems.

5.2.2. MICROCRACK PREVENTION

During the analysis of the sample cohort, we encountered several microcracks in the front part of the capillary, the part which is surrounded with sheath-liquid in the sprayer needle, when the capillary was not removed over night from the MS-sprayer. The capillary was shortened in this case (to 60 cm) and could be used regularly afterwards. Storage of the capillary tip outside of the sprayer over night avoided such negative effects, as well as swelling effects of the polyimide coating. This effect was pronounced when acetonitrile was main the constituent of the buffer system applied.

5.2.3. CHEMICALS

Water was purified with a Millipore Milli-Q (Bedford, MA, USA) ultrafiltration unit (resistance > 18 MΩ). 2-propanol (99.9%), was purchased from Sigma Aldrich. Acetic acid (99%), and methanol (LC-MS grade) were purchased from Merck, acetonitrile was purchased from Honeywell (Morristown, NJ, USA). Ammonium acetate and ammonium formate were purchased from BDH Prolabo (Darmstadt, Germany). All opium and heroin standard substances were provided by the German Federal Criminal Police Office and of analytical grade.

5.2.4. SAMPLES AND SAMPLE PREPARATION

5.2.4.1. CONDITIONS AND BUFFER FOR THE ANALYSIS OF KRATOM

For the profiling of Kratom, we can rely on our previously developed NACE methodology [102,103] (see Chapters II and III). The interested reader is referred to these publications for the detailed method development, as well as for the advantages and limitations of the method. In short: A non aqueous buffer system using 60 mM ammonium formate in a mixture of acetonitrile and glacial acetic acid (975:60 v/v) was used in a bare fused silica capillary (50 µm inner diameter, 365 µm out diameter, 67 cm length, Polymicro Technologies®). A mixture of isopropanol, water, and acetic acid (64/31/5) was used as sheath-liquid at a flow rate of 7 µL/min.

5.2.4.2. ORIGIN OF THE KRATOM SAMPLES

The Kratom samples analyzed in this study were provided by the Criminal Institute of North Rhine-Westphalia. They were part of a seizure from the investigation of a vendor for biogenic drugs. In total, 40 samples were analyzed in duplicates, including mostly powdered samples, but also resins and powdered extracts. Two fresh *Mitragyna speciosa* plants (so called Red Vein Kratom & Rifat Kratom) were included in the study, as we suspected a lot of analytes to be artifacts, resulting from a prolonged storage time and isomerization, based on our experience with this biogenic drug [103]. Accordingly they should differ from the other samples. Additionally, the plants were from different endemic origins, which should allow for a sample classification according to their geographic origin, as described previously [3].

During the analysis we had to exclude three samples as they were not identified as Kratom and created severe memory effects on the capillary wall, probably as result of an oily matrix.

5.2.4.3. SAMPLE PREPARATION KRATOM

The sample preparation for Kratom was a simple methanolic extraction, 50 mg of sample were extracted with 500 µL of methanol for 15 min in an ultrasonic bath and subsequently stored over night below 6°C [277]. The samples were centrifuged on the next morning at 13 000 rpm, the supernatant was transferred into a new microtube and subsequently stored in a fridge below 6°C until analysis.

5.2.4.4. ORIGIN OF THE OPIUM & HEROIN SAMPLES

The opium and heroin samples were provided by the German Federal Criminal Police Office. Their investigation was part of a project (“MIME”) for the fast analysis of drugs and counterfeit pharmaceuticals by means of spectroscopic analysis funded by the federal ministry of education and research. In total, 11 opium and 13 heroin samples were analyzed. One of the opium samples was a reference sample purchased from Merck. For method development, the analytes depicted in Figure 29, provided by the German Federal Criminal Police Office were used. All analyte standards used were of analytical reagent quality.

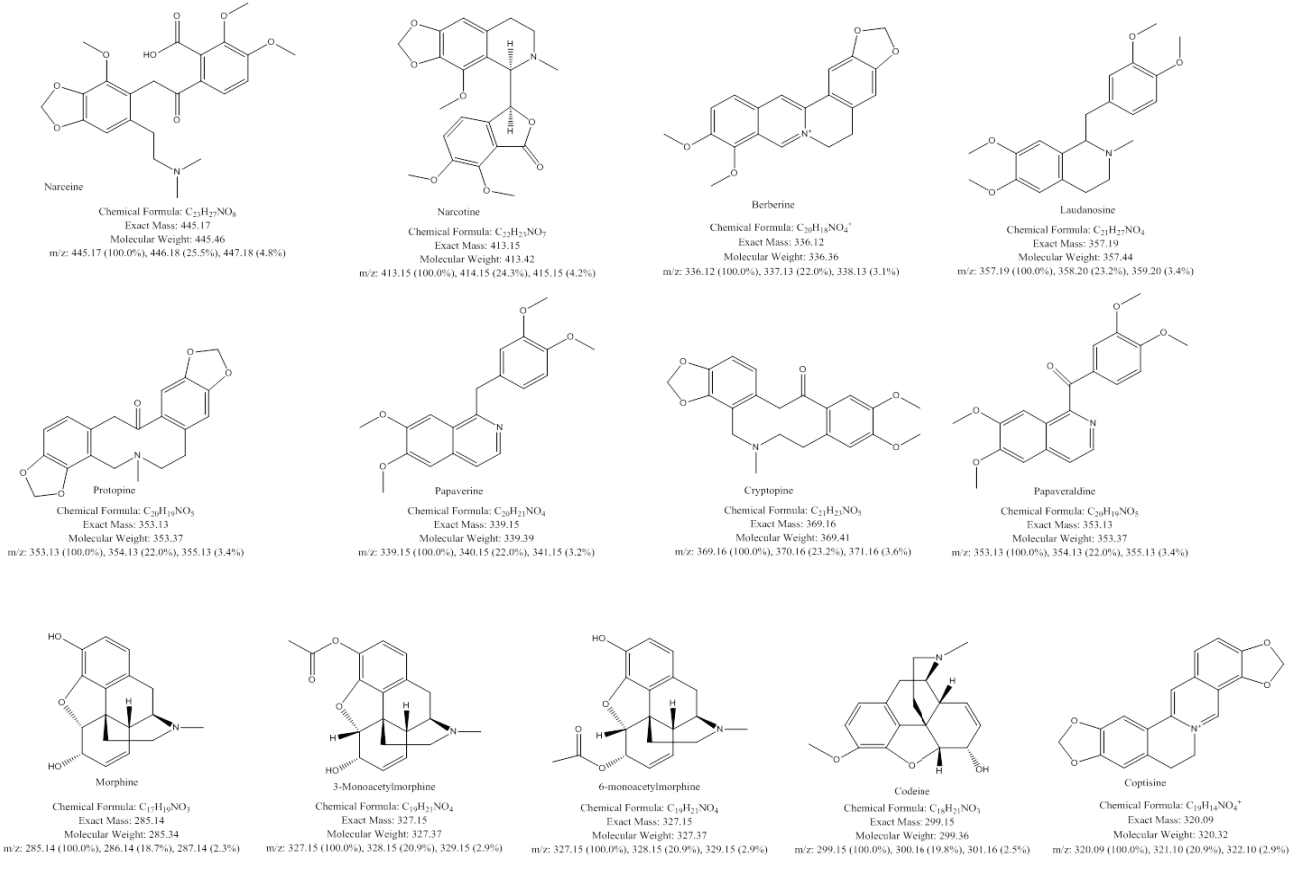


Figure 29: Reference alkaloids with their monoisotopic masses used for method development in this study

5.2.4.5. SAMPLE PREPARATION OPIUM & HEROIN

The sample preparation for opium and heroin was a simple methanolic extraction. For opium 50 mg of sample were extracted with 500 µL of methanol, for heroin 5 mg of samples were extracted with 500 µL of methanol. Both substances were subsequently placed in an ultrasonic bath for 15 min. The samples were centrifuged afterwards at 13 000 rpm and the supernatant was transferred into a new microtube. For injection 5 µL of the sample extracts were diluted with 995 µL of methanol spiked with caffeine and ajmalicine as internal standard yielding a concentration of 50 µM in the sample. Ajmalicine was used as standard, as it is not known to be part of heroin preparation, but exhibits a similar mass to cross section ratio as most opium alkaloids.

5.3. METHOD DEVELOPMENT FOR OPIUM AND HEROIN

Non aqueous capillary electrophoresis is a powerful tool for analyzing complex mixtures of plant metabolites as we demonstrated before [103]. Unfortunately, our previously developed buffer system for the analysis of psychoactive plants (60 mM ammonium formate in a mixture of acetonitrile and acetic acid (975:69) [103]) showed only limited applicability for the analysis of opium and heroin as the permanently charged analytes coptisine and berberine could not be separated using ammonium formate concentrations below 60 mM (see Figure 30). A further addition of ammonium formate (>100 mM) was able to create a partial resolution of this critical peak pair, however at the cost of total analysis time for the slowly migrating analytes narceine and narcotine (total analysis time > 40 min, data not shown). Therefore we adopted the methodology via exchanging the aprotic acetonitrile by the polar, protic methanol, which should result in less ion-pairing due to hydrogen bonding and thus ion-stabilizing capabilities of methanol.

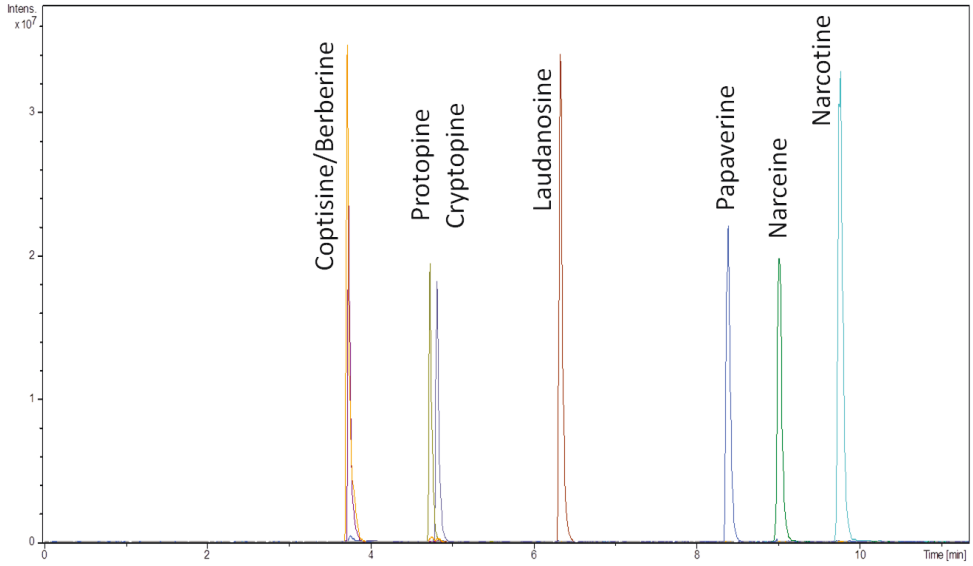


Figure 30: Analysis of opium standard compounds by NACE-MS using a buffer composed of 60 mM ammonium formate in a mixture of acetic acid and acetonitrile (95:5 v/v). Note especially the comigration of the permanently charged analytes coptisine and berberine at 3.8 min.

A mixture of opium and heroin standards was used as standard injection solution during method development for both drugs. The method was initially optimized in a univariate approach. Its robustness was assessed using a design of experiments and using the response surface methodology. Robustness assessment and definition of the optimal parameters was performed using the electrophoretic resolution as response value for the DOE. Optimal performance was defined as the maximal mean electrophoretic resolution established between all peaks in the electropherogram. The electrophoretic resolution of a peak pair is defined as:

$$R_S = 2\frac{(t_2 - t_1)}{(w_1 + w_2)}$$

Equation 7: With R_s = electrophoretic resolution, t_x = migration time of compound X, w_x = peak base width of compound X.

Three parameters were varied and tested in the DOE: The ammonium formate concentration in the range of 10-25 mM, the acetic acid content in the range of 5-25 µL, and the methanol content in the range of 975-995 µL. The results are visualized for ammonium formate and acetic acid in Figure 32. According to this, the electrophoretic resolution is purely dependent on the EOF as obviously no selectivity

changes are observed which can be seen from the absence of a curvature of the plane. Also, only the ammonium formate content has a significant influence, according to the ANOVA results. Thus, the optimization of the method needs a compromise between high resolution and analysis time. Robustness is the same over the whole range of the DOE. As no experiment conducted within the DOE exceeded 20 min run time, the maximum concentration of 25 mM ammonium formate in a mixture of methanol and acetic acid (971:29) was used as background electrolyte providing the highest resolution obtained with narcotine as the latest analyte detected at ~13.5 min (Figure 31).

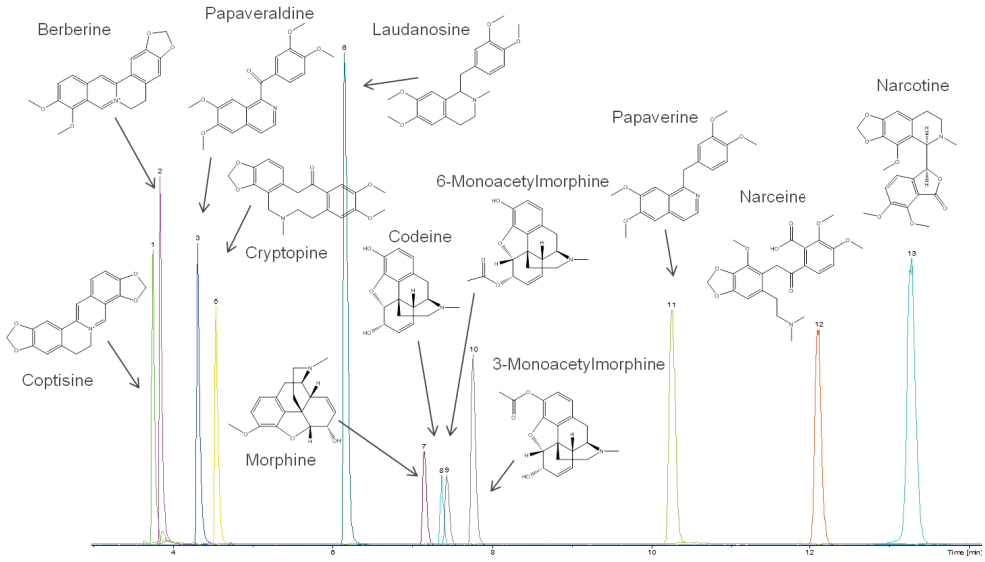


Figure 31: Electropherogram of the analysis of opium and heroin standards after univariate optimization steps. A buffer composed of 25 mM ammonium formate in a mixture of 95:5 MeOH and acetic acid was used.

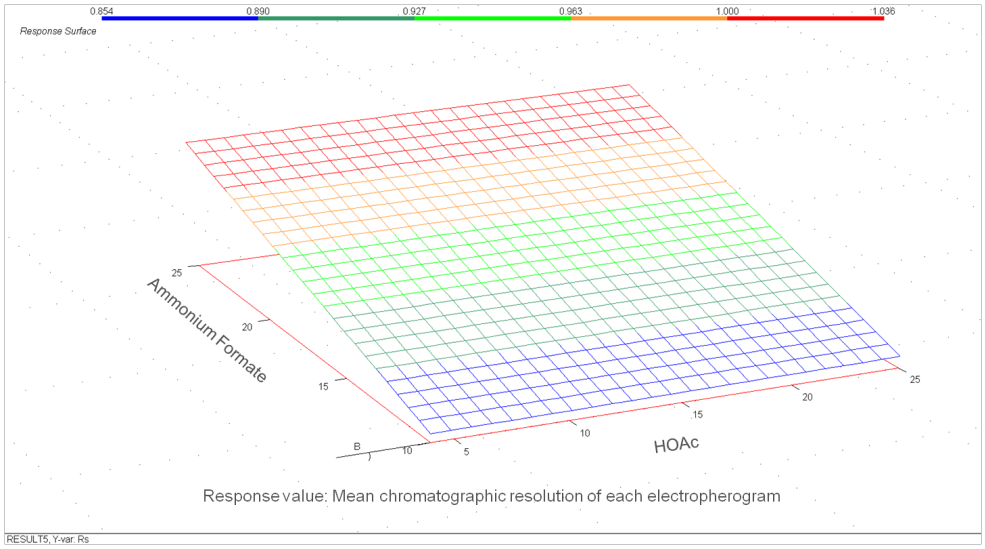


Figure 32: Assessment of robustness using the response surface methodology for the developed NACE-MS method. The figure shows the dependency of the response variable "mean electrophoretic resolution" on the parameter ammonium formate content (10-25 mM) plotted against the acetic acid content (HOAc) (5-25 µL). The higher the z-value (mean R_s), the better is the overall electrophoretic resolution. As can be seen from the absence of a curvature of the plane, the electrophoretic resolution is purely dependent on the EOF.

To verify the suitability of the developed procedure for the analysis of real opium samples, a standard reference opium sample (MERCK) was used to assess if the acquired resolution was sufficient. The results are presented in Figure 33A with Figure 33B showing an enlarged view of this analysis. Especially from Figure 33B, it becomes clear that the method has a very high separation power resolving ~ 1 peak every three seconds in the shown migration time segment (5-10 min). According to the obtained results, the method was considered to be suitable for the qualitative and relative quantitative analysis of opium samples to investigate the potential application of NACE-MS for an in-depth profiling of opium and heroin samples.

A)

B)

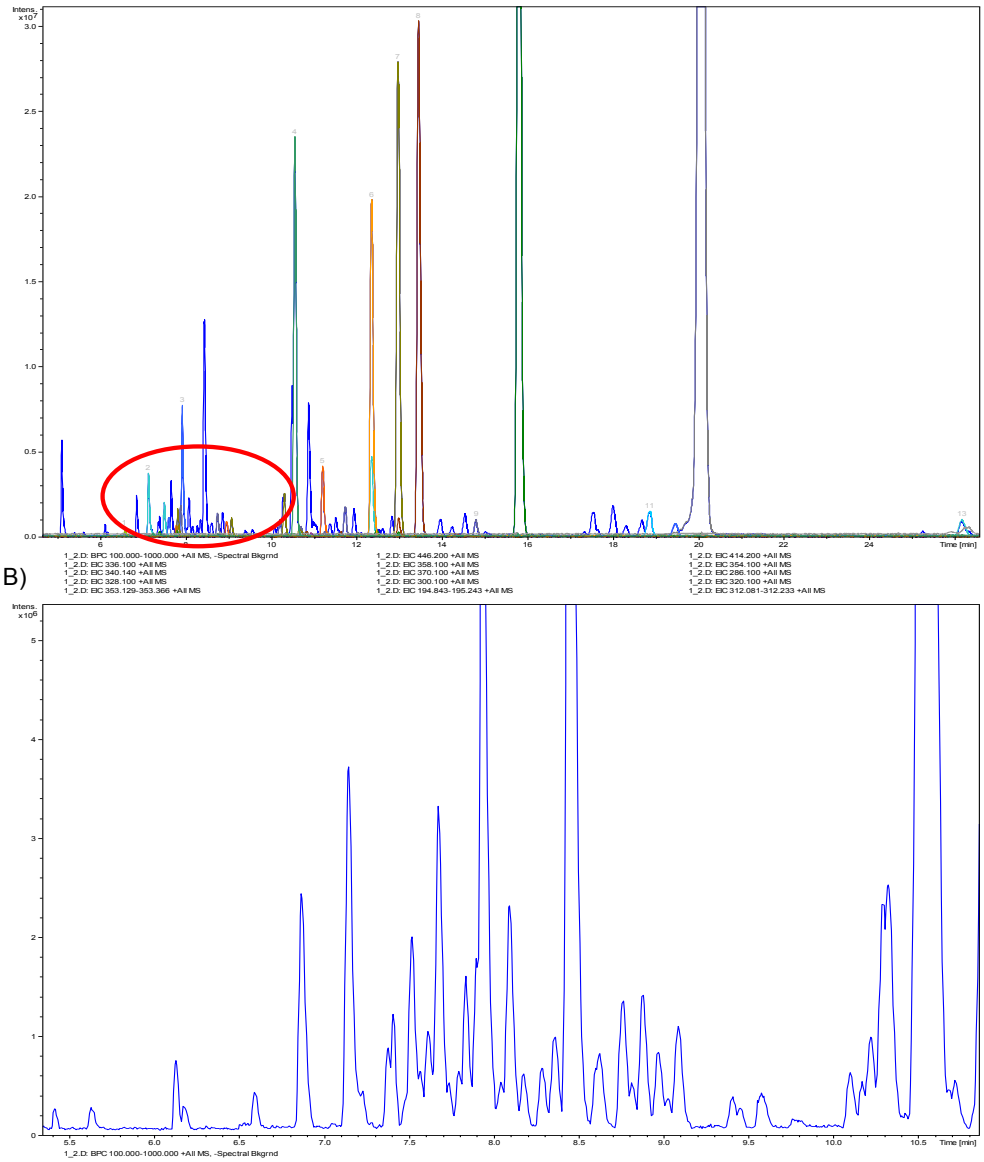


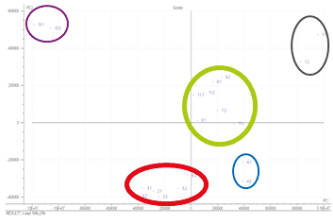
Figure 33: A) Analysis of a standard opium sample. The blue trace represents the basepeak electropherogram, the colored peaks represent the standard substances used for the establishment of the method which are also present in the sample. Note especially the accumulation of small compounds between 8 and 10 min migration time. An enlarged view of this region is shown in B) Note the high density of peaks which are still resolved and described by a sufficient number of data points, clearly showing that high performance mass spectrometric instruments with high acquisition rates are needed here.

5.4. DATA PRETREATMENT & ALIGNMENT

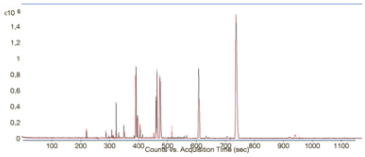
The work presented in this subchapter is the result of researchers from Jülich and Leiden, mainly Tjorben Posch, Dr. Pablo Kler and Prof. Magnus Palmblad. The software for the alignment and data extraction was developed by Prof. Palmblad; Dr. Kler was involved in the adaptation of the software for CE-MS measurements, while Mr. Posch performed the data conversion and the final analysis including also the data evaluation and the assessment of the results. The following text is an explanation of the working principle of the software.

Whenever methods for the purpose of profiling are established, it is highly desirable to achieve a very high degree in automation, as large sample cohorts have to be expected and are required to achieve a profound data base. Unfortunately software for mass spectrometers is designed for LC separations, where Gaussian shaped peaks are obtained, in contrast to the more Lorentz-type peak shape obtained by CE applications. Consequently, commercial LC-MS software shows a poor performance in automated peak picking, extraction and integration for CE-MS and especially for NACE-MS electropherograms, where extremely sharp peaks are achieved. To compensate the limitations in the currently achievable automation, an in-house software was adapted from Nevedomskaya et al. [8] to perform an alignment of electropherograms and from Selman et al. [7] for data extraction. To be able to handle the data file formats of the individual instruments and vendors, all data files were first converted into an open mass spectrometric file format .mzXML. The MSConvert program of the ProteoWizard software package was used for this purpose [320].

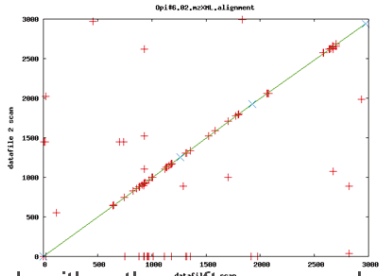
The whole data analysis pipeline of the in-house software is illustrated in a flow chart (Figure 34) (simplified). The whole process consists of three individual steps, which will be further explained below. In short: 1) an alignment of the electropherograms to a masterfile to compensate for shifts in migration times between the individual measurements. 2) An extraction of the peak height or peak area in all electropherograms for all m/z-values of interest. 3) The merge of all relevant data in one table for further data treatment and evaluation including chemometric analysis.



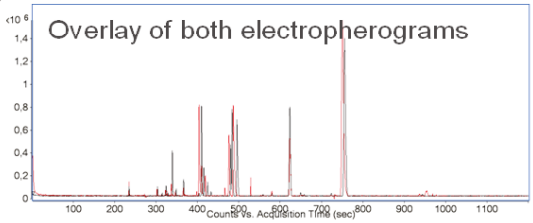
4. The extracted information from the data pretreatment was further analyzed in a principle component analysis using the Unscrambler® Software.



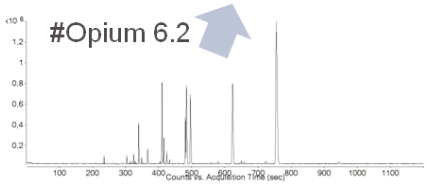
3. The software aligns the two electropherograms and automatically extracts peak heights or areas and merges all information in one table.



2. Using a genetic algorithm the software searches for a fitting curve which represents the temporal relation between the two electropherograms.



1. An electropherogram of a CE-MS run is compared to a predefined Masterfile according to characteristic *m/z*-values.



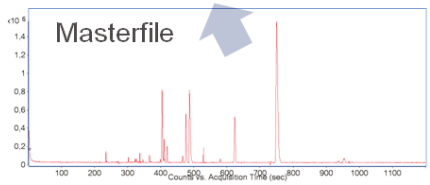


Figure 34: Flow chart illustrating the subsequent working steps of the data pretreatment.

5.4.1. ALIGNMENT

The software first aligns all electropherograms of the NACE-MS measurements to a masterfile. The masterfile is selected manually based on the expertise of the operator according to different factors: 1) the masterfile should be free from matrix effects, ghost peaks and of overall high quality regarding peak shape and separation. 2) The migration times of the individual peaks of the masterfile should match the mean migration times of the respective peaks in the data set, thus representing a sort of average electropherogram in the population. 3) The masterfile should represent an ideal separation and representation for this type of analysis to ensure an easy and reliable data extraction afterwards. The software will use the masterfile as template and align all other electropherograms of the dataset to correct for migration time shifts of peaks using the precise m/z-information for each peak and trying to match its migration time with same compound (same m/z-value) in the masterfile using a genetic algorithm. Consequently, the migration time axes of all electropherograms are stretched or clinched at certain breakpoints, calculated by the software. This is shown in Figure 35: Each red cross represents a peak. Red crosses on the x and y-axes represent peaks which are present only in one data file. The green line represents the line of best fit for the alignment. The blue crosses represent the calculated breakpoints, which are used for the correction of the electropherogram. In consequence and in an ideal case, the time axis of the electropherogram to be aligned is stretched or clinched until the peaks from the masterfile fully overlap with the peaks of the other electropherogram. The diagram A in Figure 35 shows the alignment of two measurements with very different and non-linear migration behavior, while Figure 35B shows the alignment of two very similar analytical runs.

A) B)

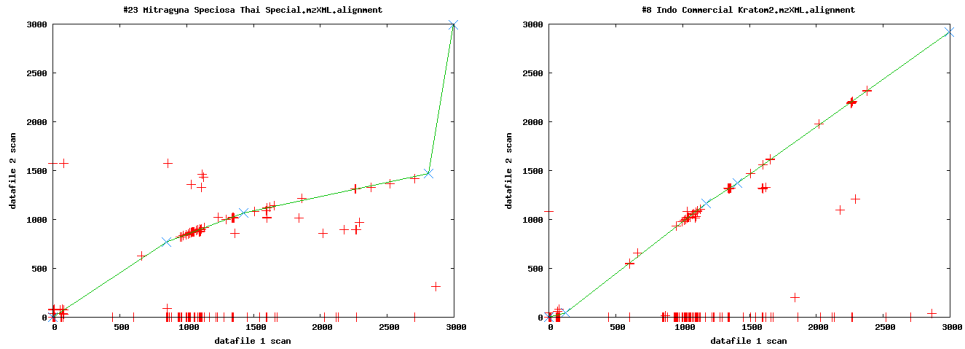


Figure 35: Results of the alignment of two measurements from the Kratom sample cohort. The x-axis represents the migration time in scan numbers in the predefined masterfile, the y-axis represents the migration time in scan numbers of the data file to be aligned. Diagram A shows the alignment of two measurements with very different and non-linear migration behavior, while diagram B shows the alignment of two very similar analytical runs. For further explanation see text.

5.4.2. DATA EXTRACTION

After the alignment, the peak heights or peak areas from all electropherograms are extracted according to a manually predefined list of analytes of interest, regarding their mass over charge ratios and defined migration time windows. This list includes the *m/z*-value, charge, migration time window and a maximal allowed mass deviation. The operator defines this list according to a visual inspection of the masterfile and a short overview on the whole population to assess migration time shifts and the resulting migration time windows. The software can then extract either the maximum height of each analyte peak within the migration time window or it can integrate the peak area within the migration time window. Figure 36A illustrates the working principle of the process in the peak area mode. The software will integrate the complete peak area below the predefined electropherogram (black line) within the red migration time window. This includes the background signal to the left and right at the base of the peak. The percentage of background co-extracted with each peak is insignificant, although it could be circumvented by background subtraction, which is generally possible with the software. Although Figure 36A depicts a basepeak electropherogram, the software will create an extracted ion electropherogram in the background for the data extraction for each individual analyte. Figure 36B shows the working principle for the peak height mode. The software will extract the maximum height for a specific m/z-value within the complete migration time window, by creating

an extracted ion electropherogram in the background. The clear advantage of this method over commercial software packages is that it does not rely on peak shape for peak recognition, thus making it ideal for CE-MS measurements in contrast to vendor-based LC-MS software packages.

The extraction of the peak height turned out to be more robust against background effects and was thus preferred throughout this study. However, as the same migration time window is used for all measurements, the definition of this window becomes the most crucial parameter for the data evaluation. Therefore, the time windows were set after a visual inspection of all electropherograms, using the widest timeframe possible, carefully excluding possible interfering analytes with a similar m/z-ratio, especially important with regard to the large number of diastereomers.

A)

B)

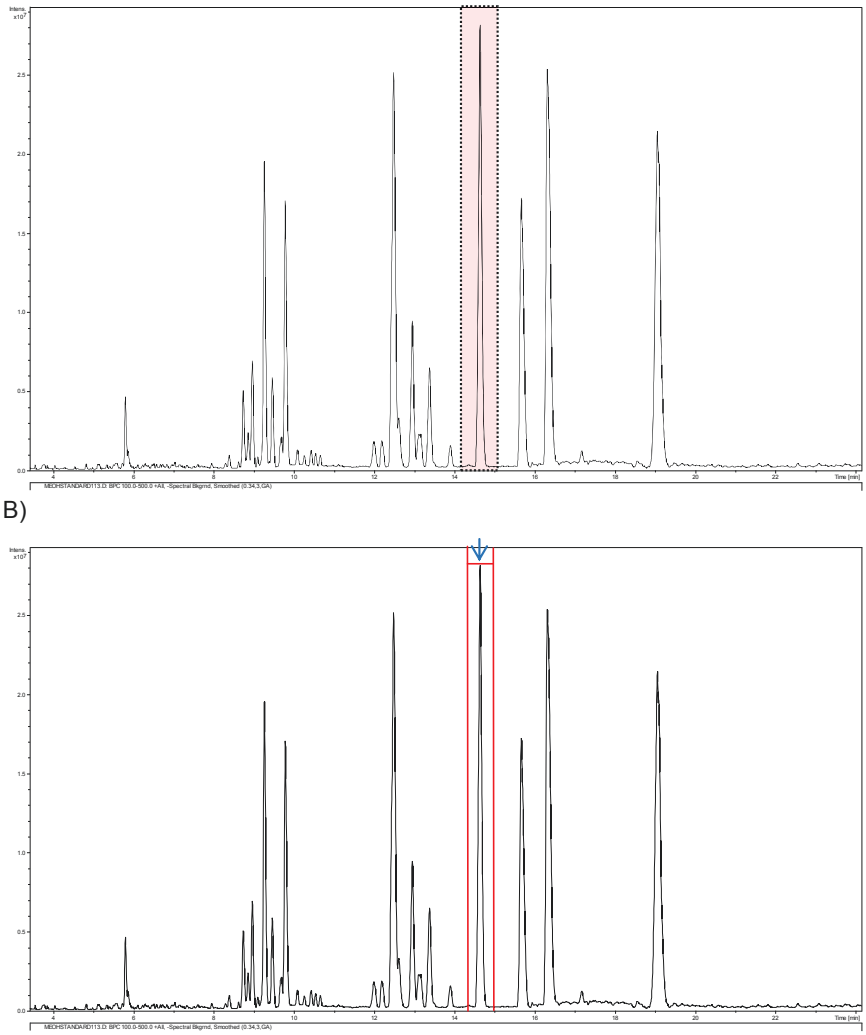


Figure 36: Basepeak electropherogram of an opium sample measured. A) Illustration of the working principle of the peak picking using the peak area. B) Illustration of the working principle of the peak picking using the peak height. For further explanation see text.

5.4.3. VISUAL FEEDBACK & MERGE OF THE DATA

After extraction of the peak heights or peak area, the extracted data is merged into a report table for further data treatment with statistical software. For an easy inspection and control of the alignment and the integration results, the software also converts the electropherograms into a heatmap and highlights the integrated peaks. For a better understanding of the creation of the heatmap an illustrative example is shown in Figure 37. As can be seen, a heatmap is a simple graphic, presenting three-

dimensional data in a two dimensional manner. From a comparison with the original electropherogram, one can see that the time axis has not changed and remains the same. The m/z-data is plotted on the y-axis while the intensity of each signal is expressed in terms of brightness.

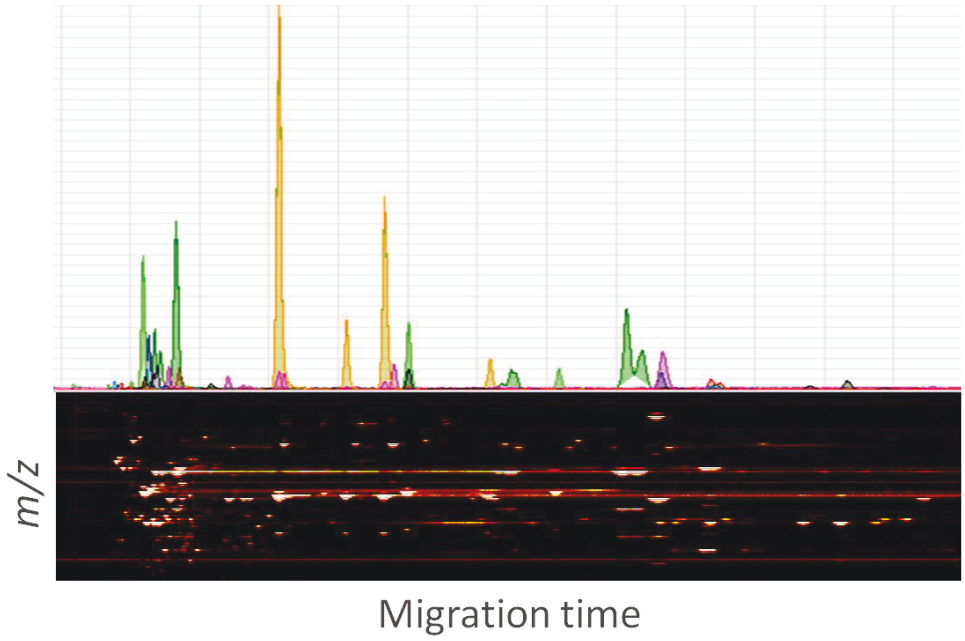


Figure 37: Example of a heatmap with the corresponding electropherogram above.

Figure 38 shows an example of a heatmap for the visual inspection of the integration. The x-axis represents m/z-values and the y-axis represents the migration time in scan numbers from the alignment. The green lines and blots represent the electropherogram as explained above. Only the red blots were extracted as peak area in the depicted example. The green blots are excluded.

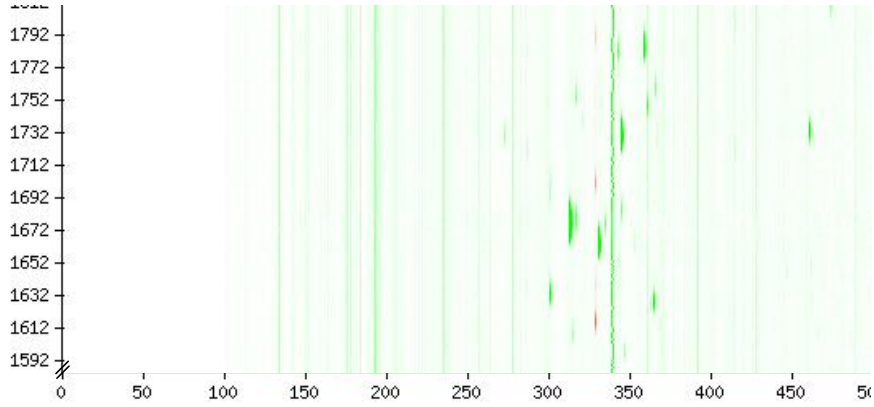


Figure 38: Example of a heatmap for the visual inspection of the measurements. The analysis shown in this figure is part of the opium measurements conducted within this study. As can be seen, only three peaks of one mass over charge ratio were extracted. The solid green line around m/z 335 represents the dominant base peak, a constant background signal.

5.4.4. ADVANTAGES AND LIMITATIONS OF THE IN-HOUSE SOFTWARE

A major drawback of the whole process originates from the open data file format. Once the files are converted they can't be converted into the file format of the MS-instruments back again. Therefore all further data analysis has to be performed with open source software packages. Consequently, one can not only perform one step of the pretreatment and extract the desired information manually afterwards, although this would be of great importance as can be seen in the following problem:

The basic idea was to use the in house software for the data evaluation of the analysis of heroin, opium, and Kratom. However, the Kratom analysis turned out to be the most challenging, as a characteristic of the Kratom samples is the very high number of diastereomers and other isobaric substances [3-5,102,103,317,318]. This has been reported for the main alkaloids however, we observed this for nearly all investigated compounds. This is problematic for all automated data extraction tools applied so far however, it is especially unfortunate for the in house software regarding the alignment process and peak area/peak height determination. As can exemplarily be seen in Figure 39, the peak density for the compounds with a mass over charge ratio of 413 is very high in the region between 6-7 minutes.

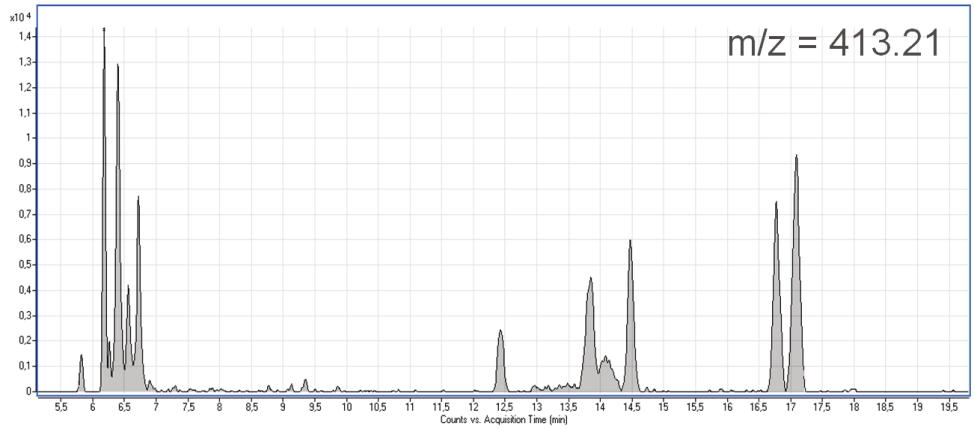


Figure 39: Extracted ion electropherogram for the mass trace m/z = 413.21 ± 0.02 for the analysis of a typical Kratom sample. At least 11 isobaric peaks can be observed in this measurement. Note especially the high number of peaks in the small migration time window between 6 and 7 min.

This causes two serious problems: 1) an extraction of the peak area by the software is impossible in this datafile, as the definition of the migration time window would need a precision that goes beyond the capabilities of the alignment, although it is impressive. Consequently, also peak height fails to deliver the correct information, because only the maximum is selected within the migration time window, hence all other peaks are missed. 2) The alignment itself might fail here, since the high peak density for isobaric compounds makes it impossible for the program to differentiate the individual peaks and subsequently match them with the masterfile. Also, differences in the number of isobaric peaks can lead to serious distortions of the alignment process. In such cases it would be desirable to perform only the alignment and perform the data extraction manually afterwards, as the software was indeed capable to correct also for non-linear migration time shifts in several measurements of Kratom (see Figure 35A). Unfortunately, this is impossible in the current state of the open source software packages. Consequently, it was decided to apply this workflow for the analysis of opium alone.

During the conduction of this series of analysis, the commercial software package Molecular Feature Extractor (MFE) (Agilent Technologies) was updated and improved. These updates turned out to be capable of dealing with NACE-MS peaks and peak shape, thus the implementation of a data evaluation by means of the MFE were evaluated in the analysis of heroin and for opium, as a cross validation experiment.

5.4.5. PRINCIPAL COMPONENT ANALYSIS

For the chemometric assessment of the results from the different data extraction tools, the principal component analysis is used in this study. The principal component analysis is a powerful tool in multivariate data analysis to identify patterns in data sets, derive characteristic differences or similarities in a set of related samples and to enable data reduction without a loss of information [321]. To achieve this overall goal, related and connected variables are identified and pooled in a new variable, the principle component. Plotting the newly established principal components against each other in the score room leads to an improved highlighting of differences between the samples, which might have been hidden in the noise before. The numerical values for each principal component are expressed in the corresponding loadings, showing the connected variables. Loadingsplots represent the impact of each original variable in the linear combination for the determination of the principal components.

In the present case, PCA is used for the reduction of the variables and identification of the so called qualifiers, which are the characteristic analytes in the samples, responsible for the clustering of similar samples in the score room. The resulting models can be used to match new sample seizures to the specific, known groups. As the loading plots show the most important qualifier substances for the assignment of a sample to a certain group in the score room, it is then possible to focus on these substances only for further analysis in the future in a targeted approach.

However, to verify such a reduction of the input data, one has to start with the whole dataset, to ensure that no important marker substance is missed. It is also advisable to repeat the whole process after the model was used for a certain time with additional, more recent samples to validate its applicability. Also a cross validation with new samples is advisable, to make sure that new developments or changes in the samples (like adulterants, impurities, etc.) do not interfere with the model or can be included as new potential qualifiers.

5.5. RESULTS: KRATOM

5.5.1. DATA PRETREATMENT & MANUAL EVALUATION

As previously discussed, no automated data evaluation routine could be employed for the analysis of Kratom. Therefore, manual data extraction was performed. However, the very high number of analytes per samples, especially those being isobaric made data pretreatment inevitable (see Figure 39 and Figure 40).

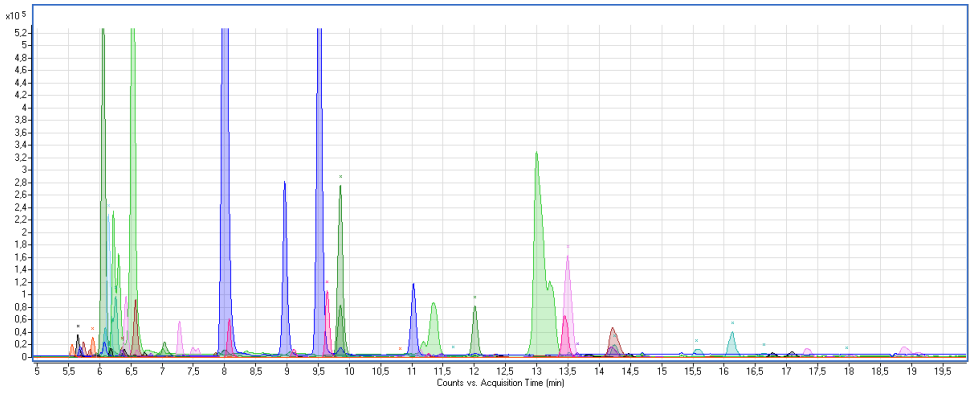


Figure 40: Electropherogram of a typical Kratom sample. Same peak colors indicate same mass-to-charge ratio. The analysis was performed using the standard conditions: 60 mM ammonium formate in a mixture of acetonitrile and glacial acetic acid (975:60 v/v) was used as BGE in a bare fused silica capillary (50 µm inner diameter, 365 µm out diameter, 67 cm length). A mixture of isopropanol, water, and acetic acid (64/31/5) was used as sheath-liquid at a flow rate of 7 µL/min.

A list of mass over charge ratios with at least one peak with an area larger than 50 000 in at least one sample was created. If the peak area of one compound was less than 50 000, its value was set to 0. The relatively large cutoff was chosen as many dehydro-compounds were detected for each analyte, creating an excessive number of very small peaks. As some of them may present storage artifacts and their origin and identity is unknown, we excluded them via this threshold and, thus focused on the side compounds, from which we expected a high differentiation potential, due to our experience from previous experiments: Figure 41A shows five measurements of Kratom samples, with a very high similarity in the distribution of the main compounds, whereas clear differences in the corresponding side compounds can be easily assessed (Figure 41B). Hence, a differentiation based on these analytes seems to be likely.

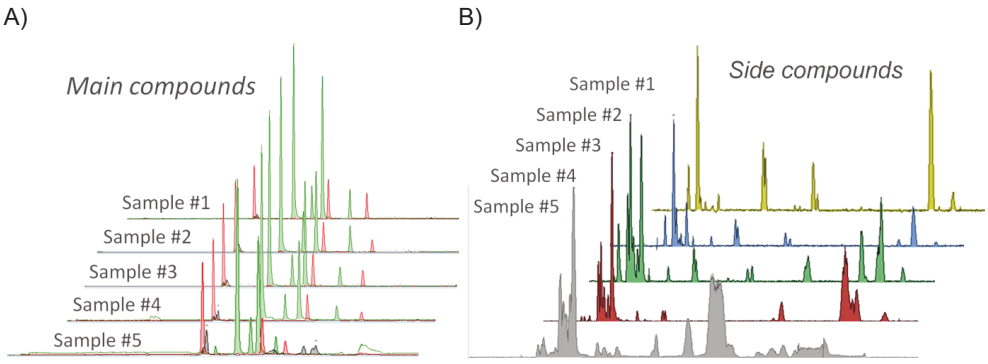


Figure 41: Extracted ion electropherograms of the main compounds of five Kratom measurements (A) and the corresponding side compounds of the same measurement (B), present only in minor proportions in comparison to the major ones. Only few differences can visually be spotted when comparing the main compounds, but an entirely different pattern can be found within the corresponding side compounds.

For those peaks above the threshold the corresponding extracted ion electropherograms (EICs) were created for each sample file. The EICs were smoothed with a Gaussian filter (9 points) and the peaks automatically integrated after smoothing, resulting in 103 individual peaks per sample, characterized by migration time, mass over charge ratio and peak area. The data set was then further pretreated. For the data analysis of Kratom we here compare and apply combinations of three different strategies: 1) a normalization of the peak area to the peak area of the internal standard (ajmalicine) was applied for all peaks within each individual electropherogram. This will reduce any injection bias and changes in the MS performance 2) all peak areas were normalized relative to the peak with the largest area within each electropherogram, resulting in relative peak areas between 0 and 1. This step will eliminate potential influence from absolute concentration differences between samples, e.g. extraction efficiency in duplicate samples

We are aware of the fact that for sample comparison the absolute amount of active ingredient is of high importance for differentiation, however, as long as no real quantification of all investigated analytes can be established (lack of available standards), we prefer to rely on relative quantification. Especially for biological samples, this is helpful with regard to homogeneity of materials and is common for e.g. metabolomic analysis [322].

The migration time was not included for further data evaluation, but was used for a correct assignment of isobaric signals in different samples. The isobaric peaks were then labeled with numbers according to the order of their appearance in the electropherogram (e.g. 399_1, 399_2 etc.). To prevent contortions in the PCA, signals detected in only in one sample were neglected.

5.5.2. PCA RESULTS

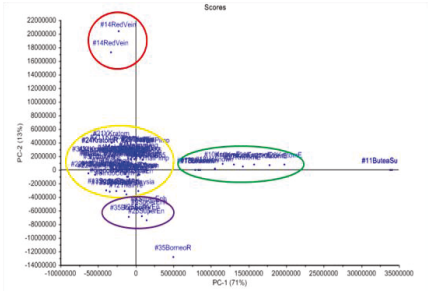
The PCA was conducted using the peak area or peak height respectively for each individual run. Duplicate measurements were consequently treated like individual samples in the PCA. This can be used as intermediate quality control for the whole analysis process and gives an indication about the standard deviation of the measurements and the effect of the normalization procedures. In the beginning, all analytes of each individual electropherogram were included into the PCA. A reduction of the input variables was performed according to the results of the analysis. Peaks which appeared only in one or two samples were eliminated from the analysis later on, as they severely distorted the model, although they are an important unique characteristic for theses samples. Leverage correction was used.

The results of the PCA without normalization procedure are presented in Figure 42A. A clear differentiation of four groups is visible. According to the loadings plot (not shown), this is a result of the distribution of the four main alkaloids (mitragynine (MG), speciogynine (SG), mitraciliatine (MC) and speciociliatine (SC)), dominating PC1 and PC2.

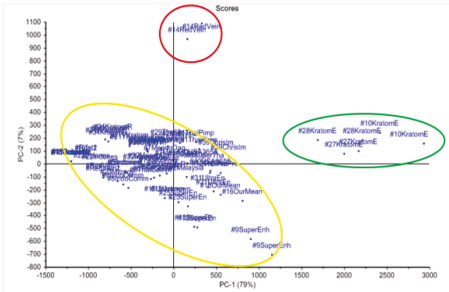
However, it is also visible that duplicate measurements differ in their position in the score plot (see Figure 42A “#14RedVein”-sample), hence preprocessing in form of correction via internal standards was performed to reduce influence probably from injection biases or instrumental performance. The resulting new scoreplot (Figure 42B) shows less discrimination power, but a clear increase in the repeatability for the duplicate measurements. To further improve the significance of the PCA, all data was set in reference to the largest peak (set to 1) in every individual electropherogram to pronounce differences relative to each other, instead of absolute values. By doing so, an improved grouping in the sample set could be achieved, however on cost of a decrease in the explained variance of each PC (Figure 42C). While PC 1 & PC 2 explained 84% of the variance of the raw sample set and 86% in the corrected

sample set, the normalization led to a reduced value of only 56% combined for PC 1 & PC 2. However, this can be easily explained, as the differences in absolute values were removed, which were responsible for a large part of the variance It is also evident that in this data set, no sample can be located in the center of the score plot, as a result of the pretreatment of setting everything into relation to the largest peak, resulting in a model which relies on the “point of view” of the largest peak. As can be seen, the data pretreatment allows here for an increase in the precision in the assignment of the sample towards distinct groups (compare Figure 42A & C). Also, the benefits from the combination of different strategies is evident, as the number of assignable clusters was reduced when only the normalization towards the internal standard was performed, but further pretreatment increased this differentiation potential which is similar compared to the unpretreated dataset, but with increased precision and clear differentiation in the score room.

A)



B)



C)

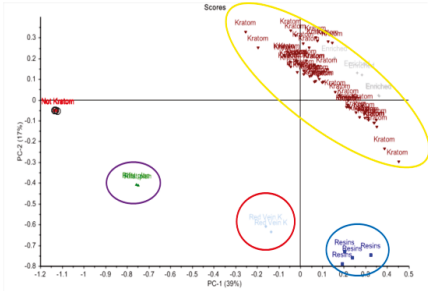


Figure 42: A) Score plot for the analysis of Kratom of the results of the PCA of the raw data without data pretreatment. The PCA is based on the peak area of the individual variables. B) Score plot of the results of the PCA of the Kratom analysis corrected for peak area by an internal standard. C) Score plot of the PCA of the analysis after correction with an internal standard and normalization to the highest peak in each individual electropherogram.

As mentioned above, the loadings plot (Figure 43), corresponding to Figure 42C reveals that the differentiation is mainly based on the distribution in the peak area of the main alkaloids, mitragynine (MG), speciogynine (SG), mitraciliatine (MC), speciociliatine (SC), paynantheine and hydroxymitragynine relative to each other. Consequently, an absolute quantification of these analytes would greatly improve the significance of the profiling.

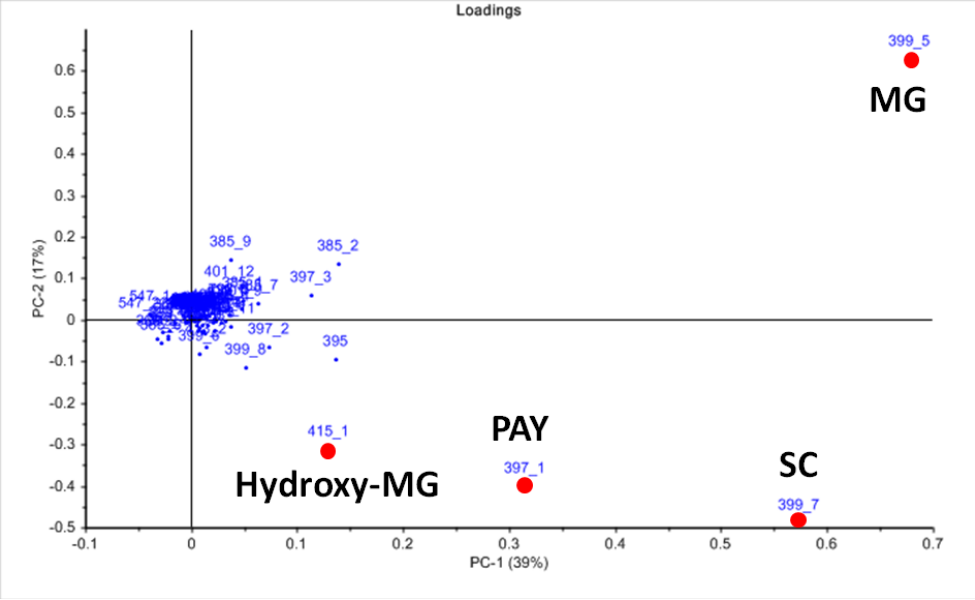


Figure 43: Loadings plot of the PCA based on the normalized and corrected data set (Figure 42C) The numbers in the plot represent the m/z-values of the individual variables (front) and the number behind the underscore represents the order of the peak appearance in the electropherograms in case of isobaric substances, sorted according to their migration times from the fastest to the slowest isobaric analyte.

The comparison of the groups found in Figure 42C with the original labeling of the samples by producer and their physical appearance shows interesting details: The group marked in blue (Figure 42C, PC 1 high, PC 2 very low) represents resins, characterized by a relatively larger peak of speciociliatine instead of mitragynine, according to our tentative identification. The group marked in red (Figure 42C, PC 1 low, PC 2 very low) represents the duplicate measurement of a sample, labeled with "Red Vein" with properties that are entirely different from all other samples, called the

Red Vein Kratom. This Kratom is similar to the resins as the content of the speciociliatine is higher than the mitragynine content, however the overall succession of the relative amounts of the main alkaloids is SC > MG > MC > SG, while the order in the resins is SC > MG > SG > MC. Despite this unique characteristic, it cannot be concluded that Red Vein Kratom generally shows this distribution, as the fresh Red Vein plant included in this study showed a distribution pattern, which matched the general score of most of the analyzed samples (yellow circle, PC 1 high, PC 2 high). The main content of their alkaloids followed the order MG > SC > SG > MC, which is the one most often encountered. The group highlighted in purple (Figure 42C, PC 1 very low, PC 2 low) represents the analysis of the fresh Rifat plant, which is different from any sample within this set. The red group (PC 1 low, PC 2 medium) represents the samples which are definitely not Kratom. The analysis of these samples showed no peaks at all, expect for the internal standard, although the samples had like physical appearance, smell and behavior during sample preparation as the Kratom samples. Only one of these samples was labeled as Kratom ("Brown Kratom"), the others were labeled as unknown by the Criminal Police Office.

A further differentiation of the sample set based on PC 1 & PC 2 was not possible. However, when PC 3 was plotted against PC 1, another subgroup can be observed: the enriched Kratom samples plotted in grey (see Figure 44A). This group shows no real unique characteristics in the PCA, only the difference in relative concentrations leads to their differentiation. According to their labels from producer, they are enriched in some unknown way (labeled as "special", "enhanced", or "ultra enhanced"). However, other samples also labeled as "enriched" can be found in the main population (Figure 44A PC 1 high, PC 2 high). The peaks responsible for the further differentiation are mostly related to the m/z-value 385 in addition to the main alkaloids (Figure 44B). In this case, it was helpful to make use of PC 4 plotted against PC 1 (Figure 45A). The loadingsplot (Figure 45B) corresponding to this scoreplot clearly shows that PC 4 is dominated by peaks of the m/z-value 385 similar to PC 3, however, the achieved differentiation is stronger, although PC 3 represents 13% of the variance and PC 4 only represents 9% of the variance. The comparison of the results from the loadings plot with the electropherograms shows that the peaks for the m/z-value 385 are very small or even absent in the "normal" Kratom samples, but up to nine peaks exhibiting peak areas larger than 50 000 were found in samples labeled as "enhanced", hence a classification based on these substances is justified.

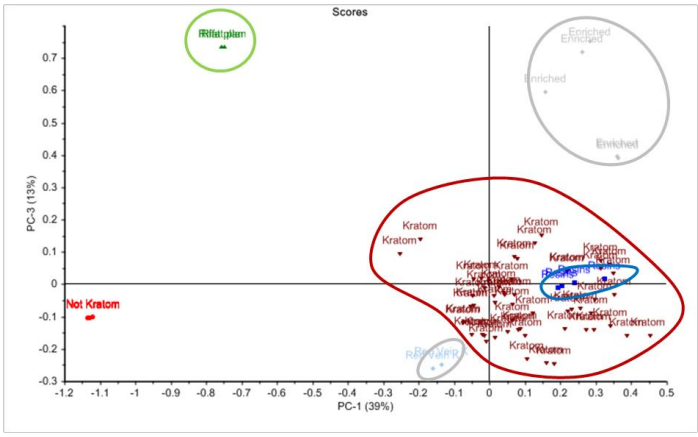
Interestingly, the samples with high peak counts for m/z = 385 were mostly labeled as “enhanced” or “extracts”, also including the resins (samples with positive values for PC 4 (Figure 45A), while the other ones were called “premium”, “commercial” or named according to their origin stated by the vendor. The boundary between those groups can be described as diffuse and some samples fall into the transition zone between those groups. Hence a real classification of Kratom according to this grouping is not robust, but can be used as a marker for the degree of processing, as an indication for a possible further differentiation. According to literature results on the study of the Phase I and Phase II metabolites of the main alkaloids of Kratom [317,318,323-325], the most common metabolites are *O*-demethyl derivates of mitragynine and its diastereomers, exhibiting an m/z-value of 385. As the distribution of these analytes varies strongly over all samples, this can be used as an indicator that these analytes are results of decomposition processes, probably originating from UV-instability or prolonged oxidation and microbiological processes. The increased appearance of these analytes in processed products (resins, extracts, etc.) supports this, as one could expect increased decomposition when the plant material is extracted, enriched or dosed with some artificial substance, like the scents (e.g. Christmas edition, cinnamon smell).

The most important outcome of this analysis of the Kratom samples is that the main alkaloids and therefore only the mass to charge ratios 385, 397, 399 and 415 are sufficient for a differentiation of all Kratom samples. Although several differences could be observed during analysis in the content and composition of the side compounds, these differences cannot be used as robust markers for a profiling. Their distribution seems to be random to some extent as the strong grouping of these parameters in the center of all loading plots shows. In terms of data reduction and workload reduction this conclusion is of high importance, as the comprehensive investigation of all parameters in this study allows us to identify the most important marker substances. Consequently, the side compounds can be excluded from investigations in the future, as they do not provide useful and robust additional information. This will also reduce the demands for the instrumental setup, as these compounds should be easily accessible using simple UV-detection.

When focusing on mitragynine and its diastereomers only, it also seems to be likely to achieve a rough differentiation just by the observation of their distribution. A closer

inspection of Figure 46 shows that there is indeed a strong variation in the content of the main alkaloids. The most important one can be observed in the comparison of Figure 46 C & E with A, B and D. In the case of C & E the speciociliatine is the most dominant alkaloid, while usually mitragynine is the main alkaloid, as visible in case of A, B and D. A further distinction of A, B and D based on the ratio of the main alkaloids to each other seems to be likely. However, although we were able to present these five very different and distinct samples with different patterns, one has to keep in mind that at least 80% of the all samples show the major alkaloid pattern of Figure 46A with MG >SC > SG > MC, hence the additional information originating from the other alkaloids is required.

A)



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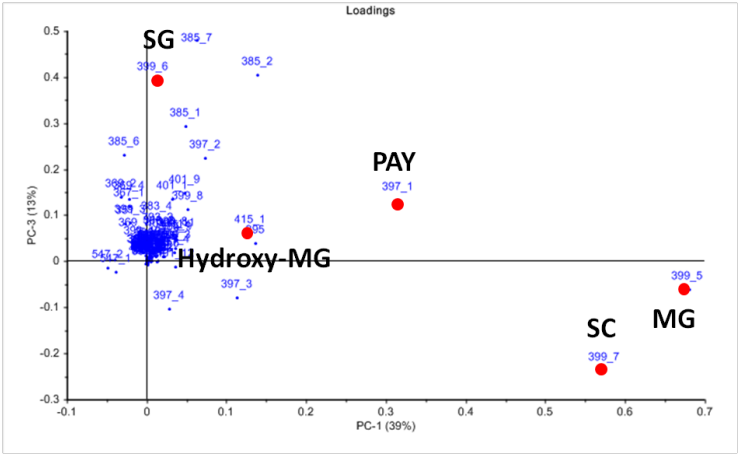
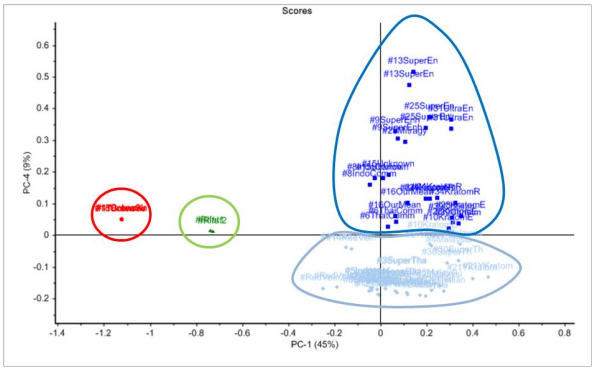


Figure 44: A) Score plot of the PCA from the analysis after data correction with an internal standard and normalization to the highest peak in each individual electropherogram. A clear grouping of samples becomes visible. The differentiation of the resins (blue) from the general Kratom samples (brown) in based on the score plot of PC 1 vs. PC 2. B) Corresponding loadings plot to A plotting PC 1 vs. PC 3. The numbers in the plot represent the m/z-value of the individual variable (front) and the number behind the underscore represent the number of the peak in the electropherograms, sorted according their migration time (fastest to t slowest analyte).

A)



B)

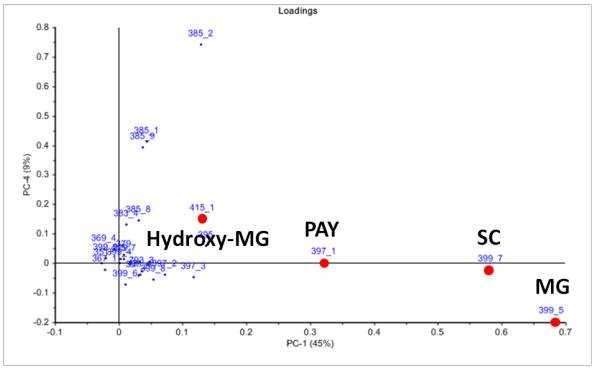


Figure 45: A) Score plot of the corrected and normalized data, showing PC 1 plotted vs. PC 4. A further differentiation of the samples in "enriched" and "normal" can be identified. The boundary between both groups is diffuse. B) The numbers in the plot represent the m/z-value of the individual variable (front) and the number behind the underscore represents the number of the peak in the electropherograms, sorted according their migration time (fastest to slowest analyte).

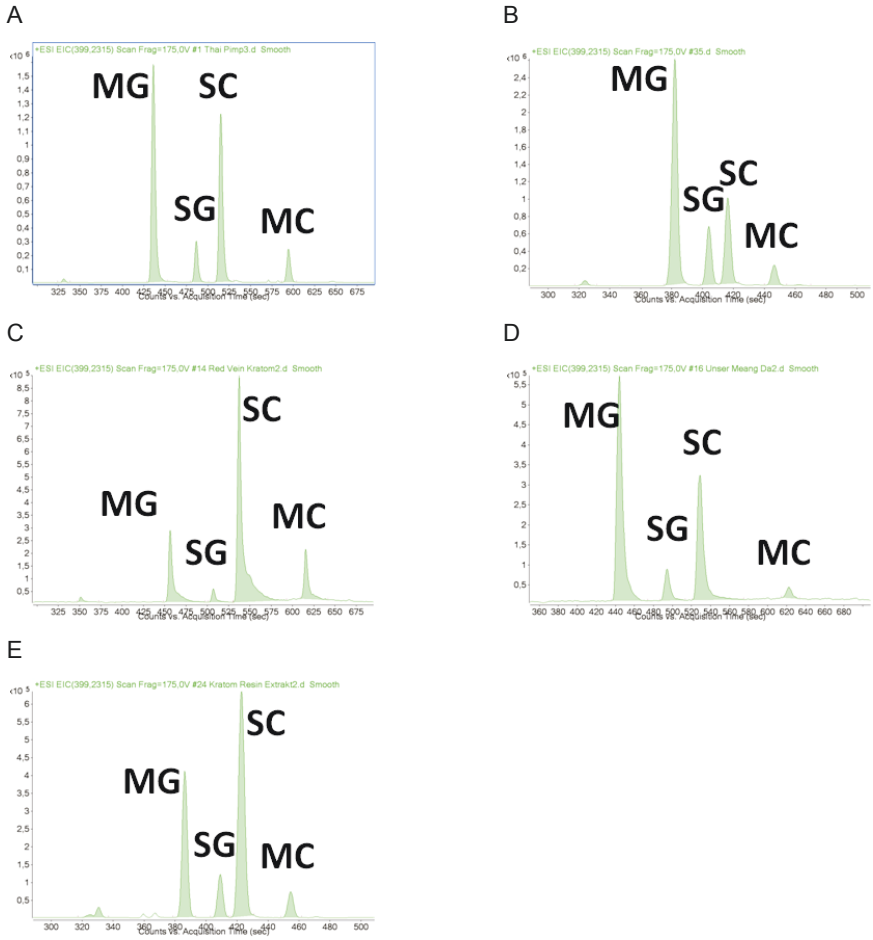


Figure 46: Exemplary extracted ion electropherograms (raw data) for the mass trace of the mitragynine diastereomers (m/z = 399). The ratio of these alkaloids to each other can already be used as an indicator for a differentiation. The migration time shift observed is the result of matrix effects in case of C and of shortening of the capillary in case of E.

5.6. CONCLUSIONS: KRATOM

The results of the Kratom analysis are in general agreement with our expectations and with previous studies [4]: The typical Kratom samples are hard to distinguish as no systematic differences can be observed or related to any possible differentiation based on origin etc., although this should be generally possible for fresh plants as explained in the work of Takayama et al. [3] and other studies from the countries of origin. Additionally, the observed concentration range of the alkaloids is large, which is in agreement with other comparative studies investigating biogenic drugs and Kratom especially. Surprisingly, previously conducted experiments (Figure 41) did

not reveal any significant differences between Kratom samples regarding major alkaloids and active ingredients, altough vendors claim them to be different and also prices and physical properties (color, solubility, etc.) of the samples varied. However, this might also be a result of a too small number of samples for a representative sample set. The measurements conducted during this study however, showed clear differences between the samples, especially with regard to the minor compounds (compare Figure 47). A differentiation of the samples based on these minor compounds was therefore likely. However, the suprisingly low influence of the minor compounds for the differentiation of the samples in the PCA can be regared as a hint that most of these componds are results of a random isomerisation processes, possibly as a result of storage, storage time and photochemistry influence, since they were detected in high amounts in the fresh-plant specimen, but only in smaller amounts within the samples, without an assignable pattern.

However, it was possible to clearly differentiate resins from the other samples. This can usually also be done by a simple visual inspection of the sample, but might be helpful in cases when the resin is very dry and crushed into smaller grains. An interesting opportunity is the identification and assessment of enriched samples, based on the content of the analytes exhibiting an m/z-value of 385 as shown in Figure 45A.

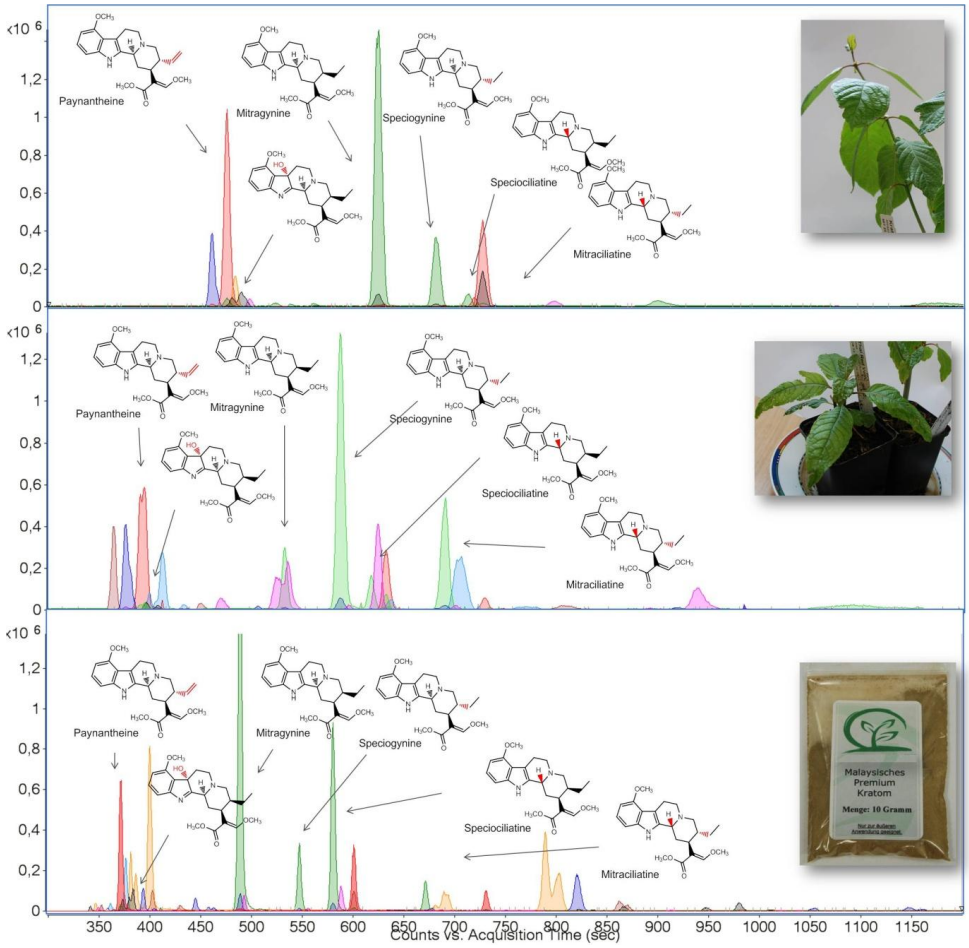


Figure 47: Electropherograms for the analysis of different Kratom samples: The first electropherogram shows the analysis of the leaves of a young "Mitragyna speciosa red vein" plant, while the second electropherogram shows the analysis of a young "Mitragyna speciosa Rifat" plant. The third figure shows the analysis of a Kratom extract bought from an online shop. The capillary length differed between the sample measurements shown here, the migration time shifts are a result of this. Additional influence from the samples on the migration time was observed in the analysis of the fresh plant material. A solid phase extraction is therefore recommended for a repetition of the experiments.

It was quite surprising that the alkaloid pattern of the extract of the fresh plant ("Rifat") did not match with any of the investigated samples, while fresh plant "Red Vein" Kratom matched with most of the Kratom samples, except for the one which was labeled as "Red Vein". Consequently, regarding the geographical correlation of the samples, no systematic pattern could be established, based on the labeling of the samples, affecting a certain geographical origin (e.g. "USA Kratom, Thai Kratom,

Malaysian, Bali" etc.). According to Takayama [3] a differentiation of the known specimen should have been possible based on the relative concentration of the main alkaloids, as the fresh Thai Kratom plant of that study showed an alkaloidal composition of 66% mitragynine, 7% speciogynine, 1% speciociliatione and 9% paynantheine (based on total alkaloid content) while the mitragynine is present only to 12% in the Malaysian plant [3]. However, no evidence for this distribution could be found. This allows three possible conclusions: 1) The main alkaloids themselves undergo random isomerisation processes upon drying and storage, which would make a profiling of this drug nearly impossible, if no stable marker compound can be found. A study observing the alteration of the alkaloidal composition over time could reveal such marker compounds. 2) Possibly the label does not give the true origin of the samples and is just for marketing aspects prohibiting a profiling with regard to geographical origin based on this sample set. 3) The determining factors for the alkaloidal composition of the plants is are only indirectly related to the geographical origin and the major influence stems from other sources e.g. soil composition, UV-light influence, age or fertilization. Plants grown in a green house should consequently exhibit an alkaloidal pattern which can not be related to any geographical origin.

The classification of the biogenic drug Kratom into the groups shown in Figure 42C & Figure 45A is reasonable based on our analysis, although most of the samples exhibit a similar pattern and are consequently assigned to a very large main population. As pointed out before, its is crucial to achieve a high resolution of isobaric compounds, especially for the mass over charge ratios 385 and 399. To the best of our knowledge, such detailed resolution of all these isobaric compounds has not been performed before. As we were able to investigate and analyze the separation principle and demonstrate the broad range applicability of NACE-MS for the analysis of biogenic drugs before [102,103,326], we can now conclude that NACE-MS can be used as a powerful tool for profiling biogenic drugs. The ease of applicability, sample preparation, orthogonality towards chromatographic methods and the low operating costs make it an excellent choice as an additional tool in forensic laboratories. Nevertheless, although the method has demonstrated its suitability, it is evident that there are several aspects to be improved: The robustness of the method was satisfactory during the optimization and analysis, however some samples caused severe problems and adsorption phenomena with the capillary wall. It is also clearly

visible in Figure 46 and Figure 47 that migration time differences occured during the measurements, possibly originating from a high matrix load. Although these effects might not be related to the plant samples, but to adulterants, artificially added to create a “special edition”, one should consider solid phase extraction (SPE) as sample preparation.

It is also well known that NACE backgroundelectrolytes are characterized by a high purity of the solvents and often a high autoprotolysis constant, hence allowing the application of high voltages with low induced currents. Consequently, changes in the ionic strength due to the sample matrix have a pronounced effect on NACE systems in contrast to aqueous buffer systems. During the analysis we encountered samples with unusually high salt contents, having a noticeable effect on the conductivity of the BGE and thus migration times. It remains unclear where this high salt load comes from, but again an SPE step should avoid the influence from such matrix compounds and further increase the overall robustness of the NACE system.

5.7. COMPARISON WITH A TYPICAL ASSESSMENT OF THE ACTIVE INGREDIENTS OF KRATOM

The reference data set used here was established and provided by H. Mahler and L. Welge from the LKA NRW and is used here for the sake of comparison.

All samples analysed within this study were already analyzed by a forensic laboratory using LC-MS/MS (LKA NRW). Here, only the main alkaloid mitragynine was quantified and its concentration was expressed in µg/g for an assessment of the potency of each type of Kratom and each dosage form. The results of this analysis are shown in Figure 48. The red boxplot and sample distribution represents all measurements, as the samples were analyzed in duplicates. The green boxplot and sample distribution represents the mean values of these duplicates. From an inspection of these plots is it obvious, that a classification based on this dataset is not possible, altough two groups may tentatively be identified. Of course, the analysis performed by the forensic laboratry did not aim at a profiling, but for an assessment of the potency of the samples. But the analysis nicely shows that most Kratom samples exhibit identical or comparable amounts of active ingredients, a result which is in agreement with our findings. As a conclusion towards the vast amount of different Kratom “types”, editions, “enriched” and “ultraenriched” samples one can

assess all these differentiations as misleading labeling and false advertisement, aiming at financial profit.

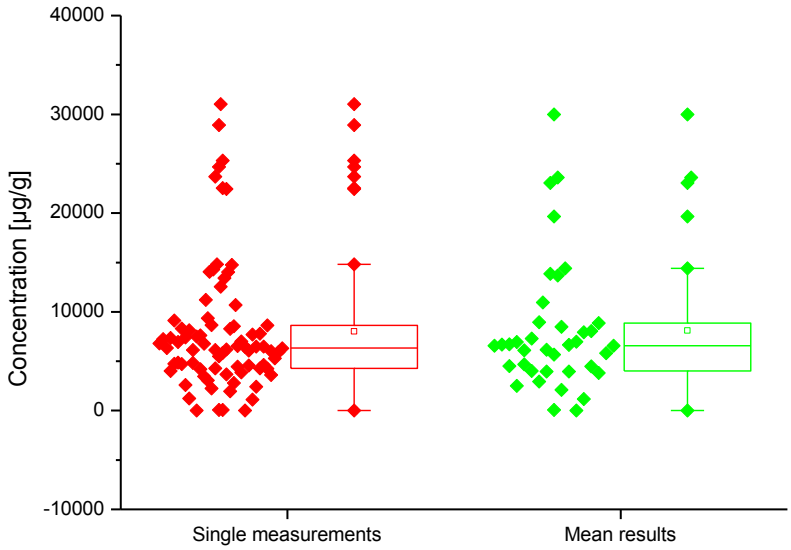


Figure 48: Boxplots of the results of the Kratom measurements made by the LKA NRW. The single measurements depict the concentration of the active ingredients in µg/g in each sample. The mean results represent the mean value of the duplicate measurements of each sample in µg/g.

5.8. SUMMARY

We here presented an indepth investigation of the biogenic drug Kratom. The results of our analysis allowed the identification of the most important qualifiers for a differentiation of Kratom samples. Further analysis can therefore be operated with less effort, in data analysis as well as in instrumental requirements, as usual UV-detection should be sufficient for the suggested analytes in a targeted approach. However, the majority of the Kratom samples was indistinguishable and represents a large group with the concentration and composition of pharmacologically acitve alkaloids differing over a large range. This is in agreement with results from other groups [281] and clearly shows that a profiling of Kratom might be impossible for these samples. For an easy comparison, Figure 49 shows the distribution of the alkaloids MG, SC and PAY in the samples analyzed in the study from Schröfel et al.

[4]. It is evident the observed concentrations show a very broad range without clear patterns, as may be expected for biological samples of similar origin.

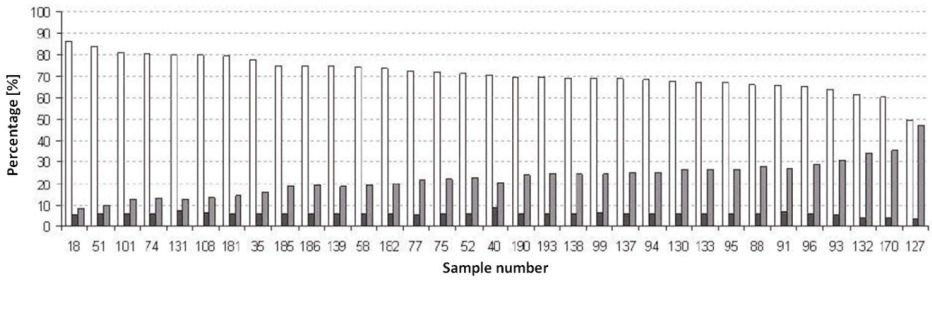


Figure 49: Distribution of the alkaloids MG, SC and PAY in Kratom samples, calculated as percent of the sum of these three alkaloids (taken and translated from [4], with permission from the authors).

As already mentioned above, we suspect decomposition processes as a major source for the alkaloid composition we encountered in this study in comparison to the alkaloid distribution from the Kratom plants in the countries of origin [3]. Similar decomposition by UV-light has already been observed in different molecules, exhibiting a structural moiety identical as the one marked in red in Figure 51 (personal communication during a conference). A study on the decomposition of mitragynine under different environmental conditions could deliver useful information about these suspected processes, but goes beyond the scope of this investigation and it is recommended for future investigations.

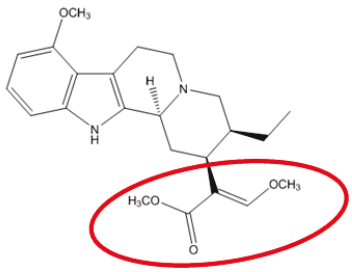


Figure 50: Mitragynine alkaloid. The structure circled in red is expected to suffer from UV-decomposition.

From our results and from [4], we can conclude that a controlled dosage of Kratom is therefore impossibe for a potential consumer. Additional problems with the Kratom

abuse arise from artificially enhanced Kratom products like the “Krypton” which contains a very high concentration of O-desmethyltramadol to fake enhanced potency [2,281]. Also, we had to exclude samples from the analysis, as they were not identified as Kratom. This has been observed in previous investigations of commercial Kratom samples [4]. A possible explanation for this can be found in the work of Maruyama et al. [327]. They investigated the botanical origin of Kratom samples on the Japanese market and found out that some samples contained plants from the same botanical tribe (Naucleeae) as *Mitragyna speciosa,* which poses further threat to the consument, as they are also reported to contain pharmacologically acitve compounds [328] which could lead to dangerous cross-reactivity and may be harmful in itself.

5.9. RESULTS: OPIUM

Here we evaluate the performance of NACE-MS also as a profiling tool for forensics of opium samples. In comparison to the Kratom cohort, for opium analysis most analytes are well known and standard substances are commercially available. Also, the alkaloid composition of opium does not exhibit as many isobaric substances. Consequently, the application of automated data extraction tools is suspectively less prone to errors in the analysis and comparison of opium samples. Therefore, two different types of automated data extraction will be compared here. The commercial software tool “molecular feature extractor” will be compared against the in-house software. We expect the in-house software to perform under ideal conditions in this analysis as the established method is very robust and no diastereomers or closely migrating isobaric substances are present, potential leading to errors in the alignment or the assessment of the peak height.

5.9.1. AUTOMATED DATA EXTRACTION USING THE IN-HOUSE SOFTWARE

Here the results from the full workflow, including data evaluation, application of the in-house software and subsequent chemometric data analysis as explained above (see Section 5.4) are presented for the analysis of the eleven opium samples. Sample preparation and method development are explained in detail above (see Section 5.2.4). The same data pretreatment steps (see Section 5.5.1) as for the analysis of Kratom were applied.

The results from the PCA of the opium samples after automated data extraction are shown in Figure 51 without any data pretreatment. A clear clustering into five groups is visible. The corresponding loadings plot (Figure 52) shows that the differentiation is mainly based on the relative concentration of papaverine, thebaine and narcotine within the samples. Further data pretreatment (correction according to the internal standard, normalization towards the largest peak in each electropherogram) led to a reduction of the explained variance in PC 1 with PC 2 combined (Figure 53A).

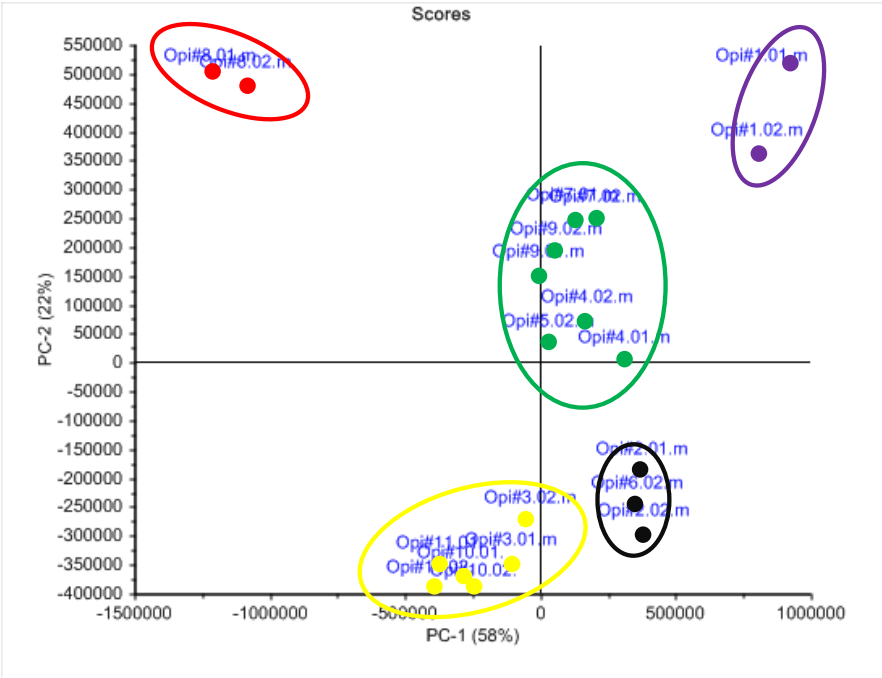


Figure 51: Score plot of the PCA from the analysis of opium samples evaluated by the in-house software without any data pretreatment. PC 1 and PC 2 explain 80% of the total variance of the sample population.

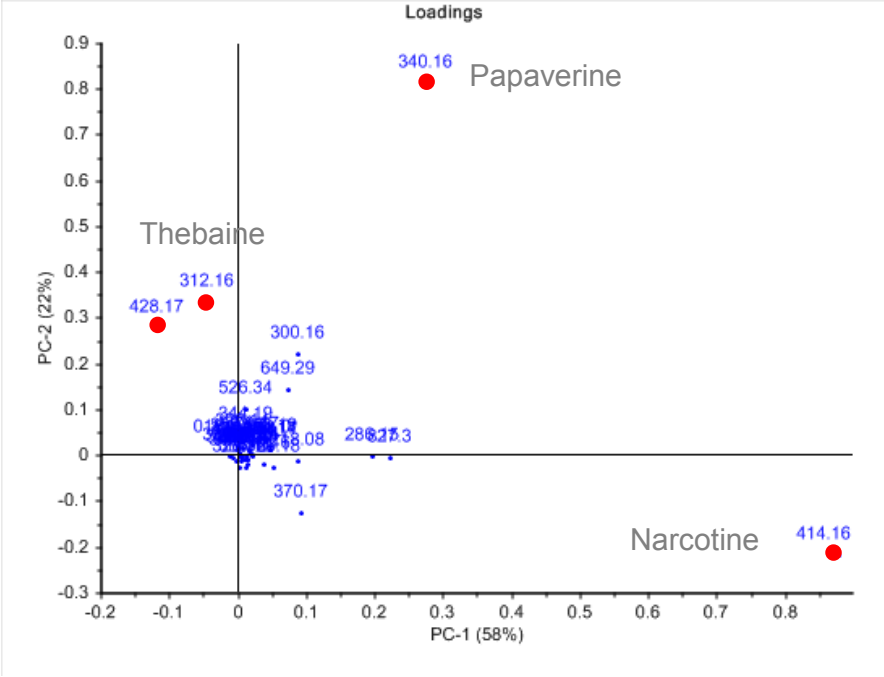
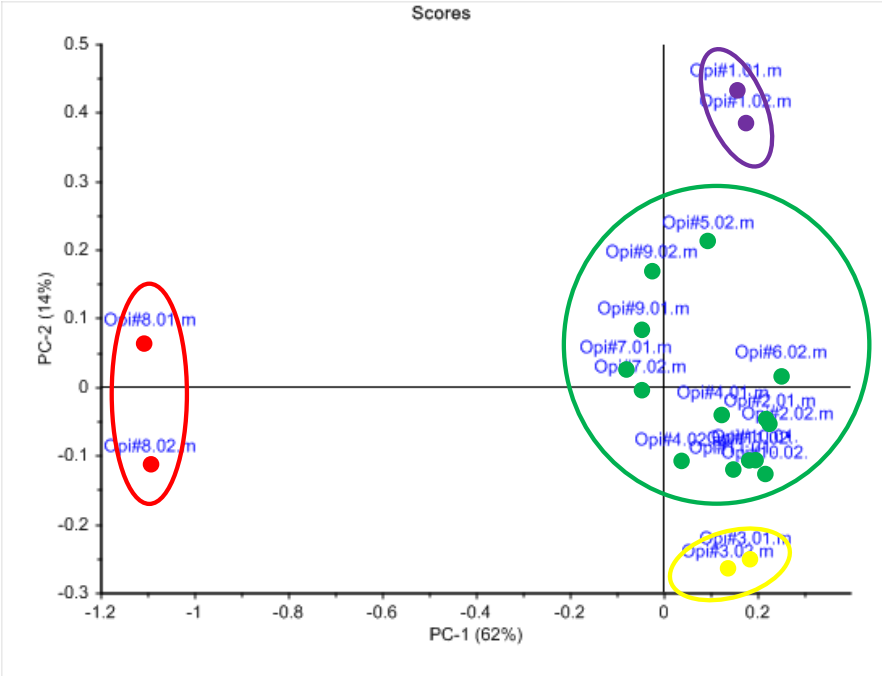


Figure 52: Loadings plot to the corresponding scoreplot in Figure 51 for the PC 1 vs. PC 2. The most important qualifiers for the differentiation in the scoreplot are highlighted in red.

Also, the boarders of the groups become more diffuse and a clear assignment towards the one or the other group becomes more difficult. Only four groups can be identified after normalization and correction. However, the corresponding loadings plot in Figure 53B clearly shows that the same qualifiers are responsible for the differentiation of the groups and, according to their position in the coordinate system, their importance should be pronounced. Hence, a sharper separation of the groups should have been the outcome of the normalization. Consequently, the separation of the opium samples into five groups seems to be a result of overinterpretation. However, it is also visible in Figure 53B that several further qualifiers have now a pronounced influence on the score plot and the underlying model as a result of the normalization and correction of the data. This is an indicator that the data pretreatment created an artificial influence, which is not beneficial. Additional problems arise for the, partially, unknown identity of these compounds. Consequently, a differentiation of the opium samples based on the data evaluation by the in-house software should be performed without normalization and correction. The problems, arising from a possible overinterpretation of the differentiating qualifiers

could easily be eliminated by the application of the whole process to a larger sample cohort.

A)



B)

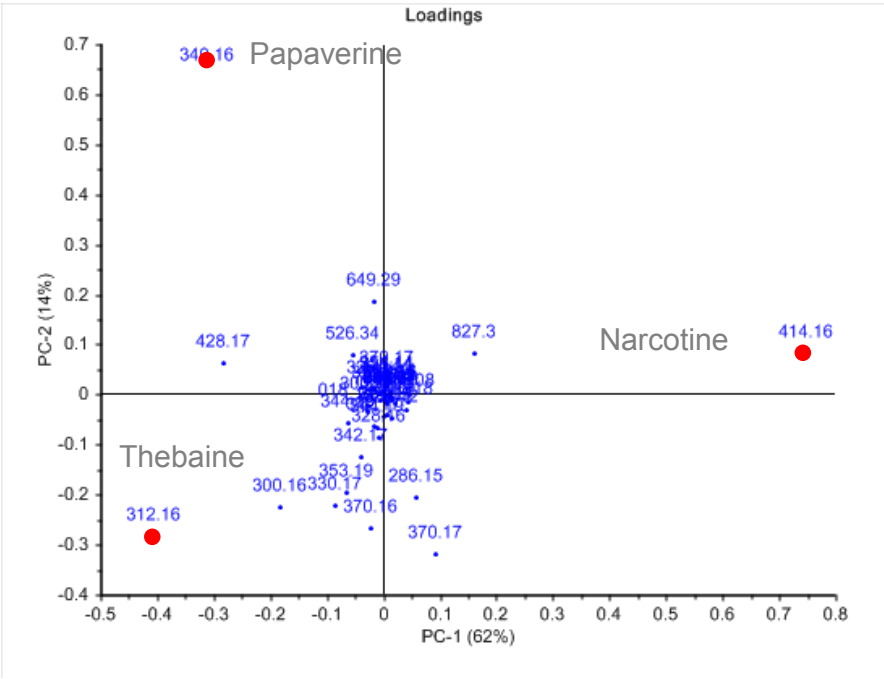


Figure 53: A) Score plot of the PCA for the analysis of opium samples evaluated by the in-house software after data pretreatment. PC 1 and PC 2 explain 76% of the total variety of the sample population. B) Loadings plot for the PC 1 vs. PC 2. The most important qualifiers for the differentiation in the scoreplot are highlighted in red.

5.9.2. AUTOMATED DATA EXTRACTION USING THE MOLECULAR FEATURE EXTRACTOR

The results of the chemometric data evaluation of the analysis performed by the MFE of the commercial software package using the peak height are depicted in Figure 54A. Similar to the results of the in-house software, five groups can easily be identified and are well separated. The established principal components PC 1 and PC 2 represent 72% of the explained variance of the sample set (compare: 80% by using the in-house software). The corresponding loadings plot identifies the qualifiers papaverine and narcotine as the most important ones. Interestingly, thebaine is not an important qualifier in this model, although the resulting scoreplot is very similar to the one in Figure 51. However, the most noticeable characteristic in Figure 54A is the isolated appearance of the opium Sample 4 in the top left corner of the scoreplot (PC 1 very low, PC 2 very high), while its duplicated measurement is part of the largest cluster in the center of the scoreplot. Hence, the duplicated measurement represents

the most different sample from the population on the one hand and the average one with very similar characteristics to sample population on the other hand. An inspection of the individual variables of the dataset reveals that the whole differentiation of these two samples is mainly based on one single factor: The molecular feature extractor failed to assess the peak at m/z = 414.2 in one of the duplicates, the most important qualifier for PC 1 and PC 2 as can be seen in the loadings plot (Figure 54B). A visual inspection of the electropherograms (Figure 55) shows that the peak is present in both electropherograms and does not differ in peak shape, intensity or retention time between both electropherograms. It is not evident why the peak was not recognized, but this serves as a very good example why automated data extraction needs verification by the operator. To remove the influence from such misinterpretation, the data file was cleaned. In the current situation, cleaning meant that the value from the manual assignment was copied into the datafile from where the assignment failed to achieve a dataset which could be compared. This was only performed when a visual inspection of the electropherogram justified such an approach. On total, this had to be done 23 times in a total number of 960 variables. Most of the variables were of very low absolute values with a very low influence on the total model. However, the failed assessment of the peak corresponding to m/z = 414.2 and three failed assessments of the peak corresponding to m/z = 314.2 show that the results of automated data extraction always need cross-validation or inspection of the results by an experienced operator. An alternative approach could have been an exclusion of such variables form the model. However, this would clearly have impaired the explanatory power of the PCA.

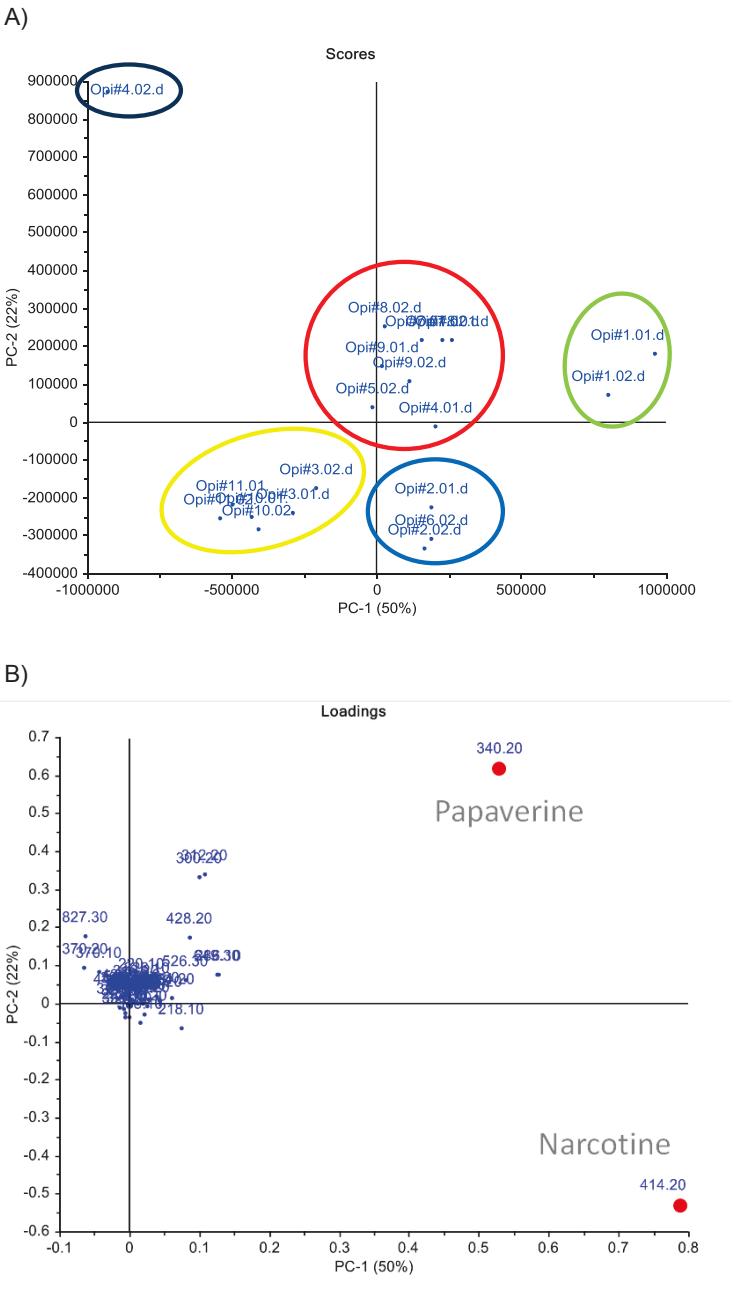


Figure 54: A) Score plot of the PCA of the analysis of opium samples evaluated by the commercial software MFE without data pretreatment. PC 1 and PC 2 explain 76% of the total variety of the sample population. B) Loadings plot to the corresponding scoreplot for the PC 1 vs. PC 2. The most important qualifiers for the differentiation in the scoreplot are highlighted in red.

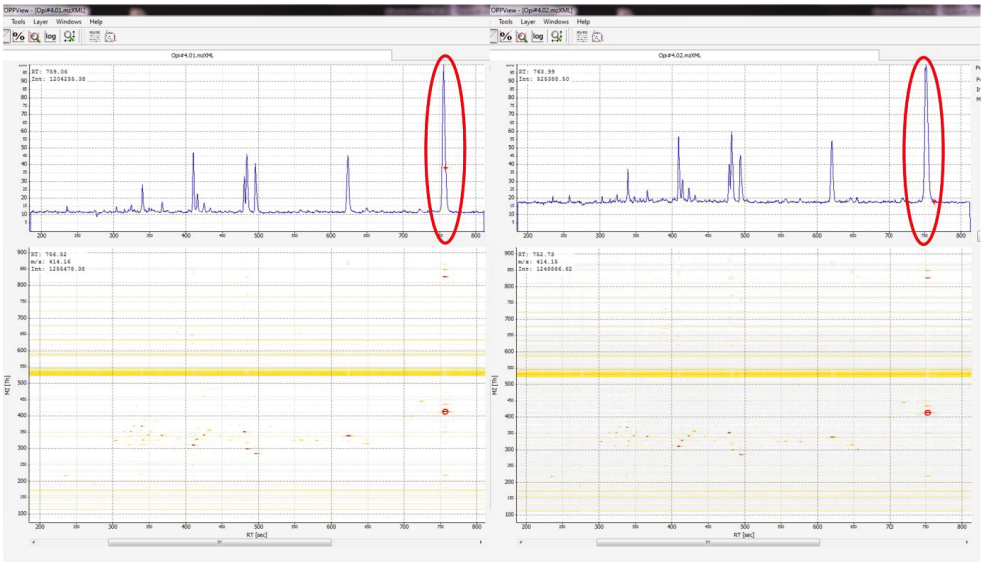


Figure 55: Electropherograms of the opium samples Number 4.01 and 4.02. The peak corresponding to m/z = 414.2 is highlighted with a red circle in both electropherograms.

The results of the principal component analysis after a correction of the dataset are shown in Figure 56A. As intended, the outlier (opium Sample 4.02) fits the general population and the differentiation was truly based on the data extraction error. The model resulting from the cleaned data set allows for a separation of the opium samples into four groups, which are well separated and corroborate the previous results from the in-house software. Also the affiliation of the samples to the groups is similar to results obtained by the in-house software. However, the corresponding loadings plot shows further qualifiers with pronounced importance for the discrimination of the samples, but the most important ones are still thebaine, narcotine and papaverine.

Consequently, the MFE has generally been proven to be applicable for a correct data extraction, fit of the analysis of electropherograms from NACE-MS. The results are comparable to the ones achieved with the in-house software.

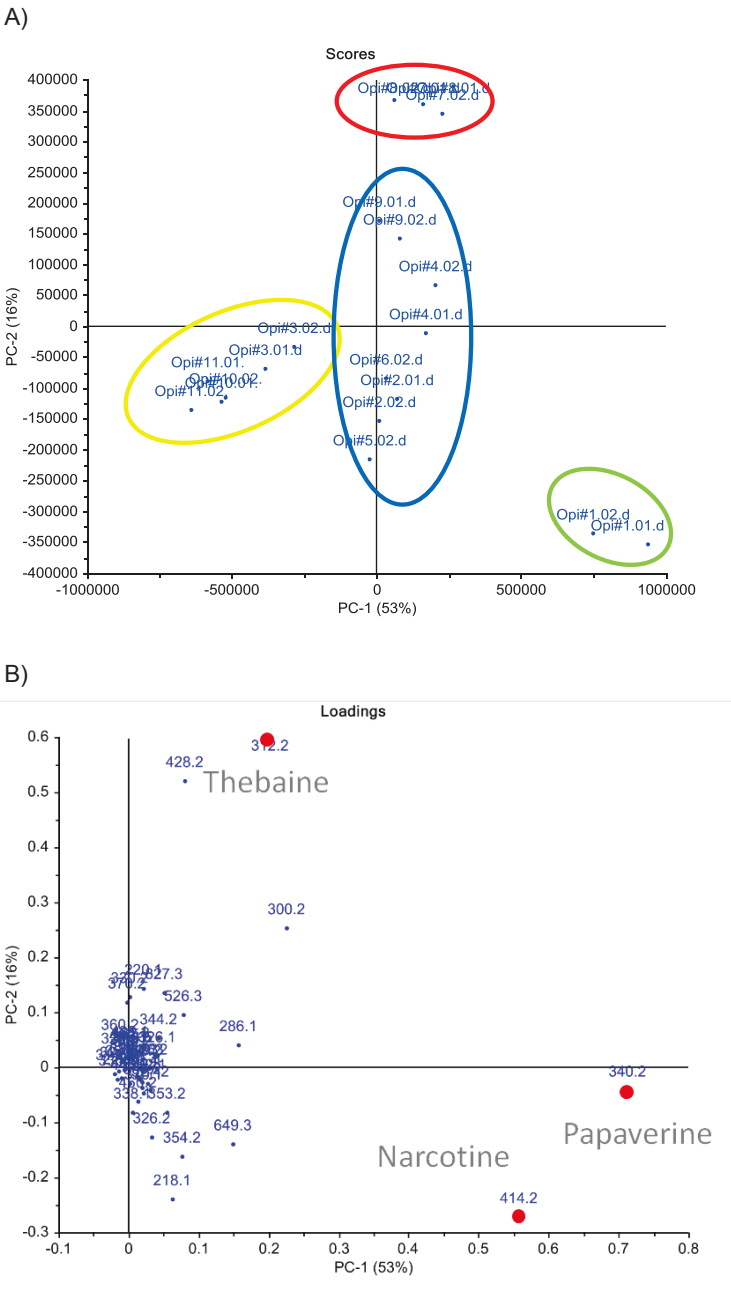


Figure 56: A) Score plot of the PCA of the analysis of opium samples evaluated by the molecular feature extractor after cleaning of the data set. B) Loadingsplot corresponding to the scoreplot.

5.10. CONCLUSION: OPIUM

A comparison of the individual extracted peak heights of the MFE with the ones from the in-house software show a very high degree of correlation between both data sets for some analytes. Figure 57 shows the correlation of the results for the peak heights of the cryptopine peaks in the whole opium data set. The numbers are identical up to the second decimal place. For other peaks, the values differ between the two data sets in the region of the very low values, but the correlation coefficient between the datasets is usually $R^2 > 0.95$. The basic operating principle of the software packages seems to be the same. The differences, especially in the lower absolute peak height ranges, might be a result of a stricter assessment of the migration time window, in which the peak is assessed in the case of the MFE.

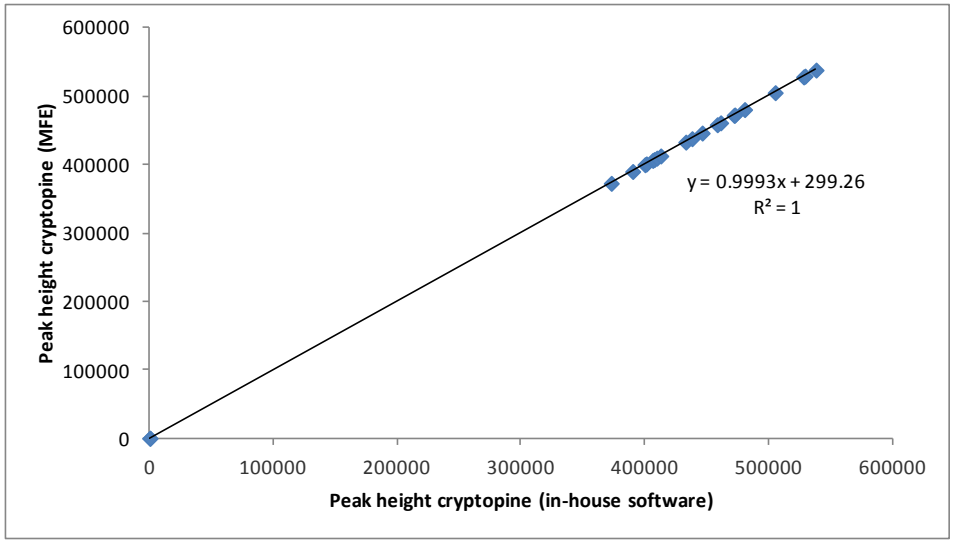


Figure 57: Correlation of the peak heights of both data extraction procedures for the peak of cryptopine for cross-validation. Note: The data point appearing close to the 0-value is an actual result from a sample measurement (Value x = 312, y = 0) with a very small cryptopine peak. A t-test showed no significant differences between both data sets.

Both extraction techniques showed a very similar performance, regarding the end result. Both were able to extract the peak heights correctly, consequently both techniques are suited for the profiling study. The results from the chemometric data evaluation are accordingly similar and the same qualifiers were identified and similar groups were assessed. In terms of handling, the MFE was superior and is therefore recommended for further investigations.

The easy and reliable analysis of opium samples for forensic profiling purposes is generally possible by using non aqueous capillary electrophoresis mass spectrometry. This was clearly demonstrated in this investigation. This is in accordance with literature results, were NACE was used for the analysis of opium samples and proven as a valuable tool [32,104,105]. However, the real application of such methods for profiling has not been investigated before, thus a proof was still missing. Based on the results achieved in this investigation, further analysis can be performed with less effort, since tools for data preparation and strategies for data evaluation have been analyzed and compared here.

5.11. RESULTS AND DISCUSSION: HEROIN

In order to establish a procedure fit for the analysis of all products of the supply chain heroin, we applied the NACE-MS method, used for the analysis of raw opium also, for the analysis of the product heroin. It was even possible to integrate cutting agents in the analysis. It is of high interest to have a method fit for the analysis of both, as it could further be used for a combined profiling of the substances, to draw conclusions about similar compositions, or, in an ideal case, to combine educt and product in one model.

During the analysis of the heroin samples we encountered several “types” of heroin which could easily be differentiated by visual inspection of the electropherograms. These included very clean samples, which contain nearly exclusively diacetylmorphine (see Figure 58), samples containing the typical residues from the processing steps such as addition of cutting agents and/or medication to reduce unwanted side effects, e.g. the tentatively identified procaine, a local anesthetic or paracetamol (see Figure 59, electropherogram on top). On total we can say that the method developed for opium and heroin analysis perfectly fulfilled its purpose and was proven to be well suited for the analysis of all types of heroin encountered, based on visual inspection of all electropherograms. As a drawback, caffeine and paracetamol migrate as neutral substances at the pH of the BGE and are consequently transported within the sample plug by the electroosmotic flow (see Figure 59). The numerical results for the compounds should therefore be evaluated

with care as these substances are not focused in the electrical field. Consequently, band broadening effects and quenching effects by comigrating substances are likely.

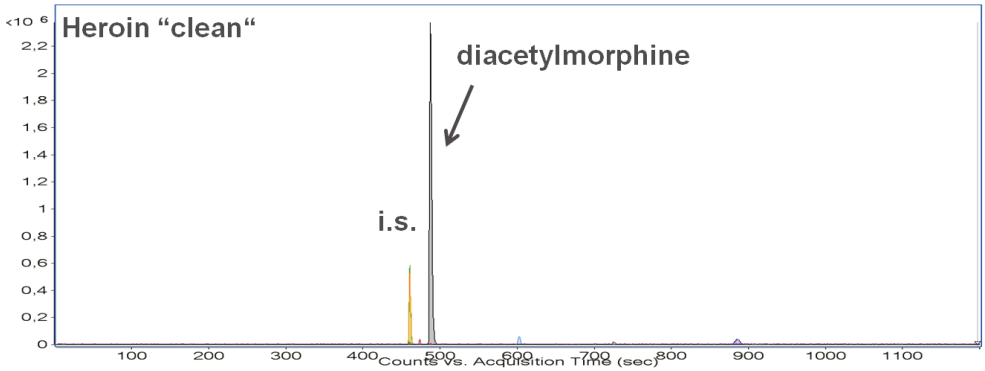


Figure 58: Electropherogram of the analysis of a very clean heroin sample. The method applied used 25 mM ammonium formate in a mixture of methanol and acetic acid (971:29) as background electrolyte in a bare fused silica capillary (60 cm long, 50 µm inner diameter). Measurements were performed at +30 kV separation voltage.

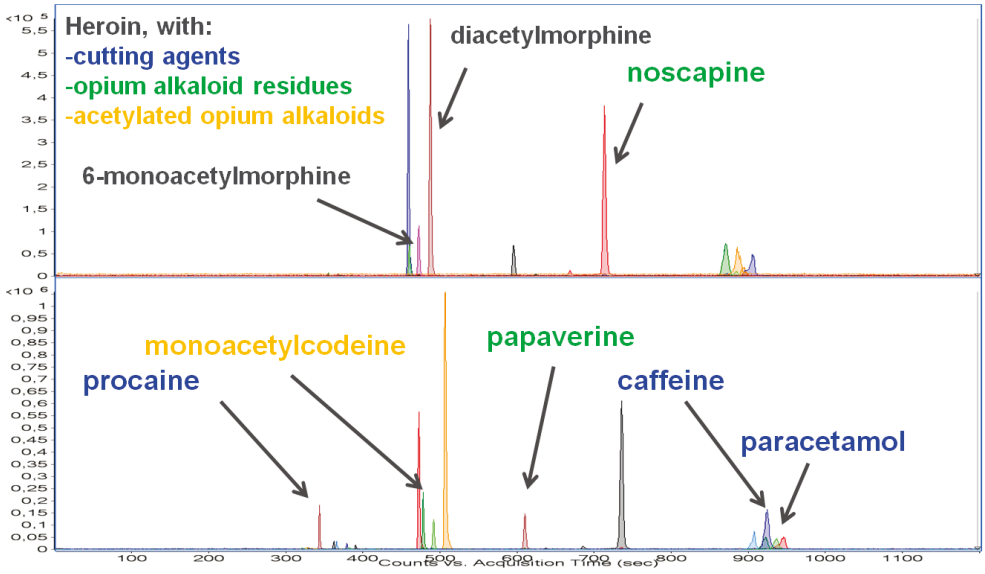


Figure 59: Different exemplary electropherograms form the measurements of the heroin samples containing different cutting agents.

5.11.1. CHEMOMETRIC ANALYSIS OF HEROIN AFTER MANUAL DATA EXTRACTION

Here the results from a manual data evaluation and subsequent chemometric data analysis as explained above (see Section 5.4.5) are presented for the analysis of the heroin. Sample preparation and method development are explained in detail above (see Section 5.2.4.5). The same data pretreatment steps (see Section 5.5.1) as for the analysis of Kratom were applied.

The chemometric evaluation of the data, manually extracted from the heroin measurements shows a clear clustering into four groups in the scoreplot of the principal component analysis (Figure 60). The depicted score plot explains 96% of the variance of the sample set-thus all characteristic differences should be included here. The corresponding loadings plot marks the analytes with the m/z-values of 414.15 (narcotine) and 370.16 (diacetylmorphine) as the most important qualifiers for the differentiation. However, it can also be seen that some duplicate measurements (e.g. heroin 1.1 and 1.2) don't exactly overlap, hence indicating a small influence from standard deviation on the analysis. To reduce such influence the data were normalized to the largest peak in each individual electropherogram before the principal component analysis was conducted. As can be seen in Figure 61, the normalization improved the repeatability and clearly increases the quality of the overall analysis as the differentiation of the groups is improved (total explained variance of PC 1 and PC 2 combined increased from 96% to 97%) and differences between duplicate measurements was reduced by the normalization. Its overall low influence demonstrated the good quality of the raw data.

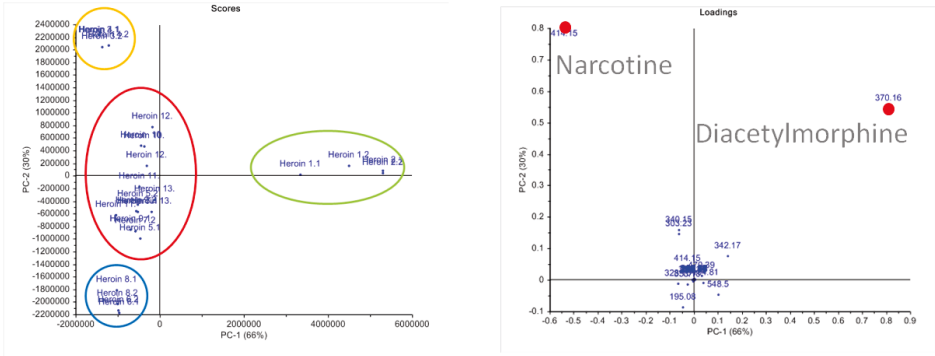


Figure 60: Results of the chemometric data evaluation of the manual data extraction from the heroin measurements.

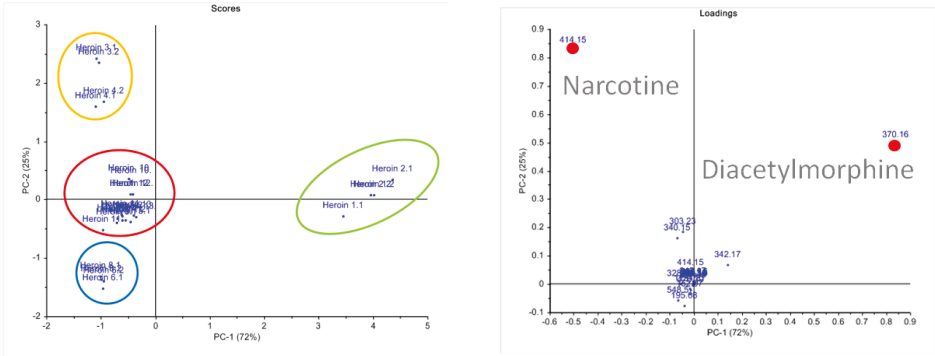


Figure 61: Results of the chemometric data evaluation of the manual data extraction from heroin measurements. The data was normalized to the largest peak in each individual electropherogram before the principal component analysis was conducted.

Further optimization of the data pretreatment or the search for additional differentiation potential in additional principal components was not successful. According to the results of this evaluation, our heroin samples can be differentiated into four different clusters, mainly based on the relative amount of diacetylmorphine and narcotine, which is in agreement with different, already established profiling programs [1,307]. In this case, no additional information could be obtained due to the application of the orthogonal electromigrative separation, but the results from classical analysis can now be cross-validated and verified by orthogonal CE-MS experiments.

5.11.2. CHEMOMETRIC ANALYSIS OF HEROIN AFTER DATA EXTRACTION BY THE MFE

The results of the analysis of the raw data of the heroin measurements evaluated with the aid of the MFE (without data pretreatment) can be found in Figure 62. PC 1 and PC 2 combined explain, 97% of the variance, which is very similar to the results obtained by manual data extraction. However, especially in the case of heroin Sample #4, it can be seen that duplicate measurements do not match very well. Inspection of the electropherograms shows that the peak picking routine has obviously trouble with the unconventional peak shape, as expected.

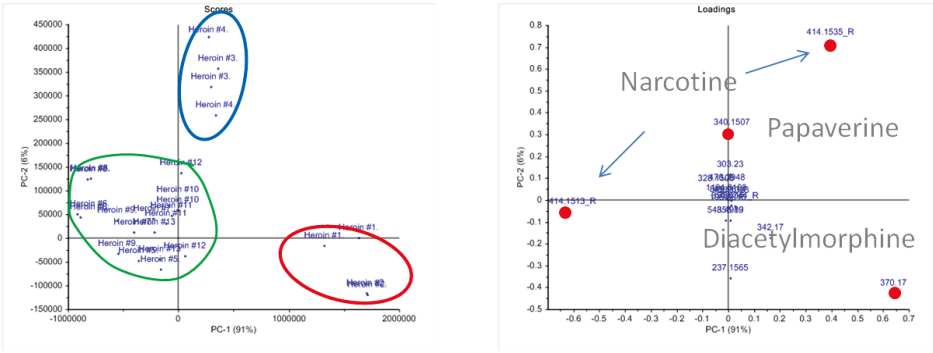
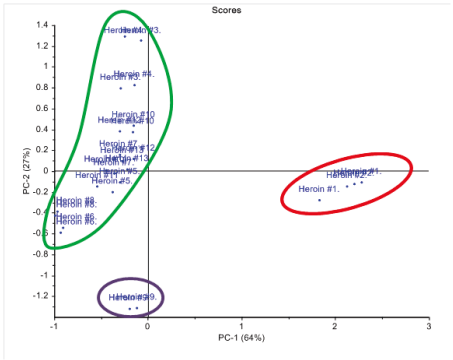


Figure 62: Results of the chemometric data evaluation after data extraction with the MFE using the raw data without pretreatment.

Correction of the peak height with the internal standard together with normalization on the largest peak in every individual electropherogram increased the quality of the principal component analysis as can be seen in Figure 63 A. A clear grouping of the samples in three distinct clusters can be observed. The inspection of Figure 63 B clearly shows that the differentiation is based on the distribution of the narcotine and the diacetylmorphine which is in agreement with the manually evaluated results. In some cases (6 out of 26), the MFE assessed two peaks for the mass 414.14, representing the m/z-value for narcotine. It remains unclear why this happened, but it happened reproducibly, especially in samples with a high content of narcotine. A possible explanation would be a decrease in mass precision in concentrated samples and thus the assignment of 2 peaks. Consequently, this factor was included into the model and surprisingly, although this analyte had a strong influence on the model (Figure 62), the outcome remains unchanged in comparison to the manual data extraction (Figure 61). The scoreplot of PC 1 against PC 3 (Figure 64) allows for a grouping of the heroin samples into four groups. Interestingly, the same qualifiers attribute to this differentiation but with alternated influence on the principal components.

A)



B)

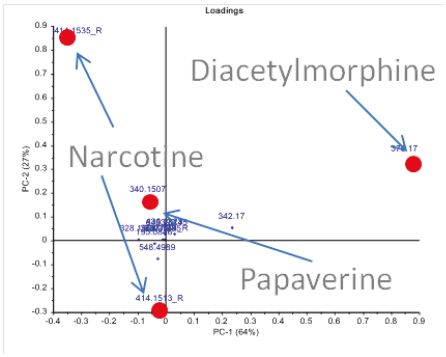
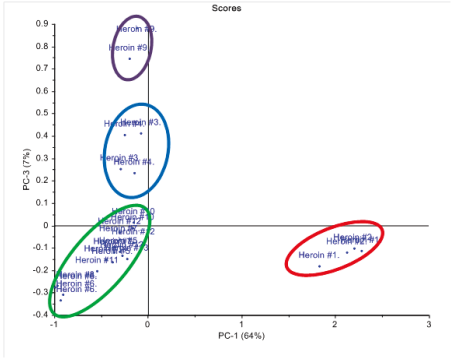


Figure 63: Results of the chemometric data evaluation after data extraction with the MFE after data pretreatment.

A)



B)

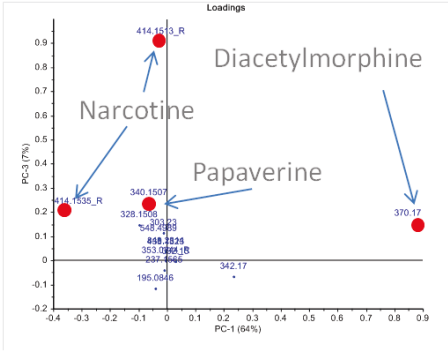


Figure 64: Score plot of the results of the chemometric data evaluation after data extraction with the MFE and data pretreatment/normalization. In these plots, PC 3 is plotted against PC 1, explaining 71% of the samples total variance.

5.11.3. COMPARISON OF THE MANUAL DATA EVALUATION VS. AUTOMATED DATA EXTRACTION

A crucial and very laborious step in the data evaluation is the data extraction, as described above. A short comparison of manual data extraction versus data extraction by the commercial molecular feature extractor is presented in this

subchapter. It will focus on the amount of gathered data, the reliability and quality of data extraction and the time consumed for data extraction.

Heroin was picked as dataset for the comparison, as the low density of peaks in the electropherogram and the overall very high quality of the measurements seemed to be ideal for an automated data extraction, as overlapping peaks and isobaric compounds are scarce.

5.11.3.1. AMOUNT OF DATA EXTRACTED

For the manual data extraction, all peak areas were assessed in each individual extracted ion electropherograms for each analyte. The MFE was programmed to extract all singly charged peaks of pseudo molecular ions, exhibiting a single charge in the electropherograms in the time region of interest (starting from 2 min to the respective end of each run). As peak filter, a threshold for the absolute height of 20 000 was chosen to prevent interferences from integrated noise, but to include all relevant analytes. A further reduction of the threshold (to 5 000) is possible and viable, however, the same algorithm was used for opium and an exclusion of several minor compounds was advisable there.

When establishing a sound database for profiling, it is always advisable to start from as many variables as possible, to subsequently excluded irrelevant signals and focus on the potentially important qualifier. We here compare the number of extracted peaks in each data extraction method, then the number of extracted peaks with a peak area/height above 20 000 arbitrary units and finally the number of peaks corrected for discrepancies. A Correction was necessary when a peak was assessed only in one measurement of the duplicated measurements. The whole variable was then excluded from further investigation. Especially the MFE was prone to such errors, however also in case of a manual data extraction one peak had to be excluded. We here regard the “supervised” manual data extraction to provide a reference for the true number of peaks to be assessed. Unfortunately, the MFE can only assess peak heights, which makes an absolute comparison difficult however trends and extreme differences should be visible.

As can be seen in Table 5, the manual data extraction clearly extracts quantitative data from more peaks than the MFE. However, a reduction of the peak number to the ones exhibiting a peak area/height above 20 000 in the manually obtained data set

shows a drastic reduction in peak numbers. This is to be expected, nevertheless the manual data extraction yields ~ 100 peaks more than the MFE extraction. A reason for this is the difference of peak height (used by the MFE) and peak area (used in manual data extraction), as a peak area is a result of an integration, representing a two-dimensional plane, while peak height is only one-dimensional. Consequently it is natural that more peaks for the manual data extraction are found. However, a closer inspection of the dataset reveals that most peaks exceed peak heights and areas of 100 000 arbitrary units rendering this argument partially invalid. Therefore it is reasonable to judge the manual data extraction to be superior in terms of the amount of data extracted. Also the corrected number of peaks supports this statement, as fewer differences between duplicate measurements were obtained.

Table 5: Comparison of the total amount of data extracted for the dataset heroin. The corrected number of peaks represents only the peaks which were found in both measurements of the duplicated measurements.

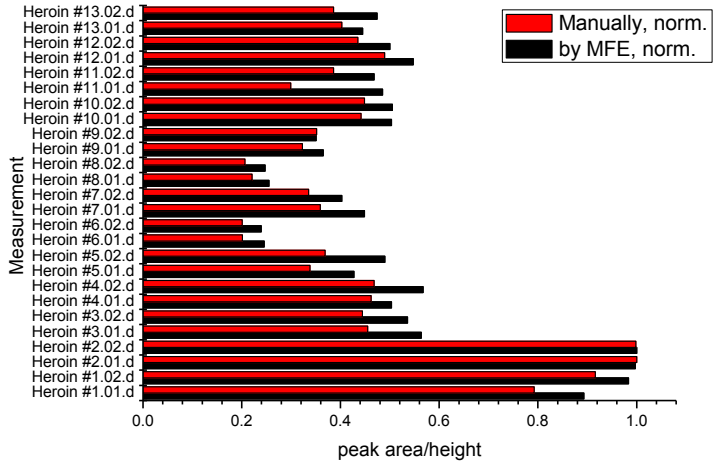
	Manually extracted	Extracted by MFE
Total number of peaks extracted	619	250
Peaks with peak area/height > 20 000	356	250
Corrected number of peaks	342	226

5.11.3.2. QUALITY OF THE EXTRACTED DATA

When assessing peak area and peak height, several error sources are possible as well as a subjective interpretation of data and measurements when performed manually. Consequently, automated data extraction is generally preferred. However, the current algorithms used for peak identification and assessment are also not error free, especially when applied to capillary electrophoresis. They often miss individual peaks as Gaussian peak shape is assumed for the peak identification algorithm. As for peak numbers, we here use the manual data extraction by integration and manual peal selection as a reference to assess the quality of the automated data extraction. Hence, the quality of the extracted data is assessed as the degree of correlation of the quantitative data from the manually extraction procedure vs. the MFE extraction

procedure. The comparison of the peak assessment (normalized peak area or height) is shown in Figure 65 for two representive compounds.

A)



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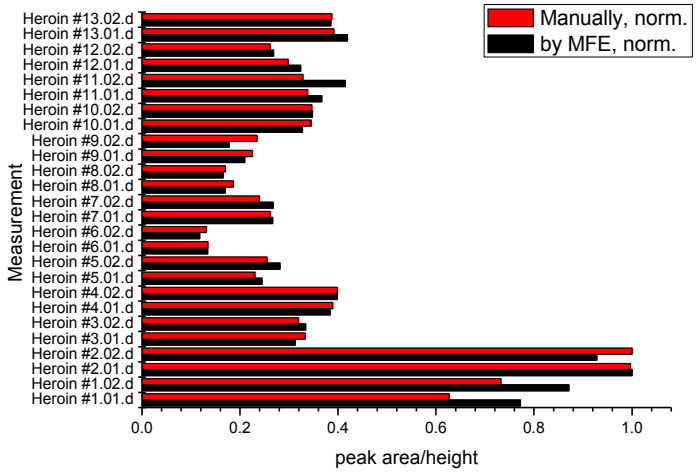


Figure 65: Comparison of the quality of the extracted data for the compounds (A) diacetylmorphine, and (B) unknown compound with m/z = 342.17. The depicted figures show the degree of agreement between the manually extracted peak area (red bars) and the peak height (black bars) extracted by the MFE. For a better comparison, the data was normalized to 1 to the largest peak in each sample set.

As visible from Figure 65 A and B, a similar distribution pattern in the samples is visible for both extraction methods. A paired t-test shows no significant difference between both data sets ($p < 0.05$). The normalized data clearly shows the high correlation between both extraction methods, showing that the values obtained by the MFE are very close to the true results for all samples, especially for compound m/z = 342.17. There are deviations for the Samples #3, #4 and #5 in the diacetylmorphine content, however as this is always the largest peak, sometimes close to reaching the detector saturation, influences especially on the peak height is likely. The overall quality of the MFE data extraction can consequently be regarded as surprisingly excellent.

5.11.3.3. TIME CONSUMPTION FOR DATA EXTRACTION:

Besides the quality and quantity of the extracted data, it is also important to evaluate the time of work spent for the data extraction. This is important with regard to larger sample cohorts for profiling and for industry, as the salary of the operator is one of the largest expense factors in routine analysis. The single working steps and the amount of time spent for each of them are listed in Table 6. The numbers mentioned are valid only for the heroin dataset under investigation (26 measurements). The whole process is described, starting from the raw data set to the final excel sheet that can be used for further data treatment.

Table 6: Comparison of the time spent for each data extraction procedure.

Manual data extraction		Data extraction by MFE	
Working step	Time consumption	Working step	Time consumption
Extraction of the individual EICs for all m/z-traces of interest	~2 h	Setup of the MFE with the critical parameters	0.3 h
Integration of the individual peaks	~3 h	Data extraction and export into an Excel file	0.1 h
Manual data transfer to excel sheets	~ 6 h	Cleanup and reorganization	1.5 h
Cleanup of the Excel sheet and reorganization	~ 3h		
Total time consumed	~ 14 h		~ 2 h

5.11.4. SUITABILITY OF THE DATA EXTRACTION METHODS FOR THE INVESTIGATION OF HEROIN

Two important questions arise: 1) does the difference in quality and quantity justify the very high time consumption for manual evaluation? 2) Is there a significant difference in the outcome of the whole investigation? As can be seen from the direct comparison of the two score plots from the manual and the MFE data extraction (Figure 66), the outcome is nearly identical for both procedures. Small differences in the total explained variance combined in PC 1 and PC 2 can be observed, but no additional information can be gathered when principal components are considered. The precision of the manual data extraction seems to be superior, also the less precise automated data extraction allows for the same conclusions in the end. This indicates increased robustness when manual data extraction is applied, however, also the working quality of the automated data extraction can be further optimized especially with regard to the duplicated assessment of the narcotine peak and missed peaks.

A) B)

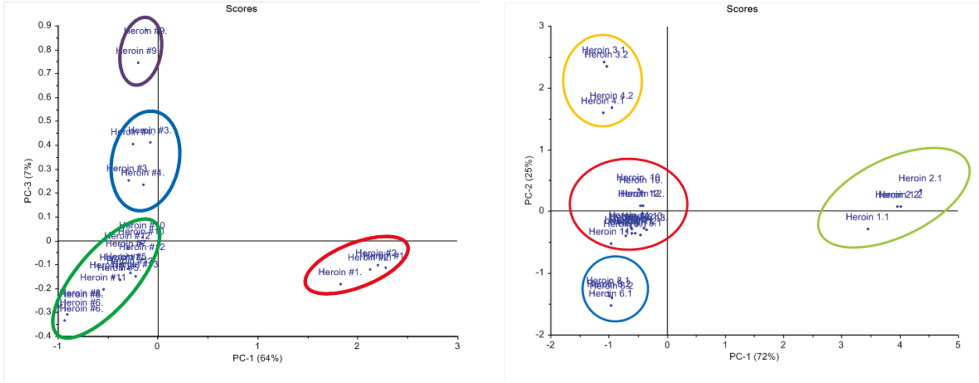


Figure 66: Comparison of the final scoreplots from the A) data extraction by MFE B) manual data extraction

Consequently, the higher workload accompanied with the manual data extraction cannot be justified based on our results. This was not expected when this comprehensive investigation was started, as all previous commercial data evaluation software failed to assess the peaks in electropherograms. With the latest

developments and an overall intensified optimization of software for the demands of high throughput analysis as needed for all fields with higher throughput (including the omics-field with proteomics, metabolomics, etc), commercial software packages greatly improved. The new software release was within the duration of this investigation, hence, the problems encountered with earlier versions of the commercial software were solved and do not negatively influence data extraction for samples like heroin anymore. This statement is true, as long as no isobaric substances are observed in the analysis, which are not fully baseline separated.

During the chemometric evaluation it became evident that principal component analysis might not be the best tool for the profiling of heroin or better to say not as the only tool. Especially in this study, where the threshold values were moderately high, artificial differences are created within the PCA when a signal of e.g. 24 000 in peak area/height is included, but a signal of 18 000 in peak area/height is excluded. This originates from the problem that PCA cannot deal with the absences of compounds corresponding to values of 0. This is problematic in analytical chemistry, as cutoff values have to be used to reduce the amount of evaluated data and to eliminate insignificant signals. The overall influence from such factors is small and does not lead to a differentiation. However, the presence of a local anesthetic or an adulterant (e.g. in case of the sample with the procaine) represents a unique characteristic in heroin for a distinct vendor or charge lot. In the PCA, the total attribution of this signal to the variance is overall low, showing that the PCA is robust against extreme contortions. This is of advantage, when the compound is an impurity without relevance for profiling. In this example however, the importance of the cutting agent as a marker is not reflected in the analysis. Consequently, it could be useful to create models with subgroups, differentiated according to the indisputable identification of adulterants or implement weighting for known cutting agents.

5.12. CONCLUSION CHAPTER 5

This chapter has impressively shown the versatility of NACE-MS and the potential of this separation technique as orthogonal complement to classical profiling techniques in forensic science. It was possible to derive robust methods for the profiling of various drugs, based on the understanding of the separation principle and the determining factors from the preceding chapter. Although the implementation of automated data extraction tools was problematic, updated standard routine from LC-

MS software packages are now fit for the analysis of CE-MS, despite some still existing errors associated with peak assessment, which can be circumvented by a careful supervision of the workflow by an experienced operator. The data pretreatment approaches and basic investigations on the differentiation of drugs can serve as a basis for an advanced profiling model, based on a larger set of samples, as the amount of samples used in this study is not sufficient for a comprehensive profiling, but clearly showed the potential and possibilities.

A potential profiling strategy for the biogenic drug Kratom has been presented here, as the first comprehensive approach, to the best of our knowledge. The applied techniques can be transferred and implemented for the profiling of the supply chain heroin and be used for verification and cross validation with classical techniques. Furthermore, there is potential to gain additional information about this drug, as several encountered additives could be easily included into the same method.

6. CHAPTER VI: THE COUPLING OF ISOTACHOPHORESIS-MASS SPECTROMETRY

Based on: T.N. Posch, P.A. Kler, D.D. Chen, C. Huhn, in preparation

6.1. CHAPTER SUMMARY

During the last years, isotachophoresis-capacitively coupled contactless conductivity detection-mass spectrometry was investigated as a promising tool for the analysis of small ions from plant extracts or preparations made thereof. The underlying idea was to use conductivity detection or MS as universal quantitative detection tool for the major plant metabolites via the isotachophoretic step length and use mass spectrometry for the unambiguous identification of all compounds accessible via electrospray ionization. Promising results for the comprehensive analysis of cationic compounds were found and linear calibration curves using the peak areas were established successfully.

However, in the case of anionic isotachophoresis, unexpected problems arise from the influence of the ionic species originating from the electrospray source with the sheath liquid, not well addressed in literature: e.g. isotachophoretic runs stopped close to the capillary outlet or the conductivity detector showed additional steps after the analytes had passed. The different influencing factors, most importantly the influence of the composition of the sheath liquid, on the performance of the ITP assays are discussed and potential solutions are presented. Successful measurements of real samples demonstrate the possibilities of this hyphenated technique for supporting research in life sciences and biotechnology, on analyzing these plant metabolites.

6.2. INTRODUCTION

Small organic ions (< 200 Da) play an important role in many biosynthetic pathways in organisms and cells, e.g. in the citric acid cycle. Often they are precursors for the synthesis of larger biomolecules, such as peptides, polypeptides, and proteins. With the impressive progress made in bioanalytical techniques, especially for the deeper understanding of the metabolome and proteome, the need for detailed and sensitive analysis and quantification of biomolecules is constantly growing. For large biomolecules such as DNA, proteins, and peptides high-throughput hyphenated analytical techniques and automated data extraction are established and well characterized. In contrast, the analysis of small organic anions and cations is still challenging, especially if the target analytes are not accessible via classical optical UV detection (the most common example is the lack of a chromophore in these small

molecules). Additionally, such low molecular weight analytes entail a challenge for most modern mass spectrometers: These devices are commonly designed to analyze a broad mass range and a consequently exhibit a poor performance in the lower m/z-region.

Small organic acids are particularly difficult to ionize in the ESI interface. Their polarity is so high, that they are hardly present at the surface of the aerosol droplets so that they are not well involved in the Coulomb explosion or fission events, so that it is hard to obtain free gas phase ions [329]. Only high detection limits evolve, irregardless of the separation method. Therefore, they are often analyzed via GC-MS which, on one hand, gives precise results; but on the other hand, it needs derivatization, thus introducing potential sources for errors and making the analysis laborious and time consuming [330-332]. Ion exchange chromatography has also been proven to be a suitable method for the analysis of such analytes, however an identification of unknown analytes would be desirable.

Several attempts using CE-MS have been described, mainly by Soga et al. [333-335] for metabolome analysis. Hagberg [246] achieved quite good results (e.g. LOD = 0.05-0.1 mg/L for malonic acid) using CE-MS for low molecular weight organic acids with a quadrupole instrument in the selected ion monitoring (SIM) mode, but reported large deviations in signal intensity associated with the required daily positioning of the capillary and a clean cut at the capillary end. Their analysis also suffered from tailing effects for the slowly migrating acids. Negative effects from of acetic acid or trimellitic acid on the ionization properties in the sheat liquid were addressed. Soga et al [336] observed unusually high limits of detection for anionic analytes using CE-MS for quantitative metabolome research. This was overcome by an increased injection volume (from 3 nL to 30 nL) and a pressure assisted CE-MS analysis to enhance quality of the peak shapes for multiply charged anions [336]. Later on, Soga et al. [333] discovered and proved that the electrospray system is severely corroded and oxidized when negative electrospray ionization is used, resulting in the formation of iron and nickel complexes with the analytes, thus decreasing ionization efficiency. An exchange of the stainless steel needle with a needle made of platinum solved some of these problems, increasing sensitivity up to 63-fold [333].

Overall, a fully satisfying approach for the analysis of small organic acids is still missing.

A so far unaddressed complex problem is the choice of the sheath-liquid. For cationic analysis via CE-MS, usually a mixture of 2-propanol and water (50:50) is used and 1% acetic acid or formic acid is added to the mixture to aid ionization and proton transfer. However, for anionic electrospray ionization, a wide variety of aqueous mixtures (2-propanol-water, methanol-water, acetonitrile-water) or non aqueous sheath-liquids (pure methanol, acetonitrile, 2-propanol) is found in the literature, using either basic substances (ammonia, triethylamine) or acids (formic acid, acetic acid) [97,337,338]. Consequently, there is a still ongoing dispute how the sheath-liquid composition can aid anionic ionization.

To circumvent the aforementioned problems in the analysis of small organic acids, we here investigate the possibility to couple the electromigrative separation technique isotachophoresis with the universal quantification capability of contactless capacitively coupled conductivity detection, and the identification potential of mass spectrometry. On the separation side, ITP as separation technique has already shown to be ideally suitable for the analysis of small organic acids [339,340]. On the detection side, C^4D will allow us to overcome the limited applicability of the UV detection by using this technique for quantification, also for substances inaccessible via ESI-MS. The coupling to the MS, will allow for identification and under certain conditions also for a quantification of all detected signals.

Basically, ITP-MS was already considered when the sheath liquid interface was introduced by Smith et al. [341], at that time, for small peptides, separated as cations in DB-17 coated capillaries without considerable EOF. However, they also showed that no considerable loss of separation quality appears at high EOF velocities in bare fused silica capillaries, given that full separation is achieved when the bands elute from the column. Since then, some attempts were presented, mostly for cationic species [341-343] using the sheath liquid interface. Due to the lack of suitable equipment, constant voltage was most often applied.

The distinct advantages of ITP-MS over CE-MS, leading to the first ITP-MS coupling, were clearly addressed in the first publications: isotachophoresis was capable to deal with large sample volumes and was therefore though to be ideal for diluted samples and trace analysis [342], as a result of the concentration adaption. Also the continuous analyte elution was mentioned at that time, so the detection system can be used to its full capacity for analyte detection and not longer time ranges void of

analytes [342]. ITP is especially useful for targeted analysis, as an appropriate choice of electrolytes can effectively cut out matrix load [341]. So far, all these advantages are characteristic for isotachophoresis, but referring to the special benefits of the coupling of ITP with MS, the especially the plateau-mode of ITP has to be stressed with its flat topped bands, which neither require fast MS detection systems, nor detectors with a broad dynamic range. Because of the concentration adaption, the quantification relies on the step length instead of the peak height [342] and is thus independent of the ionization efficiency of the analyte.

Another indisputable advantage of ITP-MS is that it doesn't need any new instrumentation or equipment when CE-MS equipment is already available. Generally, no special requirements or differences between CE-MS and ITP-MS were discussed in the few available publications, except for the requirements mentioned by Udseth et al. in 1989 [342], who highlighted the importance of a small capillary inner diameter and that the "[…] ionic strength of the analyte band must be at least 2 orders of magnitude below that of the buffer medium […]" to circumvent perturbation of the local electrical field strength [342]. However, these remarks were given mostly in order to prevent joule heating effects. Only Smith et al [341] highlighted that the application of ITP-MS can be more difficult compared to CE-MS, depending on the sample and the electrolyte system, but no further discussion was given

From this and more recent literature, it is evident that the interfacing of ITP with MS has not been fully thought through.

We here want to show that ITP-MS coupling has an enormous potential in the analysis of small organic ions, especially anions, based on its nature of decoupling the quantitative information from the signal height. Thus, it may solve many of the problems described above for CE-MS.

For this, we first want to discuss some basic features of ITP-MS: For identification purposes including MS^n experiments, ITP may be used as an alternative to direct infusion techniques as e.g. salt can effectively be eliminated and ideally, only analyte and counterion are present upon the detection: In an ITP assay the sample is introduced between two working electrolytes: the leading electrolyte with the highest effective electrophoretic mobility, and the terminating electrolyte with the lowest effective electrophoretic mobility. A counterion (CI) is added for buffering the

electrolyte system, keeping the working pH of the electrolyte solutions stable. In general, ITP is excellently suited for coupling with MS: the separation of the analytes in self-sharpening zones, allows for longer observation times in the detector, hence it is ideal for ITP-MS/MS or ITP-MS^n-experiments. ITP theory describes that under ideal conditions each ITP zone only consists of the analyte ion with the common counter ion, thus quenching effects should be minimal and an observation of the pure compound and the comparison of ionization efficiencies should be possible.

Quantification via ITP separation can be performed either in the plateau (or step) mode, or the peak mode: in the first case analytes achieve sufficiently high concentrations to form distinct and separate zones and their concentration is directly related with the length (detection time segment) of such plateau. In the second case, in the peak mode, one or more analytes focus at the sharp electric field gradient between the leader and terminating electrolyte zones, and have sufficiently low concentrations such that their effect on the electric field is negligible. Peak mode ITP is the typical focusing mode for trace analysis of biological molecules [344], however, when samples stem from real biological systems, salts and impurities normally tend to form additional plateaus, while the target analytes focus in the peak mode, resulting in a mixed ITP mode [345]. Peterson et al. [343] performed cationic ITP-MS experiments and mentioned that the peak mode, with its inherently narrow bands, no longer evolved when eluting into the sheath liquid, which makes detection difficult as analytes then co-elute. Spacers were claimed to solve this problem, which is, however difficult, if analytes of similar electrophoretic mobilities are analyzed. Instead, one may think of deuterated standards to first reach the plateau-mode with a longer step length and a better separation as well as a higher quantitative precision from isotope ratios. Nevertheless, Peterson et al. [343] proposed a real, comprehensive ITP-CE coupling, achieving impressive separations of human angiotensins.

To the best of our knowledge, only the group of Boček et al. achieved real anionic ITP-MS [346-348]. They proposed models to calculate the stacking capabilities of their ITP-systems and verified the validity experimentally. The achieved limits of detection are in the ng/L-region, shown to be suitable for trace analysis of pharmaceuticals in surface water using ITP in peak mode [346]. In contrast, anionic ITP-MS in the plateau or mixed mode have not yet been reported in the literature.

The model and experiments presented by Boček et al. [346-348] did not consider any particular difference between the standard ITP-C^4D or -UV assay and the ITP-MS coupling. In our study here, however, we will show that additional problems will arise from the most obvious difference between ITP and ITP-MS, especially at low EOF conditions: the absence of the outlet vial. In CE-MS, this has only a minor influence (except from moving boundaries migrating into the capillary from the sheath liquid [203]) , merely only the electrical conductivity for a closed electric circuit has to be guaranteed. The situation is entirely different for ITP-MS. Under standard experimental conditions for ITP, the outlet vial contains a large volume of leading electrolyte with its counterion compared to the capillary, which is filled with LE at the beginning of the isotachophoretic run, thus the determining factors for the velocity of the isotachophoretic steps as well as the relative concentration are kept constant throughout the run as LE and CI ions are provided from the outlet vial.

In this work, we first show the possibilities of cationic ITP both with a classical sheath liquid interface, but also with an interface with a micro flow-through vial in a junction-at-the-tip design [11,95]. We proceed with anionic ITP focusing on the different strategies to overcome the present difficulties in ITP-MS coupling for the analysis of small anions - Finally with real samples we demonstrate the potential of ITP-C^4D-MS for applications in biotechnology and life sciences.

6.3. MATERIALS & METHODS

6.3.1. INSTRUMENTATION

An Agilent HP-3D CE (Waldbronn, Germany) operated with Agilent 3D-CE ChemStation (Rev. B.01.03-SR2) was used, coupled to an Agilent 6340 Ion Trap (Bruker Daltonik GmbH, Bremen, Germany) operated with Bruker TrapControl Version 6.2 for the mass spectrometric detection via the Agilent CE-MS-adapter-kit. Sheath-liquid was added by an Agilent isocratic pump 1260 (Agilent Technologies, Waldbronn, Germany). Alternatively, a nebulizer-free sprayer interface designed and fabricated at the University of British Columbia (Vancouver, Canada) exhibiting a micro flow-through vial in a junction-at-the-tip design sprayer was applied [11,95]. When the NFS interface was used, the mass spectrometer was operated with the spray chamber opened and the nebulizer was set to the lowest possible volumetric flow. A capillary C^4D detector head (TraceDec®, Innovative Sensor Technologies

GmbH, Strasshof, Austria) was used for the contactless conductivity detection, operated by the TraceDec Monitor software (Version 0.07a). A schematic diagram of the instrumental setup used for the different ITP-C^4D-MS experiments is shown in Figure 67.

Problems arose from the interface, as the sheath-liquid flow, as well as the nebulizer pressure induced a pressure drop in the capillary outlet due to the Venturi effect generating an undesired fluid flow towards the ESI interface. In order to reduce this effect, the nebulizer flow rate was set to the minimum value of 4 psi with which stable electrospray conditions were reached.

The ion trap was calibrated daily before the first measurements and set to the parameters shown in Table 7. All measurements were conducted in the ultrascan mode, using a full scan in the range annotated in Table 7.

Table 7: Operating parameter for the Ion Trap

	Cationic ITP mode (sheath liquid interface)	**Cationic ITP mode (NFS interface)**	**Anionic ITP mode (sheath liquid interface)**
Polarity	Positive	Positive	Negative
Capillary voltage	-3500 V	0 V	+3500 V
Skimmer	+40 V	+40 V	-40 V
Cap Exit	+121 V	+121 V	-121 V
Nebulizer	5 psi	(2 psi)	5 psi
Dry Gas	5 L/min	5 L/min	5 L/min
Dry Temp	325°C	250°C	325°C
ICC*	500 000	500 000	50 000
Scan Range	m/z = 50-500	m/z = 50-500	m/z = 50-500 (1000)
Max. Accu Time	200 ms	200 ms	200 ms

**Ion Charge Control, limits the amount of charges inside the trap chamber to reduce space-charge effects, potentially artificially reducing the mass spectrometric resolution*

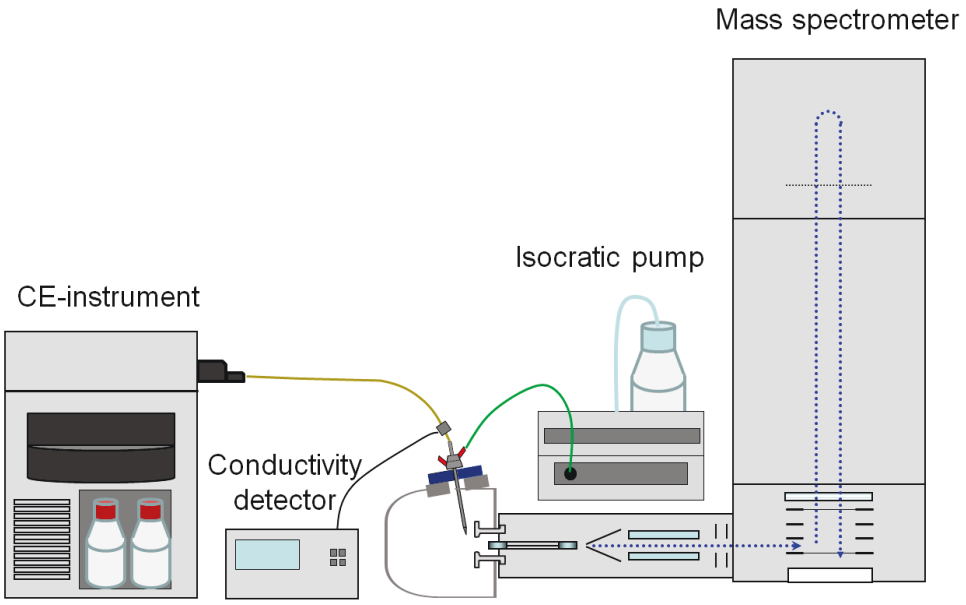


Figure 67: Instrumental setup for the ITP-C^{4}D-MS setup.

6.3.2. CAPILLARIES

Capillaries (inner diameter: 100 µm, outer diameter 365 µm, length: 60 cm) were purchased from Polymicro Technologies (Phoenix, AZ, USA) and coated with EOTrolLN (Target Discovery, Paolo Alto, CA, USA) to reduce the electroosmotic flow close to nearly zero: The initial capillary conditioning and coating were performed as follows: rinsing steps were performed for 10 min each with methanol, water, 1 M HCl, 1 M NaOH, water, EOTrolLN solution, water. Approximately 8 mm of the outer polyimide coating of the capillary was manually removed when the Agilent sheath liquid assisted interface was used. If not in use, the capillaries were flushed and stored in pure water.

6.3.3. CHEMICALS

Water was purified with a Millipore Milli-Q (Bedford, MA, USA) ultrafiltration unit (resistance > 18 MΩ). 2-propanol (99.9%), citric acid (pro analysis), hexanoic acid (99%), histidine, aniline, lysine, arginine, imidazole and nicotine were purchased from Sigma Aldrich. Acetic acid (99%), ethanol (LC-MS grade) and methanol (LC-MS grade) were purchased from Merck, acetonitrile was bought from Honeywell (Morristown, NJ, USA). Shikimic acid (98%) was obtained from Alfa Aesar, succinic

acid (pro analysis), salicylic acid (99%), glycolic (98%) and glyoxylic acid (98%) were purchased from Merck Millipore.

6.3.4. BUFFER SYSTEMS

For the cationic ITP-MS system, a 10 mM ammonium acetate solution, titrated to a pH-value of 4.7 with 1% acetic acid solution was used as LE. 0.1% acetic acid was used as TE. A mixture of 49.5:49.5:1 (v:v:v) of water: 2-propanol: glacial acetic acid was used as sheath-liquid at a flow rate of 4 µL/min.

After optimization of the anionic ITP-MS system, a 10 mM aqueous hydrochloric acid solution served as LE, titrated with a 1 M aqueous ß-alanine solution to a pH of 3.1. 100 mM hexanoic (caproic) acid with 300 mM ß-alanine in water was used as TE. The composition of the sheath liquid was a 2:1:1 (v:v:v) mixture of water, 2-propanol and acetonitrile. All solutions were stored in PET Falcon tubes to prevent potential leaching of metal ions from glassware into the buffer systems. Fresh buffers were prepared daily. All deviations from these parameters are marked in the consecutive text and in the captions to the figures. The capillary was flushed with leading electrolyte between runs until a stable signal from the C^4D detector was obtained (approx. 1.2 min).

6.3.5. SAMPLES

6.3.5.1. CATIONIC ITP

A mixture of histidine, imidazole, lysine, nicotine and arginine was used for experiments regarding the plateau mode (10 mM each). The sample solution was diluted 1:100 for injection.

A mixture of histidine, nicotine, aniline and imidazole was used for experiments regarding the peak mode (0.25 mM each). The solution was diluted 1:1 000 for injection.

6.3.5.2. ANIONIC ITP

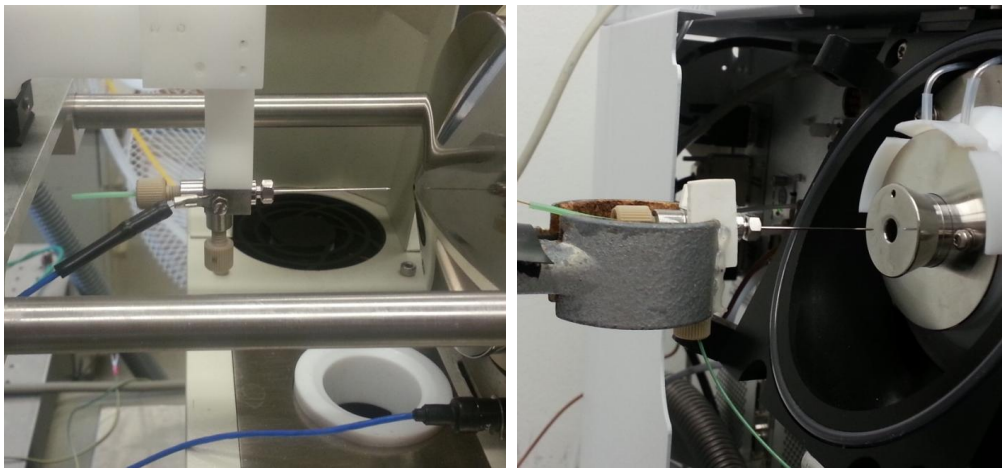
A mixture of oxalic, salicylic, citric, glycolic, succinic and shikimic acid (10 mM each) was used as test mixture for the system. The sample solution was diluted 1:100 for injection.

As an application example three different types of extracts (aqueous, ethanolic, methanolic, 2 mg/mL each) made from the plant *Arabidopsis thalina* were analyzed. The samples were prepared by the group of Ingar Janzik, a cooperation partner from the Forschungszentrum Jülich.

6.4. RESULTS: CATIONIC ITP

Although the analysis of cationic compounds with ITP-MS is rather straightforward, surprisingly few applications based on this hyphenated technique have been presented so far. Consequently, the knowledge on basic operating parameters and influencing parameters are scarce and not well attributed in the literature. To obtain first insights in the coupling of isotachophoresis with mass spectrometry, a cationic ITP-MS coupling was established to evaluate some characteristics about the two interfaces and whether they are applicable to ITP-MS. It was especially important to investigate if any of the interfaces produces turbulences at the tip of the capillary, which is expected to have a negative influence on the sharp boundaries for the samples eluting from the column that are characteristic for ITP. Some general results are presented here to show the advantages and disadvantages of the interfaces. Figure 68 shows the NFS-interface as used in the study in comparison with the classical Agilent interface. As the total flow rate for the NFS interface is around 100-1000 nL/min, the tip of the interface has to be in close proximity to the MS entrance. As the spray chamber of the mass spectrometer has to be open for this arrangement, the system is very prone to influence from the environment, like air flow or vibrations which sometimes hindered stable ESI conditions. However, a stable system could be reached if these disturbances were ruled out.

A) B)



C)

Figure 68: The NFS interface coupled to two different mass spectrometers in comparison to the Agilent interface. A) NFS interface coupled to an ABI Sciex 3000 Triple Quadrupole MS, as operated in the group of the inventors of the interface (Prof. D. Chen's group, University of British Columbia, Canada) and B) NFS interface coupled to an Agilent 6340 IonTrap as operated in this study. C) Agilent interface coupled to an Agilent 6340 IonTrap.

Figure 69 shows the analysis of four substances (nicotine, aniline, imidazole and histidine) detected in the peak mode of isotachophoresis using the NFS interface. It can be seen that three peaks are well described and no tailing is observed. Imidazole, however, only gave rise to a very small signal. It is interesting to note, that all analytes were of the same concentration in the sample, thus the differences in peak height are a direct result of differences in ionization efficiency. Consequently, peak mode ITP has to be regarded as a double-edged sword, as the capability to

focus all substances in a sample into extremely sharp bands optimal for the detection of trace compounds on the one hand, may lead to severe suppression effects from comigrating substances on the other hand as distortions of the sharp boundaries occur upon flow turbulences at the capillary outlet. The suitability of the peak mode for trace analysis is therefore strongly dependent on the composition of the samples under investigation.

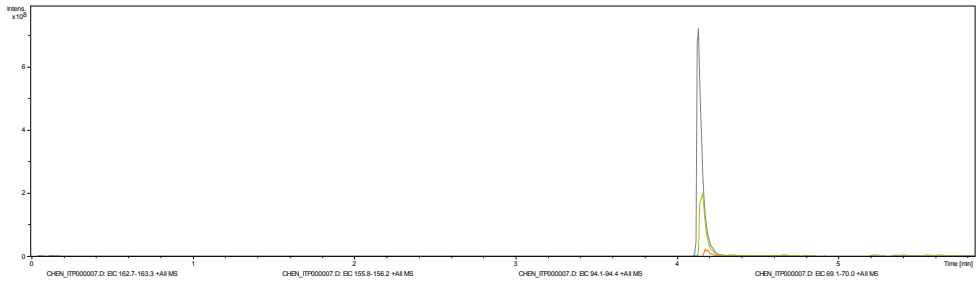


Figure 69: Peak mode isotachophoresis using the NFS interface. Note the very sharp and precisely described peaks of nicotine (grey), histidine (green) and aniline (orange). LE: 10 mM ammonium acetate, pH = 4.75, TE: acetic acid solution (0.1%), +30kV separation voltage, 0.25 µM sample concentration.

Figure 70 shows the separation of lysine (blue), arginine (green) and histidine (red) using the NFS interface in plateau mode. The very sharp boundaries at the beginning of each step show the potential of this interface for the ITP-MS coupling. Arginine shows some tailing and migrates also in the terminating electrolyte, which could be the result of adsorption to the bare fused silica capillary surface.

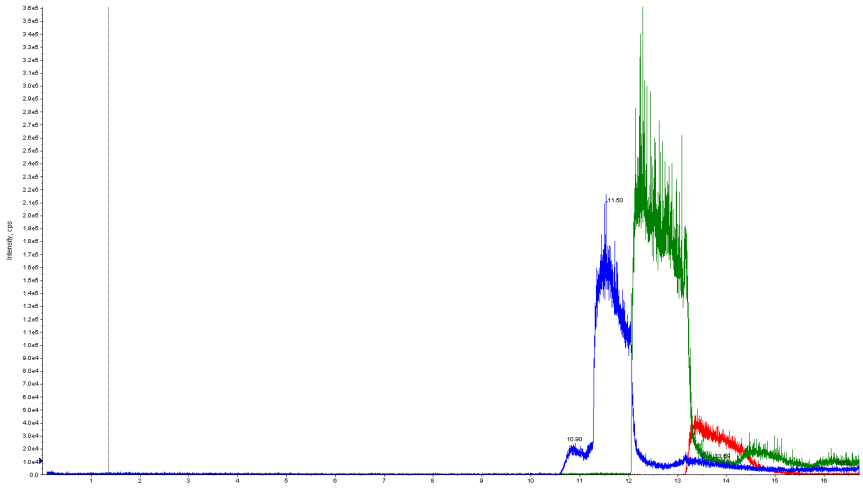


Figure 70: Plateau mode isotachophoresis using the NFS-interface. Note the very precise beginning of each step. The three steps are lysine (blue), arginine (green) and histidine (red) at a concentration of 0.05 mM, injected for 10 s at 5 psi. The downward drift within the steps is a result of the EOF, as a bare fused silica capillary was used in this experiment, instead of the usual, LN-coated capillary. LE: 10 mM ammonium acetate, pH = 4.75, TE: acetic acid solution (0.1%), +30kV separation voltage.

We can conclude that the NFS-interface shows great potential in the application of cationic isotachophoresis-mass spectrometry. However we did not achieve stable spray conditions for this interface in the negative ionization mode.

Further work was thus only conducted using the sheath liquid interface, which proved to be surprisingly robust against the expected flow turbulences at the sprayer tip as visible from Figure 71 showing the separation of five analytes in cationic ITP, using the plateau mode. The individual steps are well defined and separated with sharp boundaries. It is important to note that the peak, labeled with 1 represents an impurity and is not an analyte from the sample system. In fact, the signal from the analyte imidazole is present as the very small step, labeled with 2 in the isotachopherogram. The signal intensity from this analyte is very low, as it exhibits a small m/z-value and is therefore at the lower end of the acquisition range of the IonTrap. The other analyte steps are well separated and exhibit a similar step width, as expected.

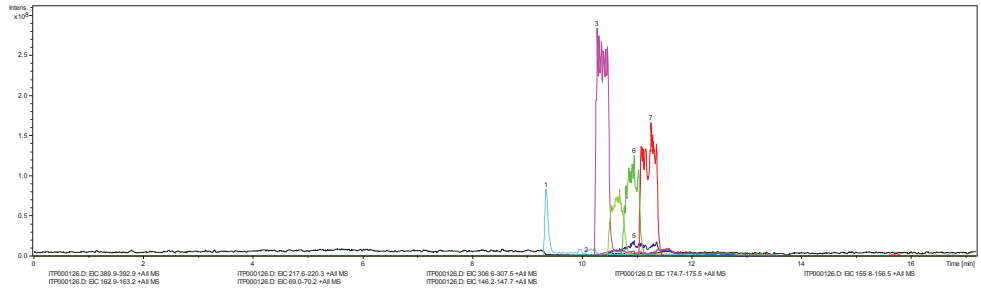


Figure 71: Plateau mode isotachophoresis of five model compounds using the Agilent interface. The steps are 1) an impurity 2) imidazole (blue, barely visible), 3) nicotine (purple), 4) lysine (dark green), 6) histidine (light green) and 7) arginine (red). Sample concentration 0.25 µM injected for 10 s at 50 mbar.

Figure 72 shows the isotachopherogram of two peptides (sequence = H-Trp-Glu-His-His-OH and H-Trp-Tyr-Lys-Arg-OH). Both peptides are well focused and can easily be analyzed, also using MS/MS experiments.

The results of these experiments show, that it is generally possible to analyse peptides and other analytes using cationic ITP-MS. No additional difficulties were observed relative to CE-MS, except for the necessary coating of the capillaries.

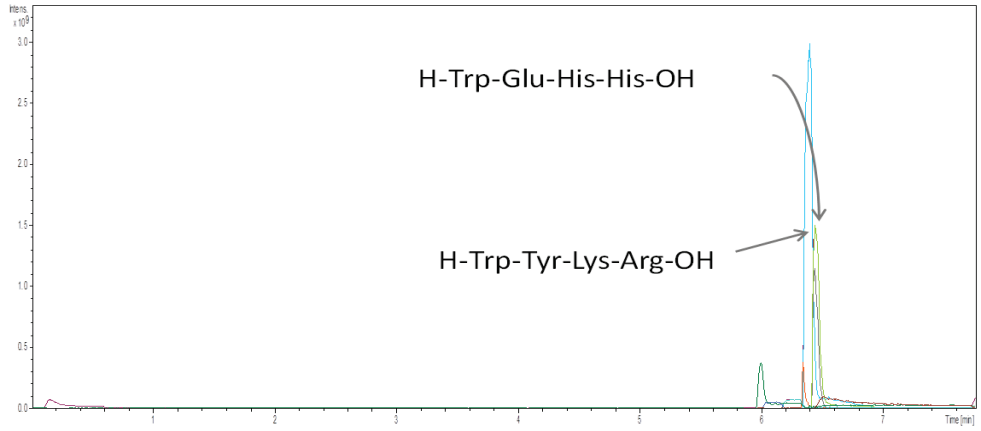


Figure 72: Plateau mode isotachophoresis of two peptides using the Agilent interface. Peptide 1 (light blue step, m/z = 326.7): tryptophan-tyrosine-lysine-arginine, peptide 2 (light green step, m/z = 304.6): tryptophan-glutamic acid-histidine-histidine, both detected as doubly charged analytes. The identity of the other steps is unknown. Sample concentration 1 µg/mL, injected for 10 s at 50 mbar.

6.5. RESULTS: ANIONIC ITP

The results presented in this subchapter stem from a cooperation of Dr. Pablo A. Kler and Tjorben Posch. All experiments, data handling and evaluation were performed by Tjorben Posch. Dr. Kler was involved in the theoretical considerations about the effect of the countermigrating H^+-ions and in the development of the strategies for step mobilization together with Tjorben Posch.

As a prerequisite for isotachophoresis, especially with regard to quantification via the step length, we consider the absence of a hydrodynamic flow through the capillary. Hence, electroosmotic or pressure driven flows (either with a positive pressure from the inlet or suction from the capillary outlet at the MS side) have to be suppressed. We here use a neutral coating to suppress the EOF in combination with a low pH-value of the buffer system (pH = 3.1). However, this absence of flow towards the MS is now combined with the lack of an outlet vial, thus during the time course of the ITP separation the leading electrolyte leaves the capillary and the LE properties will be converted to the properties of the sheath liquid. Especially, the continuous loss of counterions can be expected to be problematic if they are not further supplied by the sheath liquid composition (as would be the case for an outlet vial). If the LE counterion is not supplied by the sheath liquid or no proper buffering is provided, protons can be expected to quickly migration into the capillary and disturb the isotachophoretic conditions. However, a compromise has to be found between optimal separation and detection parameters, as many sheath liquid additives were shown to induce severe quenching effects in anion analysis.

The choice of a suitable buffer system for anionic ITP-MS is a crucial step, as it determines the width of the ITP window (mobility range) as well as the velocity of the whole experiment in combination with the separation selectivity and resolution. Also, as in conventional ITP, the concentration of leading electrolyte determines the concentration of the consecutive stacks according to the moving boundary equation [70]. Our analytes include some of very high electrophoretic mobility such as oxalic and glycolic acid. We tested some small and suspectively fast, multivalent acids such as oxalic acid or citric acid as leading ion, but only chloride proved to be fast enough and most effective at a concentration of 10 mmol/L. We here accepted non-ideal MS conditions as HCl is aggressive towards the mass spectrometer and the sprayer interface, however negative ionization is always connected to aggressive conditions

for the sprayer needle [333]. As terminating electrolyte we chose hexanoic acid due to its low effective electrophoretic mobility that allows the preconcentration of many different analytes of interest. Correct isotachophoretic conditions and the width of the mobility window were determined with ITP-C^4D (data not shown). It is important to note that the influence of LE and TE ions on the analyte ionization in the electrospray is rather low as ideally they are fully separated upon detection.

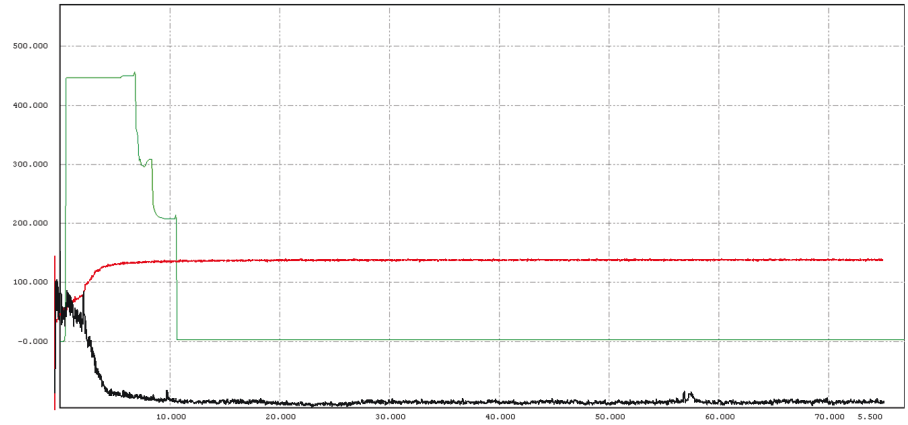
This is in contrast to the counterion, for which we have to ensure a high volatility and low interfere with analyte ionization. Depending on the pH regime ammonia may be a suitable counterion as has been used for anionic ITP-MS previously (e.g. [347]). However, ammonia buffers only in the basic region and is known to cause severe impact on anion signal intensity, especially when added to the sheath liquid, which is vital if no EOF or pressure flow is used. Otherwise depletion of CI during the run would occur and the CI would have to be replaced by protons.

Although ammonia has already been used in the sheath liquid in ITP-MS experiments by other groups, the addition of ammonia caused a dramatic drop in signal intensity, which was unacceptable for further experiments in our instrumental setup, irregardless of the used concentration. It is worth mentioning here that the results presented by Gebauer et al. [347], who successfully used 2 mM ammonium acetate in the sheath-liquid for a quadrupole MS in SIM mode, did not describe adverse effects on the detection of their analytes in contrast to our observations.

We tested several counterions as ammonia proved not to be suitable for our ITP-C^4D-MS analysis: Ion suppression is especially critical for the analysis of complex samples with unknown compounds and concentrations. We experienced that low pH values outside the buffering range of ammonia were more suitable for the fast and highly charged analytes of interest. A low pH-value had two distinct advantages: 1) as the buffer system was suspected to be non-ideal for the capillary coating, a low pH-value assured a very low EOF if the coating is not ideal anymore. 2) The low pH-value protected the BGE from carbonate, which would have been formed. This happened in previous experiments and is a well known problem in ITP. However, the usual countermeasures are not MS-compatible. Finally, in our optimized method we decided for ß-alanine for its low effective electrophoretic mobility [349], good buffering capabilities at low pH and no memory effects compared to other good buffers such as MES or MOPS.

During our optimization procedure, we encountered two very surprising effects: First, there were isotachophoretic runs with a clear evolution of isotachophoretic conditions visible in the C^4D trace (capillary length 60 cm, conductivity detection at ~50 cm). However, as visible in Figure 73A, the ITP step never arrived at the CE-MS interface, even not when using the fast chloride as leading ion. Only upon pumping, we observed the analytes and the terminating electrolyte by MS detection (mostly more or less immediately after applying pressure). It became clear that the movement of the ITP stack simply stopped short before reaching the MS. Second, in some cases, even a countermigrating cationic ITP was observed in the conductivity detector shortly after the anionic ITP stack passed the detector as visible in Figure 73.

A)



B)

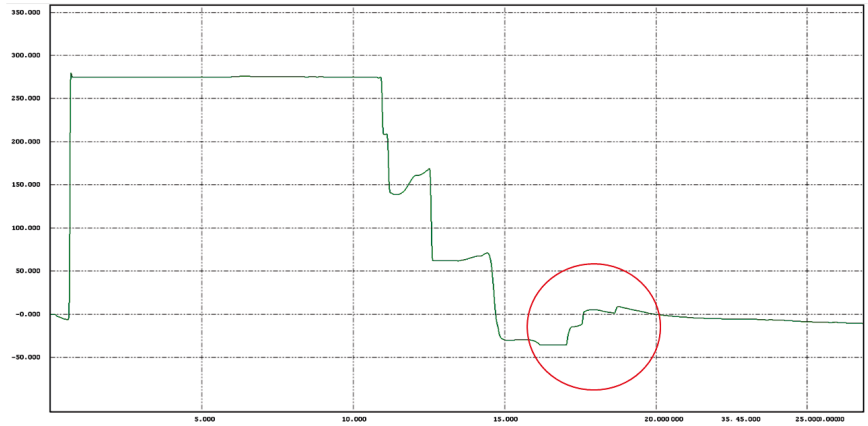


Figure 73: A) ITP-MS measurement of a standard mixture. LE = 10 mmol/L oxalic acid (pH 3.5), TE = 20 mmol/L MES. The black line represents the TIC, the red line represents the current, the green line represents the signal from the conductivity detection. B) Isotachopherogram of the analysis of a mixture of acids. LE = 10 mmol/L HCl + 300 mmol/L β-alanine (pH 3.1). TE = 100 mM hexanoic (caproic) acid. Note the countermigrating ITP stack which migrates into the system from the sprayer tip.

During the first experiments, it became evident that the coupling of ITP with MS suffers from effects which are known from CE-MS: the migration of hydronium ions from the sheath-liquid into the capillary. While the effects in CE are minor and can be circumvented, they make ITP-MS effectively impossible. The highly mobile hydronium ions migrate from the sheath-liquid interface into the capillary with a severe effect on the pH-value, leading to a protonation of the analytes and even of the leading electrolyte. Consequently, the velocity of the isotachophoretic stack decreases over time, leading to an immobilization of the analytes. It is clear that

these phenomena stem from the lack of an outlet vial and thus the depletion of CI during the ITP-MS run. With the lack of CI, H^+ takes over the role of the counterion, thus changing the pH regime in the ITP stack. An obvious and straightforward solution for this problem would be the use of the leading electrolyte as sheath-liquid, which has been tested within this study, but results in a very poor electrospray (results not shown).

In order to solve this problem, we first have to understand the underlying phenomena: For ITP-MS additional considerations have to be made, for a more realistic electrophoretic representation: For a description of ITP, we need to consider the fundamental relationship of equal speed of LE and analytes ($\mathbf{v}_{LE}=\mathbf{v}_{A1}$(with the migration velocities of LE ($\mathbf{v}_{LE}$) and the first analyte ($\mathbf{v}_{A1}$); $\mathbf{v}_{LE}= \mu_{LE}\ \mathbf{E}_{LE}$; $\mathbf{v}_{A1}= \mu_{A1}\ \mathbf{E}_{A1}$ with the electric field strength in the LE and A_1 zones $\mathbf{E}_{LE}$ and $\mathbf{E}_{A1}$). For the LE zone, we must consider the conductivities of LE(κ_{LE}), CI (κ_{Cl}), H^+ (κ_{H+}), and OH^- (κ_{OH-}), as follows (Equation 8):

$$\sigma_{LE} = K_{LE} + K_{Cl} + K_{H^+} + K_{OH^-}$$

In our study we work under acidic conditions (pH: 2-3.8), so we can neglect the contribution of the OH^- ions to the global conductivity. According to the considerations by Kler et al. [Kler, Huhn to be published] using the above mentioned fundamental equations and the condition $\mu_{LE}>\mu_{A1}$, we can write the following relationship for ITP, derived (Equation 9):

$$LE >> (K_{Cl} + K_{H^+}) > 0$$

This Condition 9 is satisfied when using CI's with low effective electrophoretic mobilities (e.g. near their buffering or isoelectric point) or at low concentration. But also a moderate contribution of H^+ has to be assured, which is normally achieved by working at a pH inside the well-known "safe ITP region" between pH 5 and 10 [350]. In ITP-MS the lack of an outlet electrolyte with CI may hinder the fulfillment of Equation 9: Ions migrate out of the capillary with LE, analyte and TE ions. In addition, ions from the sheath liquid migrate into the capillary (in anionic ITP any counterion present, depending on their relative effective electrophoretic mobilities) first of all in

the sheath liquid, but having entered the capillary, also in the electrolyte present at the end of the capillary. In anionic ITP the H^+ ions will migrate into the capillary at higher velocities than any other ion, especially for aqueous-organic sheath liquids with protic solvents and e.g. acetic acid added for enhanced ionization (e.g. 1% acid in 1:1 isopropanol:water). As a consequence, in the vicinity of the capillary outlet, the local concentration of H^+ will drastically increase, increasing κ_{H+}, possibly violating Condition 9. Together with some titration effects neutralizing analytes of interest, the ITP stack simply stops moving further through the capillary. In electrolyte combination as in Figure 73B, even a cationic counter-ITP may evolve with the original CI being the leading ion and H^+ being the termination ion. Such a bidirectional ITP has been developed previously [351,352], but is impractical for our purposes here.

How can this problem be solved? We can address either term of Condition 9, thus either decreasing κ_{Cl} or κ_{H+}. With β-alanine we already have a counterion of low electrophoretic mobility. An increase of its concentration in the sheath liquid impairs analyte detection, but does not hinder the protons to quickly enter the capillary. Thus, we have to reduce the electrophoretic mobility of the proton in the sheath liquid. First of all this would reduce the amount of H^+ migration into the capillary but it would clearly slow down and counter migration ITP. For H^+, there is a special transport mechanism in aqueous solution, the so called Eigen--Zundel--Eigen mechanism, which evokes the extreme apparent proton mobility. The stable Eigen cation ($[H_3O(3H_2O)]^+$) is converted to the transitional Zundel cation ($[H_2O\text{--}H\text{--}OH_2]^+$) with similar bond lengths for the hydrogen bond. This further reacts to the next Eigen cation [353] leading to a bond-flipping apparent proton transport with an extremely high electrophoretic mobility of $\mu_{H+(EZE)} = 306.10^{-9}\ m^2V^{-1}s^{-1}$ compared to the normal Stokesian migration carried out by solvated protons ($\mu_{H+(Sto)}=39.10^{-9}\ m^2V^{-1}s^{-1}$). The EZE mobility is of course related to the availability of water--water hydrogen bonds [354]. These can be reduced by the addition of dipolar aprotic solvents interfering with the hydrogen bonding of the otherwise aqueous solution (compare Kler, Huhn, in preparation).

For this reason different solvent compositions for the sheath liquid were tested in order to decrease the effect of the EZE conduction over the Stokesian motion of the proton. We successfully conducted ITP-C^4D-MS using a 2:1:1 (v:v:v) mixture of

water, 2-propanol and acetonitrile. The use of a purely non-aqueous sheath-liquid was tested (methanol and acetonitrile), resulting in a very poor electrospray, not suitable for reliable analysis.

We want to note that also the creation of a pressure drop between capillary inlet and outlet either by applying external pressure to the inlet vial (10 mbar) or by increasing the suction effect increasing the nebulizer pressure from 5 to 10 psi allows to conduct successful ITP-MS with a relatively low effect from the parabolic flow profile due to the self sharpening of the ITP bands. However, as described, for quantitative analysis this is disadvantageous. Another very important fact in terms of quantification is the transition phase between peak mode and plateau mode, which is not addressed in literature so far, but has a severe impact on the applicability of ITP-MS. This will be discussed in detail below (see Section 6.7)

6.5.1. STRATEGIES FOR STEP MOBILIZATION

As explained above, the coupling of ITP with MS suffers from the migration of hydronium ions from the sheath-liquid into the capillary. While the effects in CE are minor and can be circumvented, they make ITP-MS effectively impossible. The highly mobile hydronium ions migrate via the EZE mechanism from the sheath-liquid interface into the capillary with a severe effect on the pH-value due to the higher relative flow of the sheath liquid compare to the capillary, leading to a protonation of the analytes and even of the leading electrolyte, if an organic acid was used for it. Even when hydrochloric acid was used as leading electrolyte, the isotachophoretic stack immediately stopped when the leading electrolyte had exited the capillary tip. Different strategies were tested in order to reduce this effect. These strategies comprise: 1) Addition of counterions (beta-alanine) to the sheath-liquid which could preferably enter the capillary from the interface. In this case, the extremely higher mobility of protons via the EZE mechanism suppressed the migration of the beta-alanine and consequently, no major improvement could be observed.

2) External pressure was created either by an increase of the nebulizer pressure (from 5 psi to 10 psi) or by applying external hydrodynamic pressure to the inlet vial (10 mbar). In both cases successful anionic ITP-MS measurements were possible. The expected negative influence from the parabolic velocity profile were very low,

consequently this would also be an appropriate approach, however, very often the analysis time was not sufficient for the ITP to evolve into the real isotachophoretic stacks. As a consequence mixed zones were often detected, which is not ideal for the analysis of plant metabolites later on.

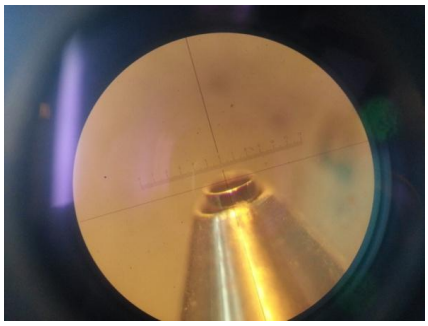
3) The addition of acetonitrile to the sheath-liquid (25%) reduced the fast EZE mechanism of the mobility of the hydronium ions, as well as the higher autoprotolysis constant of the organic solvent (overall less H+ present), due to its lower dielectric constant and its protophobic characteristics [355]. The result of this addition was that the reduction in both concentration and mobility of protons was enough to keep the migration velocity of the isotachophoretic stack sufficient for the MS analysis. Therefore the addition of acetonitrile to the sheath-liquid was chosen as an appropriate tool for anionic ITP-MS without having to rely on external pressure.

The final method uses a 10 mM HCl leading buffer with 300 mM ß-alanine as counterion at a pH-value of 3.1. The terminating electrolyte consists of 100 mM hexanoic acid. A mixture of 50% water, 25% isopropanol and 25% acetonitrile was used. The appropriate choice of a sheath liquid was unexpectedly difficult and needs further explanation as can be seen below.

6.5.2. INSTRUMENTAL CONSIDERATIONS

The mass spectrometric negative ionization mode is generally aggressive towards the used instrumentation, especially towards the ESI-source, as an electrochemical oxidation and corrosion of the stainless steel needle in the sprayer tip takes place and iron oxides are deposited within the separation capillary [333]. In combination with the aggressive buffer system used in this study, the effect was even pronounced and led to a strong corrosion of the sprayer needle (Figure 74B & C). The results for the spray stability however, are small, as the tip of the capillary protrudes out of the sprayer needle and is the determining factor for a stable electrospray, but stronger effects are to be expected if the needle is not exchanged. A straightforward approach to circumvent this problem is the implementation of platinum needles, which are resistant towards the corrosion.

A)



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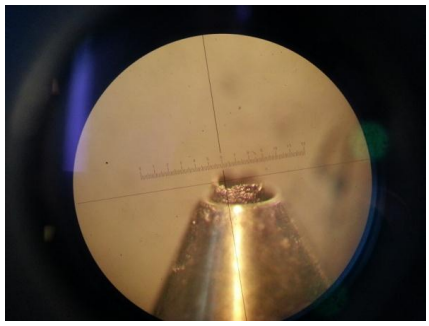

C)

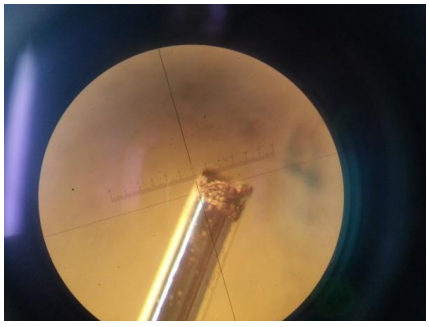

Figure 74: Microscopic images of the tip of the electrosprayer. A) Intact stainless steel needle in the sprayer, B) corroded stainless steel needle in the sprayer assembly, C) corroded stainless steel needle.

6.6. DISCUSSION ANIONIC ITP-MS

Several anionic ITP-C^4D-MS experiments were performed in order to demonstrate the applicability of the proposed method and its ability to surmount the different issues of anionic ITP-MS. Figure 75 presents the MS trace of the extracted ion isotachopherograms (EII) for an ITP experiment with the parameters of the final method. In this figure is possible to observe an efficient separation of the analytes in the ITP plateau mode. It is also worth noting the mixed migration zone between Analyte 5 and Analyte 6 at 22-23 min, and the different, ionization efficiencies for each analyte (this is discussed in the following section).

Figure 76 presents the conductivity traces measured with the C^4D device for the same experiment shown in Figure 75. For a precise reading of the inflection points in the trace, marking the transition from one step to the next one, the first derivative of

the resistance trace was build. The resulting plot was smoothed using a 5 point window Savitzky-Golay filter. Peaks were assigned using the spectroscopy toolbox of the Origin software. The resulting peak tips mark the inflection points, thus a precise reading of the beginning and the end of each step is possible (Figure 76).

6.6.1. IONIZATION EFFICIENCY & QUENCHING EFFECTS

One of major ideas of using isotachophoresis coupled to mass spectrometry was the possibility of observing analytes in an ion suppression free environment, as the individual isotachophoretic steps usually only contain the individual ion and the counter ion, respectively. Hence, matrix effects like ion suppression due to co-migrating compounds could be nearly excluded. And indeed, as can be seen from the maximum intensities in the isotachopherogram (Figure 75) the ionization efficiency on total is very high, but strongly differs from analyte to analyte, although nearly identical concentrations are present within the steps (compare MBE). However, this only holds true, if the total time of analysis is sufficient for the steps to evolve and to separate clearly in the ITP plateau mode. If the time of analysis is not sufficient, since the system was intentionally overloaded, mixed zones can be observed. Figure 75 clearly shows that such zone also exhibits very sharp boundaries, but both steps suffer from ion suppression. Also the extent of ion suppression is clearly visible and can be directly assessed from the isotachopherogram. While succinic acid experiences only minor effects, the comigration significantly reduces the ionization efficiency of the shikimic acid to less than 50%. Figure 75 also shows that the fluctuation of the MS-signal is very high for analytes with high ionization efficiency (Step 2 & 3), despite software-related averaging (the mean value of 10 scan cycles using a rolling averaging to filter out short term fluctuations. The filter creates a weighted average over the last three spectra (weighting 1:2:1).

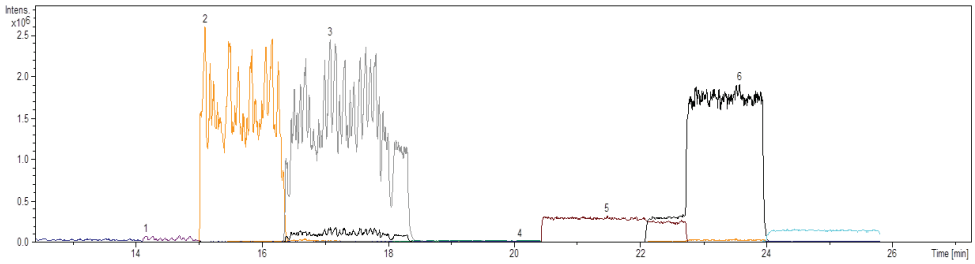

Figure 75: Isotachophoresis-mass spectrometry of six small organic acids. Note especially the mixed zone migrating between 22-23 min. Method parameters: LE: 10 mM HCl + 300 mM ß-alanine, pH = 3.1, TE = 100 mM hexanoic acid, sheath liquid = 50% water, 25% isopropanol, 25% acetonitrile.

6.6.2. QUANTIFICATION

Quantitative information can most easily be obtained from the distance in time between each peak minimum in the derivative isotachopherogram from C^4D detection in Figure 76. However, as it can be inferred from Figure 77, an implementation of this technique shows several problems: 1) The resolution of the conductivity detector is not sufficient for our purpose, due to the fact that the differentiation of individual steps is not possible when the amount of analytes in the capillary is lower than a certain level as can be seen in Figure 77, although a clear separation of the steps can be observed in the mass spectrometric trace. A new conductivity detector with a smaller distance between the two working electrodes to give rise to sharper steps due to a lower detection volume is currently being developed within our group, potentially lowering the limits of detetion by increasing the spatial resolution of the detector. However, from Figure 77, results show that the currently available conductivity detector does not provide sufficient resolution. 2) The bottom graph in Figure 77 exhibits more steps in the trace from the conductivity detector than in the mass spectrometric trace. These additional steps are mixed zones where the separation into the isotachophoretic stacks is not finished. These mixed zones can be later observed in the mass spectrometric trace in situ.

In some cases we observed a very large isotachophoretic stack in the conductivity detection system. This step cannot be identified in the mass spectrometer, as no characterisitc ion is measured, however a certain pattern is visible. This is most probably a result from carbonate, slowly diffusing into the buffer systems, creating a

carbonate step the system. This effect is well known for basic buffers in ITP and can be circumvented by the addition of barium hydroxide to the buffer systemshowever, these countermeasures are not compatible with mass spectrometry. The choice of a leading electrolyte exhibiting a low pH-value decreased this problem.

Nevertheless, the application of the combination of conductivity detection and mass spectrometry is still a valuable tool for the precise quantification of highly concentrated samples. Additional information about analytes not accessible via mass spectrometry (e.g. because their m/z-value is below the scan range (< 50) or they are not accessible via ESI-MS) can be obtained or a least, their presence can be confirmed.

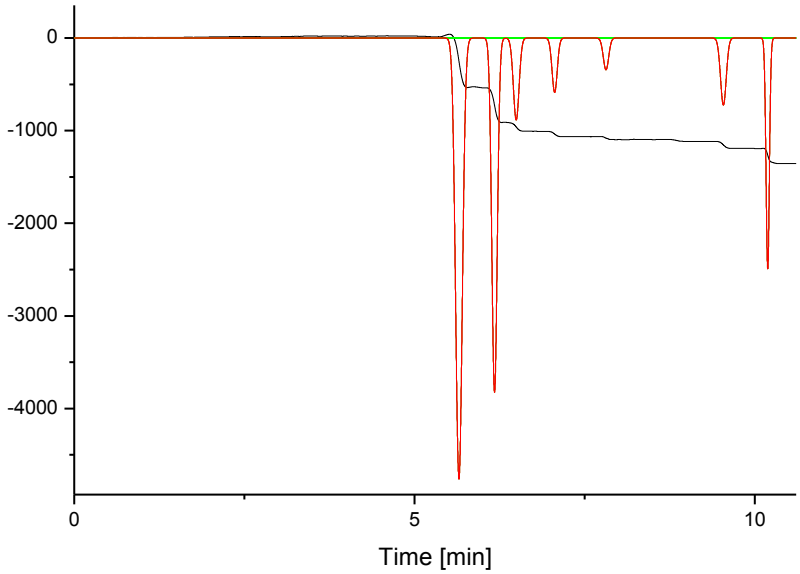

Figure 76: Isotachopherogram of the analysis of small organic acids. The solid black line indicates the resistance trace from the conductivity detector, the red line represents the smoothed first derivative thereof for a precise marking of the inflection point.

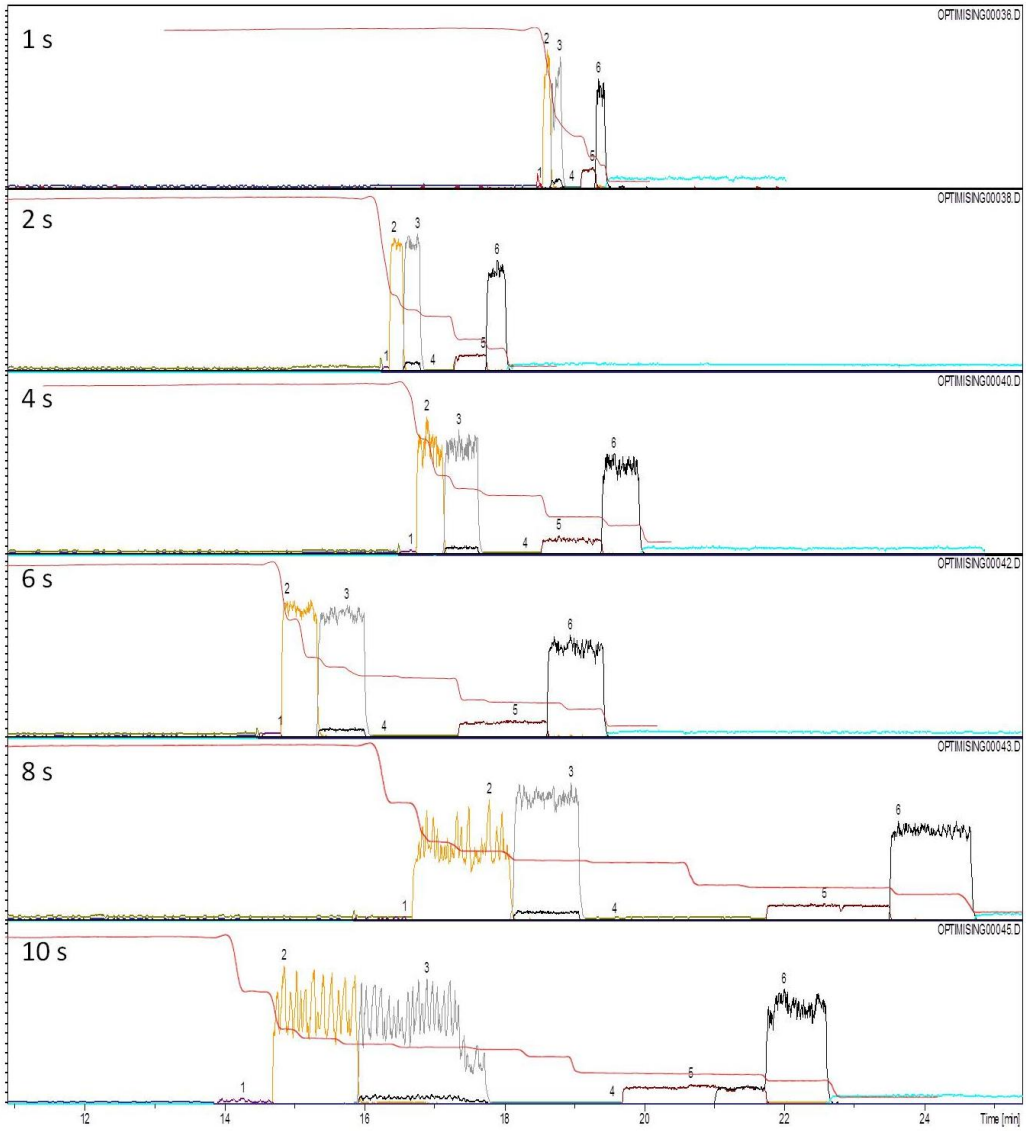

Figure 77: MS-traces varying the injection time (see figure legend). Migration order: 1 oxalic acid, 2 salicylic acid, 3 citric acid, 4 glycolic acid, 5 succinic acid, 6 shikimic acid.

During our investigation it became evident, that the achievable limits of detection of the mass spectrometer exceed the possibilities of the conductivity detection by far as surprisingly sharp boundaries of the isotachophoretic steps were obtained (see Figure 78). In this Figure we present the analysis of six organic acids via ITP-MS using the 10 mM HCl as leading electrolyte with beta-alanine as counterion at pH 3.1

with varying injection times. In this case, the mass spectrometric interface did not disturb the moving boundaries of the isotachophoresis to a noticeable extent providing an excellent platform for quantification of analytes. It is worth to note that a quantification using the C^4D would not be possible in most of these analytical runs, as signals are not resolved when an injection time of only 1s was used, and multiple steps appeared for the sample zones and the mixed zones when more analyte was injected, hence an attribution of the steps towards a specific analyte is impossible.

6.6.3. DATA EVALUATION STRATEGIES

Three different data evaluation procedures for quantification were tested: 1) The real peak range (time interval from the starting point of each plateau to the end of the plateau) of each plateau was assessed for quantitative data evaluation. 2) The peak range relative to the mean migration time of each peak was assessed. 3) The real width of each stack was assessed at half height of the maximum. By using the full width at half height (FWHM), it was expected to rule out negative influence from minor tailing effects as e.g. visible in Figure 75.

The relative peak range gave the best results for quantitative data evaluation, in terms of linearity, thus only this type of results is presented. Relative peak areas can be used to correct for band broadening effects either from a slower passage of the analyte through the detector or from migration time differences and resulting diffusion processes in electrophoretic separation techniques. As we expect a general decrease in velocity of the isotachophoretic stack each time an analyte leaves the system and thus artificially creating longer consecutive steps, the relative peak range seems to be an adequate tool to correct for these changes. This effect is even pronounced in our instrumental setup, as the CE unit is not capable of controlling a constant current at desired levels, thus constant voltage (+30 kV) was used. Figure 79 shows the calibration plots for the analysis of oxalic, tartaric, succinic and shikimic acid. According to our results, quantitative measurements with the instrumental setup are possible however, the limit of quantification is high as long as we are in the plateau mode. In contrast, the limits of detection are very low, as the stacking of the analytes still happens at the boundary to the terminating electrolyte, although the amount is not sufficient to form a plateau. This peak mode is well know to achieve very low limits of detection [346]. But, with regard to the aim of this study, the peak mode exhibits two different features, which are disadvantageous for our purpose: 1)

when operated in the peak mode, the analytes elute simultaneously, thus isobaric analytes cannot be separated and quenching effects reduce reliability of the analysis as well as reduced signal heights. Also the possibility for prolonged MS/MS experiments would be reduced. 2) In terms of quantification, problems will arise in the transition zone, when the amount of an analyte is at the boundary between peak mode and plateau mode. As the peak area is an adequate tool for the quantification of ITP stacks in the peak mode, we have shown that the relative peak range is superior for the quantification of plateau shaped ITP stacks. However, in the transition zone, the results for both techniques are not optimal, clearly requiring further work in order to reach suitable precision for the use of ITP-MS for the quantification of analytes in complex mixtures with large variations in analyte concentrations.

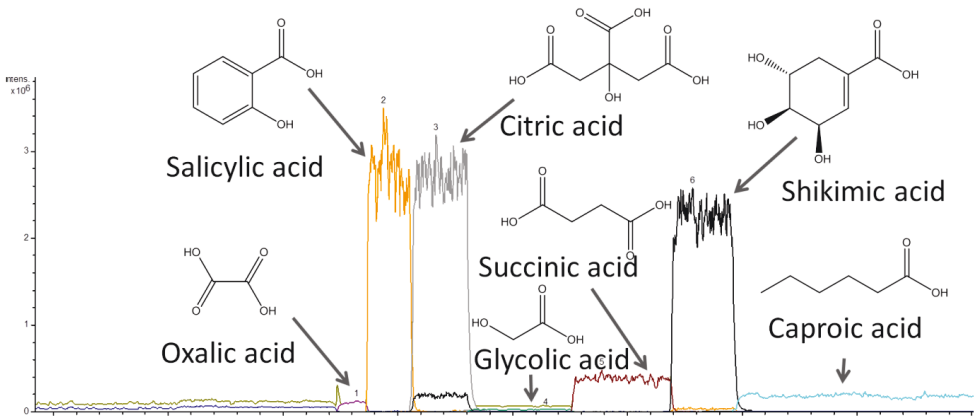

Figure 78: Isotachophoresis-mass spectrometric analysis of six standard compounds.

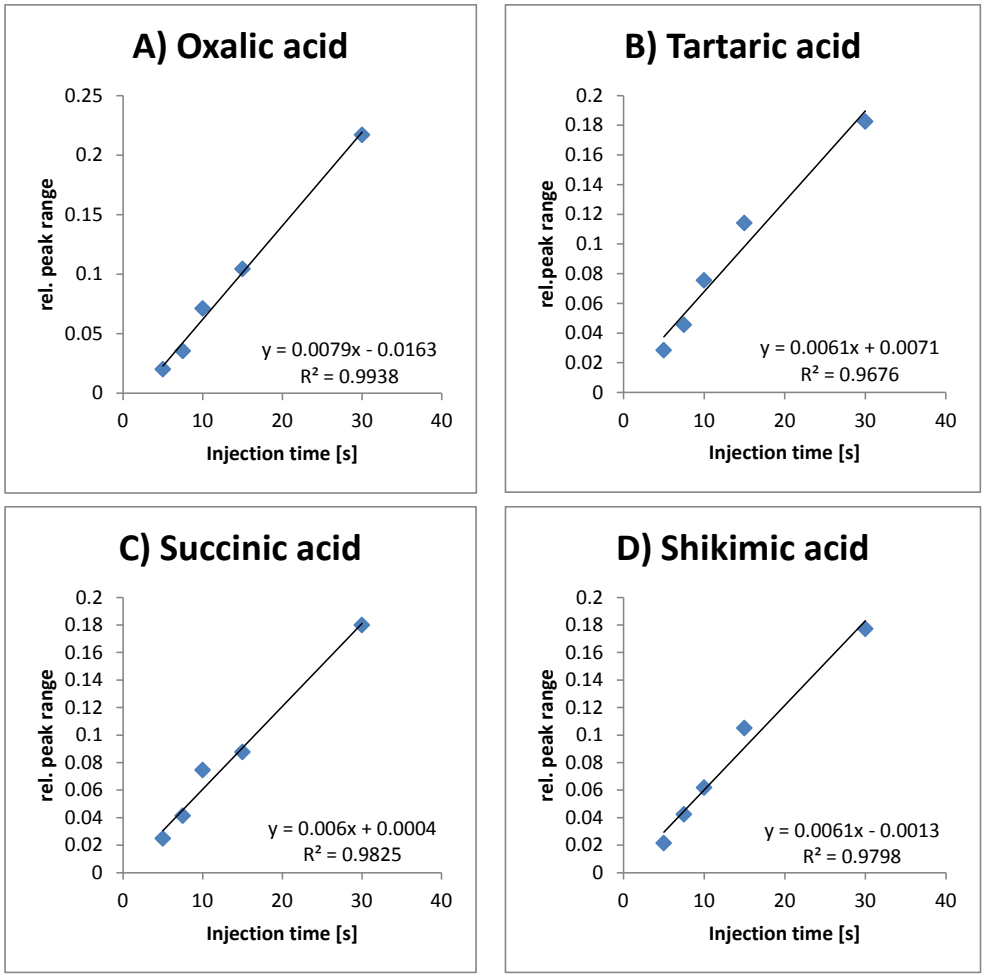

Figure 79: Calibration plots for the analysis of four small organic acids. The injected amount of analyte was varied by an increase in injection time. The y-axis represents the relative peak range, the x-axis represents the injection time. The injected sample solution had a concentration of 1 mM for each analyte.

6.6.4. REAL SAMPLES AND EXTRACTION PROCEDURES

The anionic ITP-C^4D-MS method was applied to quantification of the precursor substances of the early shikimic acid pathway, namely phosphoenolpyruvate and erythrose-4-phosphate [356]. Both substances have already been successfully analyzed via CE-MS [357], however, it was questionable if the both analytes could be stacked in the isotachophoretic window. As can be inferred from Figure 80 and Figure 81 both analytes can be stacked in the ITP window and both analytes can be detected ideally as the respective dimers in the form $[2M-H]^-$ and the monomers as

typical present in aqueous solution [358] in the anionic detection mode. The observed step with m/z = 258.9 in Figure 81 is most probably a decomposition product of erythrose-4-phosphate due to prolonged storage at -20°C and thawing for analysis. Figure 82 shows the mass spectra of the three visible stacks, the erythrose-4-phosphate at 5.6 min migration time, the mixed zone at 7.3 min migration time and the decomposition product at 7.7 min. Another important feature of ITP-MS is that the ratio of dimer to the monomer (Figure 80) is always constant through the whole step length. Consequently, both can be assessed simultaneously, as long as one signal is strong enough for identification. Figure 80 also nicely demonstrates that the ionization properties for the dimer and the monomer are independent from the ionization efficiency, as e.g. the small incision in the signal around 7.3 min can be found for the dimer and the monomer to the same extent.

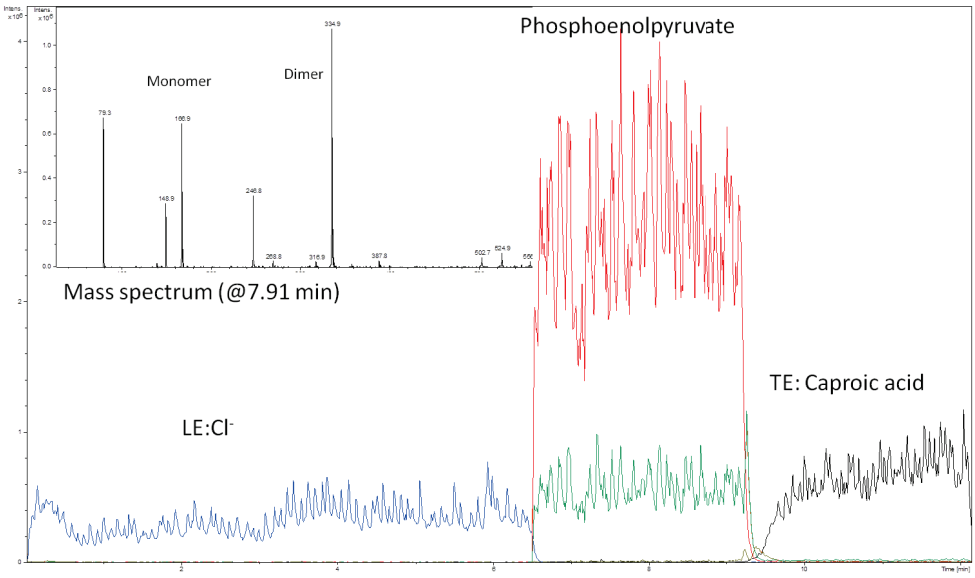

Figure 80: Isotachophoretic analysis of a standard of phosphoenolpyruvate. The window in top left corner shows the mass spectrum at 7.91 minutes migration time. LE: 10 mM HCl, titrated to pH = 3.1 with 1M ß-alanine solution, TE: 100 mM caproic acid, 300 mM ß-alanine.

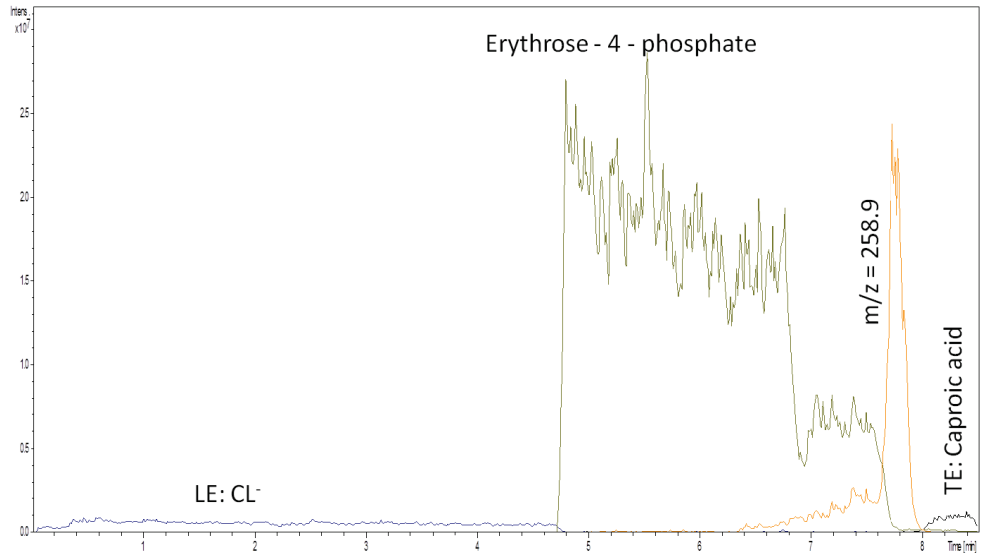

Figure 81: Isotachophoretic analysis of a standard of erythrose-4-phosphate. LE: 10 mM HCl, titrated to pH = 3.1 with 1M ß-alanine solution, TE: 100 mM caproic acid, 300 mM ß-alanine, -30kV separation voltage.

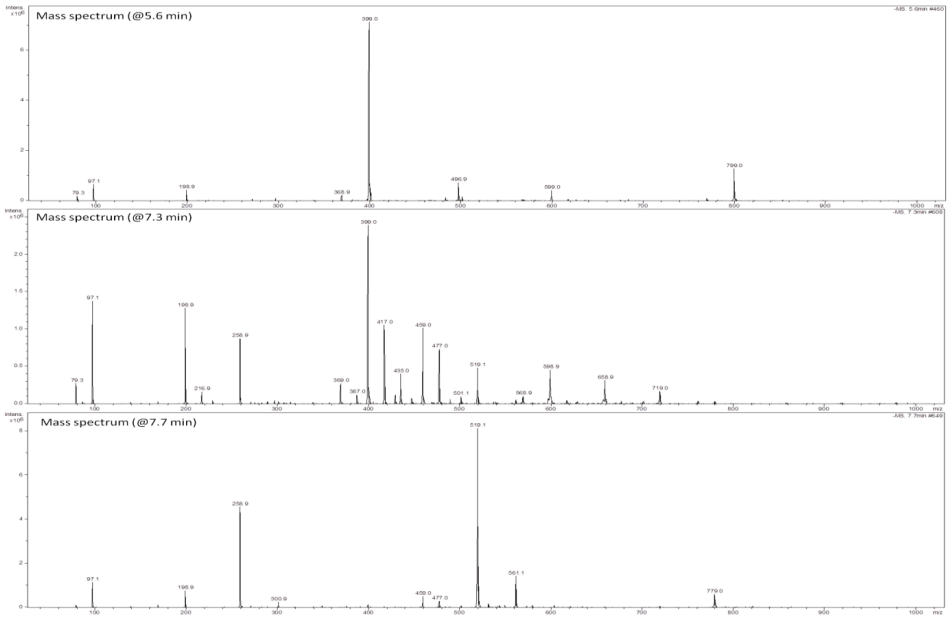

Figure 82: Mass spectra for the migration times marked in the upper left corner of each spectrum of the isotachopherogram of erythrose-4-phosphate (Figure 81).

For a comprehensive test on the suitability of our proposed method for the analysis of various plant extracts for a determination of the anionic compounds, we tested three

different types of extracts, prepared by the group of Ingar Janzik. A methanolic, an ethanolic, and an aqueous extract from *Arabidopsis thaliana* were used as samples. Each extract was injected without further sample pretreatment. Injection was performed hydrodynamically at 50 mbar for 10 s. To circumvent potential influence from the salt content in the extracts on the total ionic strength, the concentration of counterions in the leading electrolyte was increased to 1 M and 300 mM counterion were added to the terminating electrolyte.

Figure 83 shows an ethanolic extract of *Arabidopsis thaliana.* It is clearly visible that at least eight individual analytes could be stacked within the isotachophoretic stack. From the disappearance of the leading electrolyte in the isotachopherogram between 16 and 17.5 min migration time, one can suspect that substances inaccessible to the mass spectrometer are stacked and migrating here.

The depicted mass spectrum at 18 min migration time is characterized by very clean signals without much background noise and clear peaks, as we expected for ITP-MS analysis. Although at least three substances comigrate here, a clear assessment and even MS/MS experiments would be feasible here. It is noteworthy that the nature of the solvent (here: ethanol) has no negative influence on the isotachophoresis, but we suspect a strong influence from the salt content in the samples, as the migration time shifts drastically between runs, when real samples are introduced.

Figure 84 shows the isotachophoresis of a methanolic extract of *Arabidopsis thaliana.* At least, eleven substances are stacked in the isotachophoresis, most of them with the same m/z-ratio as an in the ethanolic extract, but several additional analytes can be detected here (e.g. m/z = 177, m/z = 223, m/z 132).

Figure 85 shows the analysis of an aqueous extract of *Arabidopsis thaliana.* Interestingly, the absolute intensity of the signals is reduced in comparison to the analysis of the ethanolic and the methanolic extracts. Also an increased background noise is visible in the mass spectrum. Nevertheless, around eleven individual analytes are migrating within the isotachophoretic stacks. The composition of these analytes is different to the ones isolated with the ethanolic or methanolic extraction.

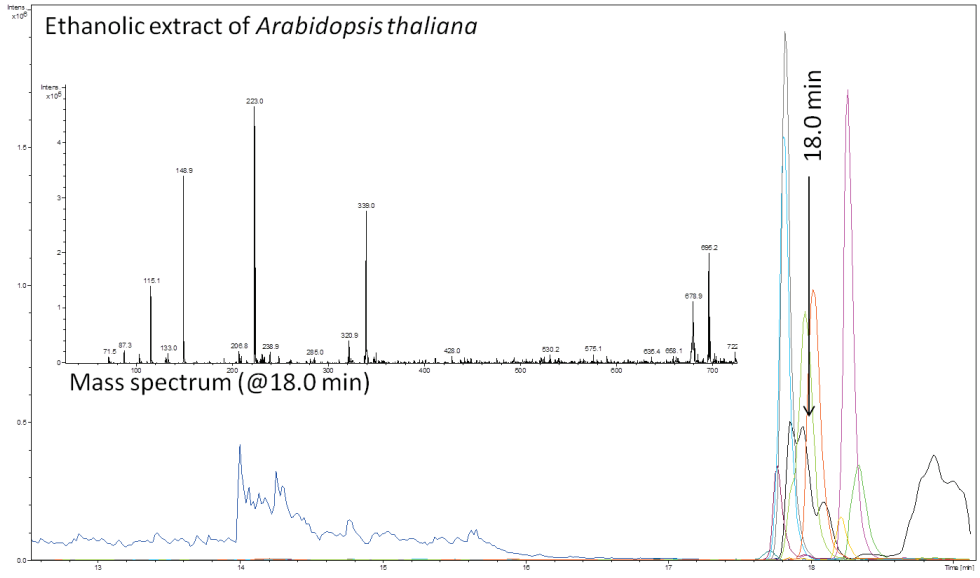

Figure 83: Isotachopherogram of the analysis of an ethanolic extract of Arabidopsis thaliana. Conditions: LE: 10 mM HCl, titrated to pH = 3.1 with 1M ß-alanine solution, TE: 100 mM caproic acid, 300 mM ß-alanine, -30kV separation voltage. Injection was performed at 50 mbar for 10 s.

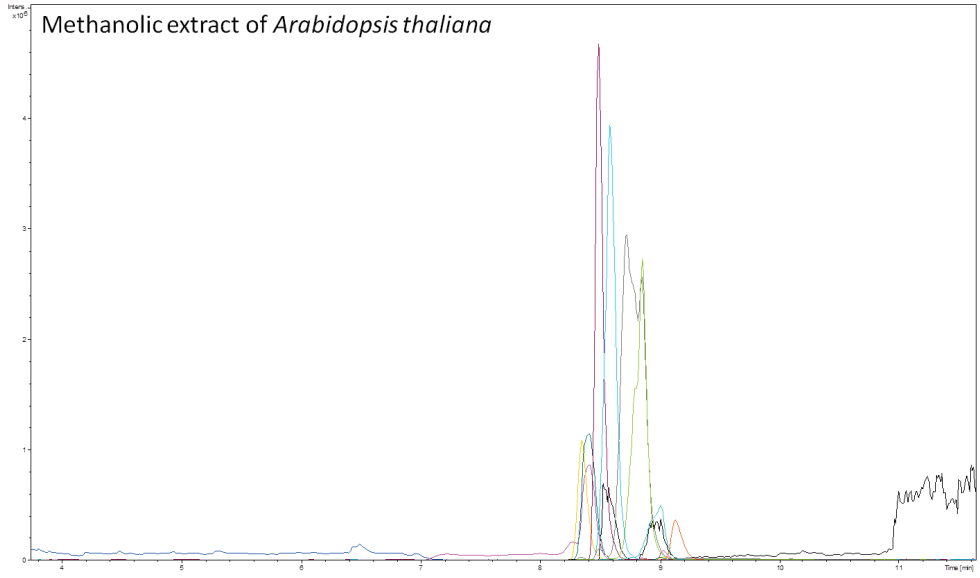

Figure 84: Isotachopherogram of the analysis of a methanolic extract of Arabidopsis thaliana. Conditions: LE: 10 mM HCl, titrated to pH = 3.1 with 1M ß-alanine solution, TE: 100 mM caproic acid, 300 mM ß-alanine, -30kV separation voltage. Injection was performed at 50 mbar for 10 s.

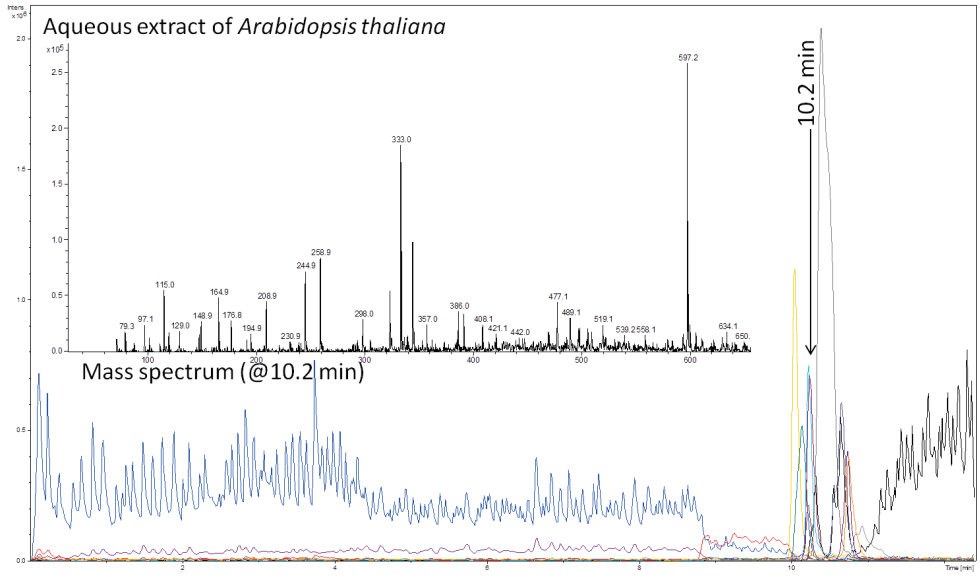

Figure 85: Isotachopherogram of the analysis of an aqueous extract of Arabidopsis thaliana. Conditions: LE: 10 mM HCl, titrated to pH = 3.1 with 1M ß-alanine solution, TE: 100 mM caproic acid, 300 mM ß-alanine, -30kV separation voltage. Injection was performed at 50 mbar for 10 s.

A detailed discussion about the nature of these analytes or a comprehensive comparison of the extraction or suitability of these extracts for the analysis of *Arabidopsis thaliana* goes beyond the scope of this study. The most interesting outcome of the analysis of the real samples is that all types of extracts could be successfully analyzed with ITP-MS, irregardless of the nature of the solvent. Therefore we can conclude that anionic ITP-MS is generally suited for the analysis of extracts from plant materials and has the potential to deliver interesting results which might be inaccessible via LC or GC analysis.

6.7. CONCLUSION

The coupling of isotachophoresis with mass spectrometry is a very challenging task, due to the many so far not addressed problems and a lack of literature results for comparison. Nevertheless, in this work one of the first real ITP-MS couplings is presented and so far unknown challenges have been solved. The so far unaddressed problem of the data evaluation is discussed and a good approach for it is presented. Only the group of Boček et al. was able to present a working anionic ITP-MS

coupling [346-348], but only in peak mode and with only two analytes in the first publication. The work presented here goes beyond this application and discusses the contra productive effects, which are evoked with this coupling. As a future perspective for quantification, a very elegant way, which could also lead to the possibility of absolute quantification, would be the application of deuterated or C-13 labeled standards. The quantity of the analyte in the sample could then directly be assessed from the peak height difference in the mass spectrum and all problems associated with ionization efficiency, determination of start and end point of the steps.

7. CONCLUSION & SUMMARY OF THE THESIS

Revisiting the scope of thesis, we can now conclude that electromigrative separation techniques have the capability to contribute significantly to the field of forensic science. **Chapter 1** impressively shows that the role of electromigrative separation techniques is currently underestimated and that the vast amount of available methods and varieties for special applications of this technique is unmatched. It has been shown that electromigrative separation techniques are indeed applied in routine analysis, performing excellent regarding robustness and precision for a broad range of difficult analytes and samples, all using one single instrument, only modifying the electrolyte composition. The distinct advantages of CE for forensic toxicology (detection of compounds in difficult matrices, detection in the presence of excess compounds, etc.) shown in this chapter will surely lead to a reconsideration of the implementation of electromigrative separation techniques into routine work or for special applications where orthogonal separation is needed.

Usage of a design of experiments in order to optimize both, separation and detection parameters in CE-MS (**Chapter 2)** went beyond a simple method development. It provided a deeper insight into the underlying separation mechanisms in non aqueous capillary electrophoresis-mass spectrometry. It was shown for the first time that the separation was based on the analytes fully engaged in ion-pairing with the co-ion in charge for the enhancement of resolution and peak capacity. The gained knowledge allows for a deeper understanding of the separation principle which was further used for the adaptation of the method towards new analytical tasks and challenges. The variability of this technique was proven in **Chapter 3,** where the broad range applicability of NACE-MS was demonstrated as a nearly generic method for the analysis of alkaloids from various psychoactive plants. With a slight modification of the background electrolyte, it was even possible to widen the analytical window towards the analysis of polyaromatic sulfur hydrocarbons (PASHs) from crude oil samples (**Chapter 4**). The impressive separation power for structurally closely related analytes (PASHs) up to diastereomers like the Kratom alkaloids is unmatched when compared to liquid chromatography. The method presented in Chapter 2 was further applied to a forensic profiling of Kratom from a large seizure (**Chapter 5**).

Chemometric analysis revealed only limited differentiation possibilities for the very similar Kratom samples. The potential of the method was further shown in **Chapter 5** for the forensic profiling of the drugs opium and heroin. For this purpose a comprehensive analytical workflow was established starting with the sample preparation, CE-MS analysis and its validation up to the implementation of new strategies for automated data extraction and chemometric data evaluation, which has not been described in literature yet. Based on the strategies achieved, the application of CE-MS for high throughput analysis in future applications is possible with reduced manual effort and increased robustness.

Finally, **Chapter 6** shows that electromigrative separation techniques, in this case the rarely applied isotachophoresis, can achieve impressive results in the solution of so far unresolved problems, here, the analysis of small organic acids. During this study, many problems so far not addressed by the scientific community in the literature were encountered, investigated and partially solved. The basic knowledge provided in this chapter goes well beyond the current state of technology and shows the impressive potential of ITP-C^4D-MS, provided that the remaining problems can be circumvented and solved.

Summarizing, this thesis represents a toolbox including a review about the possibilities of electromigrative separation techniques, a deeper investigation about the underlying principles of electromigrative separation techniques and, based on the preceding, the manifold application strategies and analytical fields, where electromigrative separation techniques can play a major role in the near future. Especially in the field of forensics, it is the author's opinion, that electromigrative separation will become a leading technique with the rise of the biopharmaceuticals, doping in sports and the "research chemicals" abused as recreational drugs. The ease of the adaption of the technique towards new types of samples (biogenic samples, xenobiotics, etc.) will always make CE a valuable alternative to liquid chromatography or gas chromatography. This also holds true for further fields, especially for many "omics" applications.

Although many questions were answered within this thesis, new questions arise that need further investigations in the near future. For example, the methods developed in this thesis allow for the analysis of cationic and anionic compounds from plant extracts and their analysis via CE-MS or ITP-MS, but no neutral compounds have

been considered. Promising approaches from the coupling of MEKC are available, e.g. using other ionization techniques than ESI or polymeric micelles, but they will need further research. The separation mechanism of NACE-MS was investigated for a particular buffer system in this thesis, but to what extend are these findings valid? Are they also valid for mixtures with water or methanol? For other compounds than indole alkaloids and PASHs? Can other ion-pairing reagents induce comparable separation of structurally closely related analytes and if so, is it possible to achieve the separation of enantiomers by using chiral ion-pairing reagents while still being compatible to mass spectrometry?

Special attention should be payed to the ITP-MS coupling, as first steps were taken and considerations made in this thesis for this promising technique. However, also several so far unknown problems were encountered and need further investigation. Key factors are the problems associated with the ion-source, but also very basic questions like the appropriate data evaluation are still unresolved.

It is the author's opinion that electromigrative separation techniques will play a pronounced role in the near future, as the new types of samples, especially native and pharmaceutical biopolymers in the (bio-)medicine (peptides, proteins, monoclonal antibodies, etc.) and the "omics" field require the flexibility and variability of CE-MS and have ideal characteristics for being separated in the electric field.

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9. REFERENCES

1. Lurie IS, Toske SG (2008) Applicability of ultra-performance liquid chromatography-tandem mass spectrometry for heroin profiling. J Chromatogr A 1188 (2):322-326.
2. Arndt T, Claussen U, Güssregen B, Schröfel S, Stürzer B, Werle A, Wolf G (2011) Kratom alkaloids and O-desmethyltramadol in urine of a “Krypton” herbal mixture consumer. Forensic Sci Int 208 (1-3):47-52.
3. Takayama H (2004) Chemistry and Pharmacology of Analgesic Indole Alkaloids from the Rubiaceous Plant, *Mitragyna speciosa*. Chem Pharm Bull 52 (8):916-928.
4. Schröfel S, Hupp A, Auwärter V, Arndt T (2011) Zur Verbreitung von Kratom (Mitragyna speciosa) in (il)legalen Kräutermischungen. Toxichem Krimtech 78 (1):45-54.
5. Philipp A, Meyer M, Wissenbach D, Weber A, Zoerntlein S, Zweipfenning P, Maurer H (2011) Monitoring of kratom or Krypton intake in urine using GC-MS in clinical and forensic toxicology. Anal Bioanal Chem 400 (1):127-135.
6. Mortishire-Smith RJ, O'Connor D, Castro-Perez JM, Kirby J (2005) Accelerated throughput metabolic route screening in early drug discovery using high-resolution liquid chromatography/quadrupole time-of-flight mass spectrometry and automated data analysis. Rapid Commun Mass Spectrom 19 (18):2659-2670.
7. Selman MH, McDonnell LA, Palmblad M, Ruhaak LR, Deelder AM, Wuhrer M (2010) Immunoglobulin G glycopeptide profiling by matrix-assisted laser desorption ionization Fourier transform ion cyclotron resonance mass spectrometry. Anal Chem 82 (3):1073-1081.
8. Nevedomskaya E, Derks R, Deelder A, Mayboroda O, Palmblad M (2009) Alignment of capillary electrophoresis–mass spectrometry datasets using accurate mass information. Anal Bioanal Chem 395 (8):2527-2533.
9. Palmblad M, Mills DJ, Bindschedler LV, Cramer R (2007) Chromatographic Alignment of LC-MS and LC-MS/MS Datasets by Genetic Algorithm Feature Extraction. Journal of the American Society for Mass Spectrometry 18 (10):1835-1843.
10. Pütz M, Martin N Application of CE-ESI-MS in forensic toxicology: Identification of piperazine-derived designer drugs in Ecstasy tablets and of food colorants in illicit drugs. In: Pragst F, Aderjan R (eds) XV. GTFCh-Symposium 2007 Aktuelle Beiträge zur Forensischen und Klinischen Toxikologie, Bad Vibel, 2008. pp 487 - 501.
11. Zhong X, Maxwell EJ, Chen DDY (2011) Mass Transport in a Micro Flow-Through Vial of a Junction-at-the-Tip Capillary Electrophoresis-Mass Spectrometry Interface. Anal Chem 83 (12):4916-4923.
12. Favretto D, Pascali JP, Tagliaro F (2013) New challenges and innovation in forensic toxicology: Focus on the “New Psychoactive Substances”. J Chromatogr A 1287:84-95.
13. Rosenbaum C, Carreiro S, Babu K (2012) Here Today, Gone Tomorrow…and Back Again? A Review of Herbal Marijuana Alternatives (K2, Spice), Synthetic Cathinones (Bath Salts), Kratom, Salvia divinorum, Methoxetamine, and Piperazines. J Med Toxicol 8 (1):15-32.
14. Tagliaro F, Bortolotti F (2006) Recent advances in the applications of CE to forensic sciences (2001-2004). Electrophoresis 27 (1):231-243.
15. Tagliaro F, Bortolotti F (2008) Recent advances in the applications of CE to forensic sciences (2005-2007). Electrophoresis 29 (1):260-268.

16. Tagliaro F, Pascali J, Fanigliulo A, Bortolotti F (2010) Recent advances in the application of CE to forensic sciences: A update over years 2007–2009. Electrophoresis 31 (1):251-259.
17. Pascali JP, Bortolotti F, Tagliaro F (2012) Recent advances in the application of CE to forensic sciences, an update over years 2009–2011. Electrophoresis 33 (1):117-126.
18. Riekkola M-L, Jönsson JÅ, Smith RM (2004) Terminology for analytical capillary electromigration techniques. Pure and Applied Chemistry 76 (2):443-451.
19. Tagliaro F, Manetto G, Crivellente F, Smith FP (1998) A brief introduction to capillary electrophoresis. Forensic Sci Int 92 (2-3):75-88.
20. Lauer HH, Rozing G (eds)High Performance Capillary Electrophoresis - A Primer. Agilent Technologies.
21. Engelhardt H, Beck W, Schmitt T, Gutnikov G (1996) Capillary electrophoresis: Methods and potentials. Vieweg, Wiesbaden.
22. Tagliaro F, Smith FP (1996) Forensic capillary electrophoresis. TrAC, Trends Anal Chem 15 (10):513-525.
23. Tagliaro F, Bortolotti F, Pascali J (2007) Current role of capillary electrophoretic/electrokinetic techniques in forensic toxicology. Anal Bioanal Chem 388 (7):1359-1364.
24. Cruces-Blanco C, García-Campaña AM (2012) Capillary electrophoresis for the analysis of drugs of abuse in biological specimens of forensic interest. TrAC, Trends Anal Chem 31:85-95.
25. Smyth WF, Brooks P (2004) A critical evaluation of high performance liquid chromatography-electrospray ionisation-mass spectrometry and capillary electrophoresis- electrospray-mass spectrometry for the detection and determination of small molecules of significance in clinical and forensic science. Electrophoresis 25 (10-11):1413-1446.
26. Fanigliulo A, Bortolotti F, Pascali J, Tagliaro F (2008) Chapter 15 Forensic toxicological screening with capillary electrophoresis and related techniques. In: Bogusz MJ (ed) Handbook of Analytical Separations, vol 6. Elsevier Science B.V., pp 513-534.
27. Petersen JR, Okorodudu AO, Mohammad A, Payne DA (2003) Capillary electrophoresis and its application in the clinical laboratory. Clin Chim Acta 330 (1–2):1-30.
28. Smyth WF, McClean S (1998) A critical evaluation of the application of capillary electrophoresis to the detection and determination of 1,4-benzodiazepine tranquilizers in formulations and body materials. Electrophoresis 19 (16-17):2870-2882.
29. Smyth WF (2006) Recent applications of capillary electrophoresis-electrospray ionisation-mass spectrometry in drug analysis. Electrophoresis 27 (11):2051-2062.
30. Smyth WF (2005) Recent applications of capillary electrophoresis-electrospray ionisation-mass spectrometry in drug analysis. Electrophoresis 26 (7-8):1334-1357.
31. Northrop DM, McCord B, Butler JM (1994) Forensic applications of capillary electrophoresis. J Capillary Electrophor 001 (2):158-168.
32. Bjørnsdottir I, Tjørnelund J, Hansen SH (1998) Nonaqueous capillary electrophoresis - its applicability in the analysis of food, pharmaceuticals and biological fluids. Electrophoresis 19 (12):2179-2186.
33. Lurie IS (1998) Capillary electrophoresis of illicit drug seizures. Forensic Sci Int 92 (2-3):125-136.
34. von Heeren F, Thormann W (1997) Capillary electrophoresis in clinical and forensic analysis. Electrophoresis 18 (12-13):2415-2426.

35. Lemos NP, Bortolotti F, Manetto G, Anderson RA, Cittadini F, Tagliaro F (2001) Capillary electrophoresis: a new tool in forensic medicine and science. Sci Justice 41 (3):203-210.
36. Deyl Z, Mikik I, Tagliaro F (1998) Advances in capillary electrophoresis. Forensic Sci Int 92 (2-3):89-124.
37. Thormann W, Aebi Y, Lanz M, Caslavska J (1998) Capillary electrophoresis in clinical toxicology. Forensic Sci Int 92 (2-3):157-183.
38. Manetto G, Crivellente F, Tagliaro F (2000) Capillary electrophoresis: A new analytical tool for forensic toxicologists. Ther Drug Monit 22 (1):84-88.
39. Boone CM, Franke J-P, de Zeeuw RA, Ensing K (1999) Evaluation of capillary electrophoretic techniques towards systematic toxicological analysis. J Chromatogr A 838 (1-2):259-272.
40. Anastos N, Barnett NW, Lewis SW (2005) Capillary electrophoresis for forensic drug analysis: A review. Talanta 67 (2):269-279.
41. Cruces-Blanco C, Gámiz–Gracia L, García-Campaña AM (2007) Applications of capillary electrophoresis in forensic analytical chemistry. TrAC, Trends Anal Chem 26 (3):215-226.
42. Thormann W, Molteni S, Caslavska J, Schmutz A (1994) Clinical and forensic applications of capillary electrophoresis. Electrophoresis 15 (1):3-12.
43. Thormann W, Caslavska J (1998) Capillary electrophoresis in drug analysis. Electrophoresis 19 (16-17):2691-2694.
44. Plaut O, Staub C (2002) Capillary Electrophoresis in Forensic Toxicology. CHIMIA International Journal for Chemistry 56 (3):96-100.
45. Porras SP, Riekkola M-L, Kenndler E (2003) The principles of migration and dispersion in capillary zone electrophoresis in nonaqueous solvents. Electrophoresis 24 (10):1485-1498.
46. Porras SP, Riekkola M-L, Kenndler E (2001) Electrophoretic mobilities of cationic analytes in non-aqueous methanol, acetonitrile and their mixtures - Influence of ionic strength and ion-pair formation. J Chromatogr A 924:31-42.
47. Porras SP, Kenndler E (2004) Capillary zone electrophoresis in non-aqueous solutions: pH of the background electrolyte. J Chromatogr A 1037 (1-2):455-465.
48. Porras SP, Kenndler E (2005) Are the asserted advantages of organic solvents in capillary electrophoresis real? A critical discussion. Electrophoresis 26 (17):3203-3220.
49. Geiser L, Veuthey J-L (2009) Non-aqueous capillary electrophoresis 2005–2008. Electrophoresis 30 (1):36-49.
50. Riekkola M-L, Jussila M, Porras SP, Valko IE (2000) Non-aqueous capillary electrophoresis. J Chromatogr A 892 (1-2):155-170.
51. Szumski M, Buszewski B (2013) Non-Aqueous Capillary Electrophoresis. In: Buszewski B, Dziubakiewicz E, Szumski M (eds) Electromigration Techniques, vol 105. Springer Series in Chemical Physics. Springer Berlin Heidelberg, pp 203-213.
52. Bjørnsdottir I, HonoréHansen S (1995) Comparison of separation selectivity in aqueous and non-aqueous capillary electrophoresis. J Chromatogr A 711 (2):313-322.
53. Amini A (2001) Recent developments in chiral capillary electrophoresis and applications of this technique to pharmaceutical and biomedical analysis. Electrophoresis 22 (15):3107-3130.
54. Chankvetadze B (1997) Separation selectivity in chiral capillary electrophoresis with charged selectors. J Chromatogr A 792 (1–2):269-295.
55. Pyell U (2006) Electrokinetic chromatography: theory, instrumentation, and applications. John Wiley & Sons, Chichester.

56. Somsen GW, Mol R, de Jong GJ (2003) On-line micellar electrokinetic chromatography–mass spectrometry: feasibility of direct introduction of non-volatile buffer and surfactant into the electrospray interface. J Chromatogr A 1000 (1–2):953-961.
57. Watarai H (1991) Microemulsion Capillary Electrophoresis. Chem Lett 20 (3):391-394.
58. Marsh A, Altria K, Clark B (2007) Microemulsion Electrokinetic Chromatography. In: Electrokinetic Chromatography. John Wiley & Sons, Ltd, pp 115-135.
59. Yang H, Ding Y, Cao J, Li P (2013) Twenty-one years of microemulsion electrokinetic chromatography (1991–2012): A powerful analytical tool. Electrophoresis 34 (9-10):1273-1294.
60. Ryan R, Donegan S, Power J, McEvoy E, Altria K (2009) Recent advances in the methodology, optimisation and application of MEEKC. Electrophoresis 30 (1):65-82.
61. Altria KD, Mahuzier P-E, Clark BJ (2003) Background and operating parameters in microemulsion electrokinetic chromatography. Electrophoresis 24 (3):315-324.
62. Liotta E, Gottardo R, Seri C, Rimondo C, Miksik I, Serpelloni G, Tagliaro F (2012) Rapid analysis of caffeine in “smart drugs” and “energy drinks” by microemulsion electrokinetic chromatography (MEEKC). Forensic Sci Int 220 (1–3):279-283.
63. Hansen SH, Sheribah ZA (2005) Comparison of CZE, MEKC, MEEKC and non-aqueous capillary electrophoresis for the determination of impurities in bromazepam. J Pharm Biomed Anal 39 (1–2):322-327.
64. Mistry K, Krull I, Grinberg N (2002) Capillary electrochromatography: An alternative to HPLC and CE. J Sep Sci 25 (15-17):935-958.
65. Bartle KD, Myers P (eds) (2001) Capillary Electrochromatography. Royal Society of Chemistry, Cambridge.
66. Deyl Z, Svec F (eds) Capillary Electrochromatography. Journal of Chromatography Library. Elsevier, Amsterdam.
67. Lurie IS, Meyers RP, Conver TS (1998) Capillary Electrochromatography of Cannabinoids. Anal Chem 70 (15):3255-3260.
68. Lim J-T, Zare RN, Bailey CG, Rakestraw DJ, Yan C (2000) Separation of related opiate compounds using capillary electrochromatography. Electrophoresis 21 (4):737-742.
69. Boček P (1981) Analytical isotachophoresis. In: Analytical Problems, vol 95. Topics in Current Chemistry. Springer Berlin Heidelberg, pp 131-177.
70. Bocek P (1988) Analytical isotachophoresis. Electrophoresis library. VCH Verl.Ges., Weinheim.
71. Everaerts FM, Becker JL, Verheggen TPEM (1976) Isotachophoresis: Theory, instrumentation, and applications. Journal of Chromatography library; v.6. Elsevier Scientific Pub. Co., Amsterdam.
72. Danková M, Kaniansky D, Fanali S, Iványi F (1999) Capillary zone electrophoresis separations of enantiomers present in complex ionic matrices with on-line isotachophoretic sample pretreatment. J Chromatogr A 838 (1-2):31-43.
73. Lurie IS, Hays PA, Parker K (2004) Capillary electrophoresis analysis of a wide variety of seized drugs using the same capillary with dynamic coatings. Electrophoresis 25 (10-11):1580-1591.
74. Tomás-Barberán FA (1995) Capillary electrophoresis: A new technique in the analysis of plant secondary metabolites. Phytochem Anal 6 (4):177-192.
75. Frost M, Köhler H (1998) Analysis of lysergic acid diethylamide: comparison of capillary electrophoresis with laser-induced fluorescence (CE-LIF) with conventional techniques. Forensic Sci Int 92 (2–3):213-218.

76. Huhn C, Pütz M, Martin N, Dahlenburg R, Pyell U (2005) Determination of tryptamine derivatives in illicit synthetic drugs by capillary electrophoresis and ultraviolet laser-induced fluorescence detection. Electrophoresis 26 (12):2391-2401.
77. Bishop SC, Lerch M, McCord BR (2007) Detection of nitrated benzodiazepines by indirect laser-induced fluorescence detection on a microfluidic device. J Chromatogr A 1154 (1–2):481-484.
78. Bailey CG, Wallenborg SR (2000) Indirect laser-induced fluorescence detection of explosive compounds using capillary electrochromatography and micellar electrokinetic chromatography. Electrophoresis 21 (15):3081-3087.
79. Melanson JE, Boulet CA, Lucy CA (2001) Indirect Laser-Induced Fluorescence Detection for Capillary Electrophoresis Using a Violet Diode Laser. Anal Chem 73 (8):1809-1813.
80. Wallenborg SR, Bailey CG (2000) Separation and Detection of Explosives on a Microchip Using Micellar Electrokinetic Chromatography and Indirect Laser-Induced Fluorescence. Anal Chem 72 (8):1872-1878.
81. Páez X, Hernández L (2001) Biomedical applications of capillary electrophoresis with laser-induced fluorescence detection. Biopharm Drug Disposition 22 (7-8):273-289.
82. Swinney K, Bornhop DJ (2000) Detection in capillary electrophoresis. Electrophoresis 21 (7):1239-1250.
83. Zemann AJ, Schnell E, Volgger D, Bonn GK (1998) Contactless Conductivity Detection for Capillary Electrophoresis. Anal Chem 70 (3):563-567.
84. Vio L, Crétier G, Chartier F, Geertsen V, Gourgiotis A, Isnard H, Rocca J-L (2012) Separation and analysis of lanthanides by isotachophoresis coupled with inductively coupled plasma mass spectrometry. Talanta 99 (0):586-593.
85. Schmitt-Kopplin P, Frommberger M (2003) Capillary electrophoresis - mass spectrometry: 15 years of developments and applications. Electrophoresis 24 (22-23):3837-3867.
86. Reinhoud NJ, Schroder E, Tjaden UR, Niessen WMA, de Brauw MCTN, Van der Greef J (1990) Static and scanning array detection in capillary electrophoresis-mass spectrometry. J Chromatogr A 516 (1):147-155.
87. Verheij ER, Tjaden UR, Niessen WMA, Van der Greef J (1991) Pseudo-electrochromatography-mass spectrometry: a new alternative. J Chromatogr A 554 (1-2):339-349.
88. Chang SY, Yeung ES (1997) Laser Vaporization/Ionization Interface for Capillary Electrophoresis-Time-of-Flight Mass Spectrometry. Anal Chem 69 (13):2251-2257.
89. Hirabayashi Y, Hirabayashi A, Koizumi H (1999) A sonic spray interface for capillary electrophoresis/mass spectrometry. Rapid Commun Mass Spectrom 13 (8):712-715.
90. Gusev AI (2000) Interfacing matrix-assisted laser desorption/ionization mass spectrometry with column and planar separations. Fresenius J Anal Chem 366 (6-7):691-700.
91. Musyimi Harrison K, Guy J, Narcisse Damien A, Soper Steven A, Murray Kermit K (2005) Direct coupling of polymer-based microchip electrophoresis to online MALDI-MS using a rotating ball inlet. Electrophoresis 26 (24):4703-4710.
92. Preisler J, Foret F, Karger BL (1998) On-Line MALDI-TOF MS using a continuous vacuum deposition interface. Anal Chem 70 (24):5278-5287.
93. Maxwell EJ, Chen DDY (2008) Twenty years of interface development for capillary electrophoresis–electrospray ionization–mass spectrometry. Anal Chim Acta 627 (1):25-33.

94. Whitt JT, Moini M (2003) Capillary electrophoresis to mass spectrometry interface using a porous junction. Anal Chem 75 (9):2188-2191.
95. Maxwell EJ, Zhong X, Zhang H, van Zeijl N, Chen DDY (2010) Decoupling CE and ESI for a more robust interface with MS. Electrophoresis 31 (7):1130-1137.
96. Schmitt-Kopplin P, Englmann M (2005) Capillary electrophoresis - mass spectrometry: Survey on developments and applications 2003-2004. Electrophoresis 26 (7-8):1209-1220.
97. Scriba GKE (2007) Nonaqueous capillary electrophoresis-mass spectrometry. J Chromatogr A 1159 (1-2):28-41.
98. Brocke Av, Nicholson G, Bayer E (2001) Recent advances in capillary electrophoresis/electrospray-mass spectrometry. Electrophoresis 22 (7):1251-1266.
99. Chee GL, Wan TSM (1993) Reproducible and high-speed separation of basic drugs by capillary zone electrophoresis. JChromatogr B 612 (1):172-177.
100. Unger M, Stöckigt D, Belder D, Stöckigt J (1997) General approach for the analysis of various alkaloid classes using capillary electrophoresis and capillary electrophoresis-mass spectrometry. J Chromatogr A 767 (1-2):263-276.
101. Unger M, Stöckigt J (1997) Improved detection of alkaloids in crude extracts applying capillary electrophoresis with field amplified sample injection. J Chromatogr A 791 (1-2):323-331.
102. Posch TN, Müller A, Schulz W, Pütz M, Huhn C (2012) Implementation of a design of experiments to study the influence of the background electrolyte on separation and detection in non-aqueous capillary electrophoresis–mass spectrometry. Electrophoresis 33 (4):583-598.
103. Posch TN, Martin N, Pütz M, Huhn C (2012) Nonaqueous capillary electrophoresis–mass spectrometry: A versatile, straightforward tool for the analysis of alkaloids from psychoactive plant extracts. Electrophoresis 33 (11):1557-1566.
104. Bjørnsdottir I, Hansen SH (1995) Determination of opium alkaloids in crude opium using non-aqueous capillary electrophoresis. J Pharm Biomed Anal 13 (12):1473-1481.
105. Bjørnsdottir I, Hansen SH (1997) Comparison of aqueous and non-aqueous capillary electrophoresis for quantitative determination of morphine in pharmaceuticals. J Pharm Biomed Anal 15 (8):1083-1089.
106. Bjørnsdottir I, Hansen SH (1999) Fast separation of 16 seizure drug substances using non-aqueous capillary electrophoresis. J Biochem Bioph Methods 38 (2):155-161.
107. Bjørnsdottir I, Hansen SH (1995) Determination of opium alkaloids in opium by capillary electrophoresis. J Pharm Biomed Anal 13 (4–5):687-693.
108. Kohler I, Schappler J, Rudaz S (2013) Highly sensitive capillary electrophoresis-mass spectrometry for rapid screening and accurate quantitation of drugs of abuse in urine. Anal Chim Acta 780 (0):101-109.
109. Nielsen MKK, Johansen SS, Dalsgaard PW, Linnet K (2010) Simultaneous screening and quantification of 52 common pharmaceuticals and drugs of abuse in hair using UPLC–TOF-MS. Forensic Sci Int 196 (1–3):85-92.
110. Badawi N, Simonsen KW, Steentoft A, Bernhoft IM, Linnet K (2009) Simultaneous screening and quantification of 29 drugs of abuse in oral fluid by solid-phase extraction and ultraperformance LC-MS/MS. Clin Chem 55 (11):2004-2018.
111. Dresen S, Ferreirós N, Gnann H, Zimmermann R, Weinmann W (2010) Detection and identification of 700 drugs by multi-target screening with a 3200 Q TRAP® LC-MS/MS system and library searching. Anal Bioanal Chem 396 (7):2425-2434.

112. Pyell U (2007) Theory of Electrokinetic Chromatography. In: Electrokinetic Chromatography. John Wiley & Sons, Ltd, pp 1-31.
113. Terabe S (1992) Selectivity manipulation in micellar electrokinetic chromatography. J Pharm Biomed Anal 10 (10–12):705-715.
114. Weinberger R, Lurie IS (1991) Micellar electrokinetic capillary chromatography of illicit drug substances. Anal Chem 63 (8):823-827.
115. Lurie IS, Hays PA, Garcia AE, Panicker S (2004) Use of dynamically coated capillaries for the determination of heroin, basic impurities and adulterants with capillary electrophoresis. J Chromatogr A 1034 (1-2):227-235.
116. Wen T, Zhao X, Luo G, Wang J, Wang Y, Yao B, Zhao J, Zhu J, Yu Z (2007) Comparison of microemulsion electrokinetic chromatography and solvent modified micellar electrokinetic chromatography on rapid separation of heroin, amphetamine and their basic impurities. Talanta 71 (2):854-860.
117. Lurie IS, Conver TS, Ford VL (1998) Simultaneous separation of acidic, basic, and neutral organic compounds, including strong and moderate acids and bases, by capillary electrochromatography. Anal Chem 70 (21):4563-4569.
118. Aturki Z, D'Orazio G, Fanali S, Rocco A, Bortolotti F, Gottardo R, Tagliaro F (2009) Capillary electrochromatographic separation of illicit drugs employing a cyano stationary phase. J Chromatogr A 1216 (17):3652-3659.
119. Aturki Z, D'Orazio G, Rocco A, Bortolotti F, Gottardo R, Tagliaro F, Fanali S (2010) CEC-ESI ion trap MS of multiple drugs of abuse. Electrophoresis 31 (7):1256-1263.
120. Beckers JL, Boček P (2000) Sample stacking in capillary zone electrophoresis: Principles, advantages and limitations. Electrophoresis 21 (14):2747-2767.
121. Malá Z, Křivánková L, Gebauer P, Boček P (2007) Contemporary sample stacking in CE: A sophisticated tool based on simple principles. Electrophoresis 28 (1-2):243-253.
122. Danková M, Kaniansky D, Fanali S, Iványi F (1999) Capillary zone electrophoresis separations of enantiomers present in complex ionic matrices with on-line isotachophoretic sample pretreatment. J Chromatogr A 838 (1–2):31-43.
123. Huhn C (2007) Optimierung von Probeninjektion und Trennung in der Kapillarelektrophorese, der micellaren elektrokinetischen Chromatographie und der Säulenkopplung Isotachophorese-Kapillarelektrophorese zur Analyse komplexer Proben in der forensischen Analytik. Philipps-Universität Marburg, Marburg an der Lahn
124. Albermann ME, Musshoff F, Madea B (2012) A High-Performance Liquid Chromatographic–Tandem Mass Spectrometric Method for the Determination of Ethyl Glucuronide and Ethyl Sulfate in Urine Validated According to Forensic Guidelines. J Chromatogr Sci 50 (1):51-56.
125. Himes S, Concheiro M, Scheidweiler K, Huestis M (2014) Validation of a novel method to identify in utero ethanol exposure: simultaneous meconium extraction of fatty acid ethyl esters, ethyl glucuronide, and ethyl sulfate followed by LC-MS/MS quantification. Anal Bioanal Chem 406 (7):1945-1955.
126. Cherkaoui S, Bekkouche K, Christen P, Veuthey J-L (2001) Non-aqueous capillary electrophoresis with diode array and electrospray mass spectrometric detection for the analysis of selected steroidal alkaloids in plant extracts. J Chromatogr A 922 (1-2):321-328.
127. Huhn C, Pütz M, Holthausen I, Pyell U (2008) Separation of very hydrophobic analytes by micellar electrokinetic chromatography. I. Optimization of the composition of the sample solution for the determination of the aromatic ingredients of sassafras and other essential oils of forensic interest. Electrophoresis 29 (2):526-537.

128. Poole CF, Poole SK (1997) Interphase model for retention and selectivity in micellar electrokinetic chromatography. J Chromatogr A 792 (1-2):89-104.
129. Poole SK, Poole CF (1997) Characterization of Surfactant Selectivity in Micellar Electrokinetic Chromatography. The Analyst 122:267-274.
130. Poole CF, Poole SK, Abraham MH (1998) Recommendations for the determination of selectivity in micellar electrokinetic chromatography. J Chromatogr A 798 (1–2):207-222.
131. Cherkaoui S, Mateus L, Christen P, Veuthey J (1999) Nonaqueous capillary electrophoresis for the analysis of selected tropane alkaloids in a plant extract. Chromatographia 49 (1):54-60.
132. Cherkaoui S, Varesio E, Christen P, Veuthey J-L (1998) Selectivity manipulation using nonaqueous capillary electrophoresis. Application to tropane alkaloids and amphetamine derivatives. Electrophoresis 19 (16-17):2900-2906.
133. Vindevogel J, Sandra P (1992) Introduction to micellar electrokinetic chromatography. Hüthig, Heidelberg.
134. Lucangioli SE, Hermida LG, Tripodi VP, Rodríguez VG, López EE, Rouge PD, Carducci CN (2000) Analysis of cis–trans isomers and enantiomers of sertraline by cyclodextrin-modified micellar electrokinetic chromatography. J Chromatogr A 871 (1–2):207-215.
135. Huhn C, Pütz M, Pyell U (2008) Separation of very hydrophobic analytes by MEKC. II. Carbon number equivalents as analyte descriptors – influence of the composition of the separation electrolyte. Electrophoresis 29:567-575.
136. Bjørnsdottir I, Hansen SH, Terabe S (1996) Chiral separation in non-aqueous media by capillary electrophoresis using the ion-pair principle. J Chromatogr A 745 (1-2):37-44.
137. Hansen SH, Bjørnsdottir I, Tjørnelund J (1997) Separation of cationic cis-trans (Z-E) isomers and diastereoisomers using non-aqueous capillary electrophoresis. J Chromatogr A 792 (1-2):49-55.
138. Iwata YT, Inoue H, Kuwayama K, Kanamori T, Tsujikawa K, Miyaguchi H, Kishi T (2006) Forensic application of chiral separation of amphetamine-type stimulants to impurity analysis of seized methamphetamine by capillary electrophoresis. Forensic Sci Int 161 (2-3):92-96.
139. Inoue H, Iwata YT, Kuwayama K (2008) Characterization and profiling of methamphetamine seizures. J Health Sci 54 (6):615-622.
140. Lee JS, Yang WK, Han EY, Lee SY, Park YH, Lim MA, Chung HS, Park JH (2007) Monitoring precursor chemicals of methamphetamine through enantiomer profiling. Forensic Sci Int 173 (1):68-72.
141. Fanali S (2000) Enantioselective determination by capillary electrophoresis with cyclodextrins as chiral selectors. J Chromatogr A 875 (1-2):89-122.
142. Zaugg S, Thormann W (2000) Enantioselective determination of drugs in body fluids by capillary electrophoresis. J Chromatogr A 875 (1-2):27-41.
143. Caslavska J, Thormann W (2011) Stereoselective determination of drugs and metabolites in body fluids, tissues and microsomal preparations by capillary electrophoresis (2000–2010). J Chromatogr A 1218 (4):588-601.
144. Fillet M, Servais A-C, Crommen J (2003) Effects of background electrolyte composition and addition of selectors on separation selectivity in nonaqueous capillary electrophoresis. Electrophoresis 24 (10):1499-1507.
145. Blanco M, Valverde I (2003) Choice of chiral selector for enantioseparation by capillary electrophoresis. TrAC, Trends Anal Chem 22 (7):428-439.

146. Sabbah S, Süß F, Scriba GKE (2001) pH-Dependence of complexation constants and complex mobility in capillary electrophoresis separations of dipeptide enantiomers. Electrophoresis 22 (15):3163-3170.
147. Sabbah S, Scriba GKE (2000) Influence of the structure of cyclodextrins and amino acid sequence of dipeptides and tripeptides on the pH-dependent reversal of the migration order in capillary electrophoresis. J Chromatogr A 894 (1–2):267-272.
148. Süß F, Poppitz W, de Griend CES-v, Scriba GKE (2001) Influence of the amino acid sequence and nature of the cyclodextrin on the separation of small peptide enantiomers by capillary electrophoresis using randomly substituted and single isomer sulfated and sulfonated cyclodextrins. Electrophoresis 22 (12):2416-2423.
149. Sidamonidze N, Süß F, Poppitz W, Scriba GKE (2001) Influence of the amino acid sequence and nature of the cyclodextrin on the separation of small peptide enantiomers by capillary electrophoresis using α-, β-, and γ-cyclodextrin and the corresponding hydroxypropyl derivatives. J Sep Sci 24 (9):777-783.
150. Loukas YL, Sabbah S, Scriba GKE (2001) Method development and validation for the chiral separation of peptides in the presence of cyclodextrins using capillary electrophoresis and experimental design. J Chromatogr A 931 (1–2):141-152.
151. Sabbah S, Scriba GKE (2001) Separation of dipeptide and tripeptide enantiomers in capillary electrophoresis using carboxymethyl-β-cyclodextrin and succinyl-β-cyclodextrin: Influence of the amino acid sequence, nature of the cyclodextrin and pH. Electrophoresis 22 (7):1385-1393.
152. Süß F, Kahle C, Holzgrabe U, Scriba GKE (2002) Studies on the chiral recognition of peptide enantiomers by neutral and sulfated β-cyclodextrin and heptakis-(2,3-di-O-acetyl)-β-cyclodextrin using capillary electrophoresis and nuclear magnetic resonance. Electrophoresis 23 (9):1301-1307.
153. Rudaz S, Calleri E, Geiser L, Cherkaoui S, Prat J, Veuthey J-L (2003) Infinite enantiomeric resolution of basic compounds using highly sulfated cyclodextrin as chiral selector in capillary electrophoresis. Electrophoresis 24 (15):2633-2641.
154. Iwamuro Y, Iio-Ishimaru R, Chinaka S, Takayama N, Kodama S, Hayakawa K (2010) Reproducible chiral capillary electrophoresis of methamphetamine and its related compounds using a chemically modified capillary having diol groups. Forensic Toxicology 28 (1):19-24.
155. Iio R, Chinaka S, Tanaka S, Takayamaa N, Hayakawab K (2003) Simultaneous chiral determination of methamphetamine and its metabolites in urine by capillary electrophoresis-mass spectrometry. The Analyst 128:646–650.
156. Iio R, Chinaka S, Takayama N, Hayakawa K (2005) Simultaneous chiral analysis of methamphetamine and related compounds by capillary electrophoresis/mass spectrometry using anionic cyclodextrin. Anal Sci 21 (1):15-19.
157. Iio R, Chinaka S, Takayama N, Hayakawa K (2005) Simultaneous chiral analysis of methamphetamine and its metabolites by capillary electrphoresis/mass spectrometry with direct injection of urine. J Health Sci 51 (6):693-701.
158. Meng L, Wang B, Luo F, Shen G, Wang Z, Guo M (2011) Application of dispersive liquid–liquid microextraction and CE with UV detection for the chiral separation and determination of the multiple illicit drugs on forensic samples. Forensic Sci Int 209 (1–3):42-47.
159. Mohr S, Pilaj S, Schmid MG (2012) Chiral separation of cathinone derivatives used as recreational drugs by cyclodextrin-modified capillary electrophoresis. Electrophoresis 33 (11):1624-1630.
160. Iwata YT, Kanamori T, Ohmae Y, Tsujikawa K, Inoue H, Kishi T (2003) Chiral analysis of amphetamine-type stimulants using reversed-polarity capillary

electrophoresis/positive ion electrospray ionization tandem mass spectrometry. Electrophoresis 24 (11):1770-1776.
161. Dieckmann S, Pütz M, Pyell U (2008) Enantiomeric identification of chiral drugs, adulterants and impurites by capillary electrophoresis-ESI-mass spectrometry (CE-ESI-MS). Paper presented at the XVI. Symposium der GTFCh, Mosbach, Germany
162. Cherkaoui S, Rudaz S, Varesio E, Veuthey J-L (2001) On-line capillary electrophoresis-electrospray mass spectrometry for the stereoselective analysis of drugs and metabolites. Electrophoresis 22 (15):3308-3315.
163. Schmitt T, Engelhardt H (1995) Optimization of enantiomeric separations in capillary electrophoresis by reversal of the migration order and using different derivatized cyclodextrins. J Chromatogr A 697 (1-2):561-570.
164. Jung B, Caslavska J, Thormann W (2009) Determination of ethyl glucuronide in human serum by capillary zone electrophoresis and an immunoassay. J Sep Sci 32 (20):3497-3506.
165. Caslavska J, Jung B, Thormann W (2011) Confirmation analysis of ethyl glucuronide and ethyl sulfate in human serum and urine by CZE-ESI-MSn after intake of alcoholic beverages. Electrophoresis 32 (13):1760-1764.
166. Iwata YT, Garcia A, Kanamori T, Inoue H, Kishi T, Lurie IS (2002) The use of a highly sulfated cyclodextrin for the simultaneous chiral separation of amphetamine-type stimulants by capillary electrophoresis. Electrophoresis 23 (9):1328-1334.
167. Ho Y-H, Wang C-C, Hsiao Y-T, Ko W-K, Wu S-M (2013) Analysis of ten abused drugs in urine by large volume sample stacking–sweeping capillary electrophoresis with an experimental design strategy. J Chromatogr A 1295 (0):136-141.
168. Dahlén J, von Eckardstein S (2006) Development of a capillary zone electrophoresis method including a factorial design and simplex optimisation for analysis of amphetamine, amphetamine analogues, cocaine, and heroin. Forensic Sci Int 157 (2-3):93-105.
169. Varesio E, Gauvrit JY, Longeray R, Lantéri P, Veuthey JL (1999) Optimization of fast CE analyses of ecstasy derivatives by use of experimental designs. Chromatographia 50 (3-4):195-201.
170. Tagliaro F, Antonioli C, Moretto S, Archetti S, Ghielmi S, Marigo M (1993) High-sensitivity low-cost methods for determination of cocaine in hair: high-performance liquid chromatography and capillary electrophoresis. Forensic Sci Int 63 (1–3):227-238.
171. Weinmann W, Schaefer P, Thierauf A, Schreiber A, Wurst FM (2004) Confirmatory analysis of ethylglucuronide in urine by liquid-chromatography/electrospray ionization/tandem mass spectrometry according to forensic guidelines. J Am Soc Mass 15 (2):188-193.
172. Jung B, Caslavska J, Thormann W (2008) Determination of ethyl sulfate in human serum and urine by capillary zone electrophoresis. J Chromatogr A 1206 (1):26-32.
173. Krivankova L, Caslavska J, Malaskova H, Gebauer P, Thormann W (2005) Analysis of ethyl glucuronide in human serum by capillary electrophoresis with sample self-stacking and indirect detection. J Chromatogr A 1081 (1):2-8.
174. Nováková M, Křivánková L (2008) Determination of ethyl glucuronide in human serum by hyphenation of capillary isotachophoresis and zone electrophoresis. Electrophoresis 29 (8):1694-1700.
175. Rittgen J, Pütz M, Zimmermann R (2012) Identification of fentanyl derivatives at trace levels with nonaqueous capillary electrophoresis-electrospray-tandem mass spectrometry (MSn, n = 2, 3): Analytical method and forensic applications. Electrophoresis 33 (11):1595-1605.

176. Tagliaro F, Manetto G, Crivellente F, Scarcella D, Marigo M (1998) Hair analysis for abused drugs by capillary zone electrophoresis with field-amplified sample stacking. Forensic Sci Int 92 (2-3):201-211.
177. Zhang C-X, Thormann W (1996) Head-column field-amplified sample stacking in binary system capillary electrophoresis: A robust approach providing over 1000-fold sensitivity enhancement. Anal Chem 68 (15):2523-2532.
178. Zhang C-X, Thormann W (1998) Head-column field-amplified sample stacking in binary system capillary electrophoresis. 2. Optimization with a preinjection plug and application to micellar electrokinetic chromatography. Anal Chem 70 (3):540-548.
179. Gottardo R, Bortolotti F, De Paoli G, Pascali JP, Miksik I, Tagliaro F (2007) Hair analysis for illicit drugs by using capillary zone electrophoresis-electrospray ionization-ion trap mass spectrometry. J Chromatogr A 1159 (1-2):185-189.
180. Wey AB, Zhang C-X, Thormann W (1999) Head-column field-amplified sample stacking in binary system capillary electrophoresis: Preparation of extracts for determination of opioids in microliter amounts of body fluids. J Chromatogr A 853 (1–2):95-106.
181. Guzman NA, Blanc T, Phillips TM (2008) Immunoaffinity capillary electrophoresis as a powerful strategy for the quantification of low-abundance biomarkers, drugs, and metabolites in biological matrices. Electrophoresis 29 (16):3259-3278.
182. Thormann W, Lanz M, Caslavska J, Siegenthaler P, Portmann R (1998) Screening for urinary methadone by capillary electrophoretic immunoassays and confirmation by capillary electrophoresis-mass spectrometry. Electrophoresis 19 (1):57-65.
183. Lanz M, Thormann W (1996) Characterization of the stereoselective metabolism of methadone and its primary metabolite via cyclodextrin capillary electrophoretic determination of their urinary enantiomers. Electrophoresis 17 (12):1945-1949.
184. Bao J, Regnier FE (1992) Ultramicro enzyme assays in a capillary electrophoretic system. J Chromatogr A 608 (1-2):217-224.
185. Wey AB, Caslavska J, Thormann W (2000) Analysis of codeine, dihydrocodeine and their glucuronides in human urine by electrokinetic capillary immunoassays and capillary electrophoresis-ion trap mass spectrometry. J Chromatogr A 895 (1-2):133-146.
186. Wey AB, Thormann W (2001) Capillary electrophoresis-electrospray ionization ion trap mass spectrometry for analysis and confirmation testing of morphine and related compounds in urine. J Chromatogr A 916 (1-2):225-238.
187. Bork W-R-, Perez SB, El-Khadra-Kluth N, Fritsch R, Hindorf G, Jacobsen-Bauer A, Klein B, Naujoks E, Standke U, Stein K, Westphal F, Zerell U (2012) Richtlinie zur Qualitätssicherung bei forensisch-chemischen Untersuchungen von Betäubungs- und Arzneimitteln. Toxichem Krimtech 79 (3):42.
188. Castaneda Penalvo G, Julien E, Fabre H (1996) Cross validation of capillary electrophoresis and high-performance liquid chromatography for cefotaxime and related impurities. Chromatographia 42 (3-4):159-164.
189. Sadeghipour F, Varesio E, Giroud C, Rivier L, Veuthey JL (1997) Analysis of amphetamines by capillary electrophoresis and liquid chromatography: application to drug seizures and cross-validation. Forensic Sci Int 86 (1-2):1-13.
190. Theobald DS, Pütz M, Schneider E, Maurer HH (2006) New designer drug 4-iodo-2,5-dimethoxy-beta-phenethylamine (2C-I): Studies on its metabolism and toxicological detection in rat urine using gas chromatographic/mass spectrometric and capillary electrophoretic/mass spectrometric techniques. J Mass Spectrom 41 (7):872-886.

191. Tagliaro F, Smith FP, Turrina S, Equisetto V, Marigo M (1996) Complementary use of capillary zone electrophoresis and micellar electrokinetic capillary chromatography for mutual confirmation of results in forensic drug analysis. J Chromatogr A 735 (1–2):227-235.
192. Schmitt-Kopplin P, Garmash AV, Kudryavtsev AV, Menzinger F, Perminova IV, Hertkorn N, Freitag D, Petrosyan VS, Kettrup A (2001) Quantitative and qualitative precision improvements by effective mobility-scale data transformation in capillary electrophoresis analysis. Electrophoresis 22 (1):77-87.
193. Schmitt-Kopplin P, Fekete A (2008) The CE way of thinking. In: Schmitt-Kopplin P (ed) Capillary Electrophoresis, vol 384. Methods In Molecular Biology. Humana Press, Totowa, pp 611-629.
194. Lewis AP, Cranny A, Harris NR, Green NG, Wharton JA, Wood RJK, Stokes KR (2013) Review on the development of truly portable and in-situ capillary electrophoresis systems. Meas Sci Technol 24 (4):042001.
195. Wallenborg SR, Lurie IS, Arnold DW, Bailey CG (2000) On-chip chiral and achiral separation of amphetamine and related compounds labeled with 4-fluoro-7-nitrobenzofurazane. Electrophoresis 21 (15):3257-3263.
196. Fernández-la-Villa A, Sánchez-Barragán D, Pozo-Ayuso DF, Castaño-Álvarez M (2012) Smart portable electrophoresis instrument based on multipurpose microfluidic chips with electrochemical detection. Electrophoresis 33 (17):2733-2742.
197. da Costa ET, Neves CA, Hotta GM, Vidal DTR, Barros MF, Ayon AA, Garcia CD, do Lago CL (2012) Unmanned platform for long-range remote analysis of volatile compounds in air samples. Electrophoresis 33 (17):2650-2659.
198. Blanco GA, Nai YH, Hilder EF, Shellie RA, Dicinoski GW, Haddad PR, Breadmore MC (2011) Identification of Inorganic Improvised Explosive Devices Using Sequential Injection Capillary Electrophoresis and Contactless Conductivity Detection. Anal Chem 83 (23):9068-9075.
199. Sarazin C, Delaunay N, Varenne A, Vial J, Costanza C, Eudes V, Minet J-J, Gareil P (2010) Identification and determination of inorganic anions in real extracts from pre- and post-blast residues by capillary electrophoresis. J Chromatogr A 1217 (44):6971-6978.
200. Sarazin C, Delaunay N, Costanza C, Eudes V, Gareil P (2013) On the use of capillary electrophoresis for the determination of inorganic anions and cations, and carbohydrates in residues collected after a simulated suicide bombing attack. Talanta 103 (0):301-305.
201. Kobrin E-G, Lees H, Fomitšenko M, Kubáň P, Kaljurand M (2014) Fingerprinting postblast explosive residues by portable capillary electrophoresis with contactless conductivity detection. ELECTROPHORESIS 35 (8):1165-1172.
202. Manetto G, Tagliaro F, Crivellente F, Pascali VL, Marigo M (2000) Field-amplified sample stacking - capillary zone electrophoresis applied to the analysis of opiate drugs in hair. Electrophoresis 21 (14):2891-2898.
203. Foret F, Thompson TJ, Vouros P, Karger BL, Gebauer P, Bocek P (1994) Liquid Sheath Effects on the Separation of Proteins in Capillary Electrophoresis/Electrospray Mass Spectrometry. Anal Chem 66 (24):4450-4458.
204. Huhn C, Pyell U (2008) Separation of very hydrophobic analytes by micellar electrokinetic chromatography: IV. Modeling of the effective electrophoretic mobility from carbon number equivalents and octanol-water partition coefficients. J Chromatogr A 1198-1199:208-214.
205. Reijenga JC, Verheggen TPEM, Martens JHPA, Everaerts FM (1996) Buffer capacity, ionic strength and heat dissipation in capillary electrophoresis. J Chromatogr A 744 (1-2):147-153.

206. Huhn C, Ramautar R, Wuhrer M, Somsen GW (2010) Relevance and use of capillary coatings in capillary electrophoresis–mass spectrometry. Anal Bioanal Chem 396:297-314.
207. Lurie IS, Panicker S, Hays PA, Garcia AD, Geer BL (2003) Use of dynamically coated capillaries with added cyclodextrins for the analysis of opium using capillary electrophoresis. J Chromatogr A 984 (1):109-120.
208. Lurie IS, Jason Bethea M, McKibben TD, Hays PA, Pellegrini P, Sahai R, Garcia AD, Weinberger R (2001) Use of dynamically coated capillaries for the routine analysis of methamphetamine, amphetamine, MDA, MDMA, MDEA, and cocaine using capillary electrophoresis. J Forensic Sci 46 (5):1025-1032.
209. Porras SP, Valkó IE, Jyske P, Riekkola M-L (1999) Effect of electrolyte and solvent composition on capillary electrophoretic separation of some pharmaceuticals in non-aqueous media. J Biochem Bioph Methods 38 (2):89-102.
210. Cherkaoui S, Veuthey J-L (1999) Separation of selected anesthetic drugs by nonaqueous capillary electrophoresis. Analusis 27 (9):765-771.
211. Porras SP, Riekkola M-L-, Kenndler E (2001) Capillary Zone Electrophoresis of Basic Drugs in Non-Aqueous Acetonitrile with Buffers Based on a Conventional pH Scale. Chromatographia 53:290-294.
212. Bjornsdottir I, Hansen SH (1995) Determination of opium alkaloids in crude opium using non-aqueous capillary electrophoresis. J Pharm Biomed Anal 13 (12):1473-1481.
213. Chiu C-W, Liang H-H, Huang H-Y (2007) Analyses of alkaloids in different products by NACE-MS. Electrophoresis 28 (22):4220-4226.
214. Sturm S, Seger C, Stuppner H (2007) Analysis of Central European Corydalis species by nonaqueous capillary electrophoresis-electrospray ion trap mass spectrometry. J Chromatogr A 1159 (1-2):42-50.
215. Walbroehl Y, Jorgenson JW (1984) On-column UV absorption detector for open tubular capillary zone electrophoresis. J Chromatogr A 315:135-143.
216. Kenndler E (2009) Organic solvents in CE. Electrophoresis 30 (S1):S101-S111.
217. Cherkaoui S, Rudaz S, Veuthey J-L (2001) Nonaqueous capillary electrophoresis-mass spectrometry for separation of venlafaxine and its phase I metabolites. Electrophoresis 22 (3):491-496.
218. Marsh A, Clark BJ, Altria KD (2004) Orthogonal separations of nicotine and nicotine-related alkaloids by various capillary electrophoretic modes. Electrophoresis 25 (9):1270-1278.
219. Tomlinson AJ, Benson LM, Gorrod JW, Naylor S (1994) Investigation of the in vitro metabolism of the H2-antagonist mifentidine by on-line capillary electrophoresis--mass spectrometry using non-aqueous separation conditions. J Chromatogr B 657 (2):373-381.
220. Tomlinson AJ, Benson LM, Naylor* S (1995) Advantages of nonaqueous solvents in the analysis of drug metabolites using CE and online-CE-MS. LC-GC Int 8 (210):6.
221. Tomlinson AJ, Benson LM, Johnson KL, Naylor S (1994) Investigation of drug metabolism using capillary electrophoresis with photodiode array detection and online mass spectrometry equipped with an array detector. Electrophoresis 15 (1):62-71.
222. Tomlinson AJ, Benson LM, Braddock WD, Oda RP, Naylor S (1994) On-line preconcentration-capillary electrophoresis-mass spectrometry (PC-CE-MS). J High Resolut Chromatogr 17 (10):729-731.

223. Barthe L, Ribet J-P, Pelissou M, Degude M-J, Fahy J, Duflos A (2002) Optimization of the separation of Vinca alkaloids by nonaqueous capillary electrophoresis. J Chromatogr A 968 (1-2):241-250.
224. Geiser L, Cherkaoui S, Veuthey J-L (2002) Potential of formamide and N-methylformamide in nonaqueous capillary electrophoresis coupled to electrospray ionization mass spectrometry: Application to the analysis of [beta]-blockers. J Chromatogr A 979 (1-2):389-398.
225. Drange E, Lundanes E (1997) Determination of long-chained fatty acids using non-aqueous capillary electrophoresis and indirect UV detection. J Chromatogr A 771 (1-2):301-309.
226. Zarbl E, Lämmerhofer M, Franco P, Petracs M, Lindner W (2001) Development of stereoselective nonaqueous capillary electrophoresis system for the resolution of cationic and amphoteric analytes. Electrophoresis 22 (15):3297-3307.
227. Karbaum A, Jira T (1999) Nonaqueous capillary electrophoresis: Application possibilities and suitability of various solvents for the separation of basic analytes. Electrophoresis 20 (17):3396-3401.
228. Vaher M, Koel M, Kaljurand M (2002) Application of 1-alkyl-3-methylimidazolium-based ionic liquids in non-aqueous capillary electrophoresis. J Chromatogr A 979 (1-2):27-32.
229. Carabias-Martínez R, Rodríguez-Gonzalo E, Hernández-Méndez J, Cruz EM, Domínguez-Álvarez J (2006) Ion-pair association and acid–base equilibria in nonaqueous capillary electrophoresis of weakly basic compounds. Electrophoresis 27 (2):423-432.
230. Descroix S, Varenne A, Geiser L, Cherkaoui S, Veuthey J-L, Gareil P (2003) Influence of electrolyte nature on the separation selectivity of amphetamines in nonaqueous capillary electrophoresis: Protonation degree versus ion pairing effects. Electrophoresis 24 (10):1577-1586.
231. Servais A-C, Fillet M, Chiap P, Abushoffa AM, Hubert P, Crommen J (2002) Optimization of the separation of β-blockers by ion-pair capillary electrophoresis in non-aqueous media using univariate and multivariate approaches. J Sep Sci 25 (15-17):1087-1095.
232. Porras SP, Jyske P, Riekkola M-L, Kenndler E (2001) Mobility and ionization constant of basic drugs in methanol. Application of nonaqueous background electrolytes for capillary zone electrophoresis based on a conventional pH scale. J Microcolumn Sep 13 (4):149-155.
233. Lämmerhofer M, Zarbl E, Lindner W (2000) tert.-Butylcarbamoylquinine as chiral ion-pair agent in non-aqueous enantioselective capillary electrophoresis applying the partial filling technique. J Chromatogr A 892 (1-2):509-521.
234. Porras SP, Kenndler E (2005) Capillary electrophoresis in N,N-dimethylformamide. Electrophoresis 26 (17):3279-3291.
235. Porras SP, Jussila M (2007) Electromigration of a heteroconjugated imidazole–acetate complex in ACN. Electrophoresis 28 (20):3590-3599.
236. Okada T (1997) Non-aqueous capillary electrophoretic separation of Brønsted acids as heteroconjugated anions. J Chromatogr A 771 (1-2):275-284.
237. Sarmini K, Kenndler E (1999) Ionization constants of weak acids and bases in organic solvents. J Biochem Bioph Methods 38 (2):123-137.
238. Cantu MD, Hillebrand S, Carrilho E (2005) Determination of the dissociation constants (pKa) of secondary and tertiary amines in organic media by capillary electrophoresis and their role in the electrophoretic mobility order inversion. J Chromatogr A 1068 (1):99-105.

239. Huhn C, Neusüß C, Pelzing M, Pyell U, Mannhardt J, Pütz M (2005) Capillary electrophoresis-laser induced fluorescence-electrospray ionization-mass spectrometry: A case study. Electrophoresis 26 (7-8):1389-1397.
240. Haynes WM (ed) (2011) CRC Handbook of Chemistry & Physics, Internet Edition. 91 edn. Taylor and Francis Group.
241. Anurukvorakun O, Suntornsuk W, Suntornsuk L (2006) Factorial design applied to a non-aqueous capillary electrophoresis method for the separation of [beta]-agonists. J Chromatogr A 1134 (1-2):326-332.
242. Cherkaoui S, Mateus L, Christen P, Veuthey J-L (1999) Nonaqueous versus aqueous capillary electrophoresis for the dosage of N-butylscopolamine in various pharmaceutical formulations. J Pharm Biomed Anal 21 (1):165-174.
243. Müller A, Flottmann D, Schulz W, Seitz W, Weber WH (2008) Assessment of robustness for an LC-MS-MS multi method by response-surface methodology, and its sensitivity. Anal Bioanal Chem 390 (5):1317-1326.
244. Müller A, Flottmann D, Schulz W, Seitz W, Weber W (2007) Alternative Validation of a LC-MS/MS-Multi-Method for Pesticides in Drinking Water. Clean 35 (4):329-338.
245. Pütz M, Huhn C, Dahlenburg R, Pyell U (2003) Bestimmung von beta-Carbolinalkaloiden und Tryptaminen in Ayahuasca und pflanzlichen Drogen mit MEKC-UV-LIF. Beiträge zum XIII GTFCh-Symposium 1:275-282.
246. Hagberg J (2003) Analysis of low-molecular-mass organic acids using capillary zone electrophoresis-electrospray ionization mass spectrometry. J Chromatogr A 988 (1):127-133.
247. Varesio E, Cherkaoui S, Veuthey JL (1998) Optimization of CE-ESI-MS Parameters for the Analysis of Ecstasy and Derivatives in Urine. J High Resolut Chromatogr 21 (12):653-657.
248. Sanz-Nebot V, Benavente F, Balaguer E, Barbosa J (2003) Capillary electrophoresis coupled to time of flight-mass spectrometry of therapeutic peptide hormones. Electrophoresis 24 (5):883-891.
249. Staub A, Giraud S, Saugy M, Rudaz S, Veuthey J-L, Schappler J (2010) CE-ESI-TOF/MS for human growth hormone analysis. Electrophoresis 31 (2):388-395.
250. Geiser L, Cherkaoui S, Veuthey J-L (2000) Simultaneous analysis of some amphetamine derivatives in urine by nonaqueous capillary electrophoresis coupled to electrospray ionization mass spectrometry. J Chromatogr A 895 (1-2):111-121.
251. Carrasco-Pancorbo A, Arráez-Román D, Segura-Carretero A, Fernández-Gutiérrez A (2006) Capillary electrophoresis-electrospray ionization-mass spectrometry method to determine the phenolic fraction of extra-virgin olive oil. Electrophoresis 27 (11):2182-2196.
252. Balaguer E, Neusüß C (2006) Glycoprotein Characterization Combining Intact Protein and Glycan Analysis by Capillary Electrophoresis-Electrospray Ionization-Mass Spectrometry. Anal Chem 78 (15):5384-5393.
253. Dolník V (1996) Selectivity, differential mobility and resolution as parameters to optimize capillary electrophoretic separation. J Chromatogr A 744 (1-2):115-121.
254. Leinweber FC, Otto M (1999) Systematic optimization of peak capacity for pesticide separation in micellar electrokinetic chromatography. J Chromatogr A 848 (1-2):347-363.
255. Chen J, Zhao H, Wang X, Lee FS-C, Yang H, Zheng L (2008) Analysis of major alkaloids in Rhizoma coptidis by capillary electrophoresis-electrospray-time of flight mass spectrometry with different background electrolytes. Electrophoresis 29 (10):2135-2147.

256. Peri-Okonny UL, Kenndler E, Stubbs RJ, Guzman NA (2003) Characterization of pharmaceutical drugs by a modified nonaqueous capillary electrophoresis - mass spectrometry method. Electrophoresis 24 (1-2):139-150.
257. Soga T, Ueno Y, Naraoka H, Matsuda K, Tomita M, Nishioka T (2002) Pressure-Assisted Capillary Electrophoresis Electrospray Ionization Mass Spectrometry for Analysis of Multivalent Anions. Anal Chem 74 (24):6224-6229.
258. Ruiz-Calero V, Moyano E, Puignou L, Galceran MT (2001) Pressure-assisted capillary electrophoresis-electrospray ion trap mass spectrometry for the analysis of heparin depolymerised disaccharides. J Chromatogr A 914 (1-2):277-291.
259. Peters FT, Hartung M, Herbold M, Schmitt G, Daldrup T, Mußhoff F (2009) Anforderungen an die Validierung von Analysemethoden. Richtlinie der GTFCh zur Qualitätssicherung bei forensisch-toxikologischen Untersuchungen 01:1-24.
260. Nilsson SL, Bylund D, Jörntén-Karlsson M, Petersson P, Markides KE (2004) A chemometric study of active parameters and their interaction effects in a nebulized sheath-liquid electrospray interface for capillary electrophoresis-mass spectrometry. Electrophoresis 25 (13):2100-2107.
261. Rafati AA, Bagheri A, Najafi M (2010) Experimental Data and Correlation of Surface Tensions of the Binary and Ternary Systems of Water + Acetonitrile + 2-Propanol at 298.15 K and Atmospheric Pressure. J Chem Eng Data 55 (9):4039-4043.
262. Huhn C, Pyell U (2010) Diffusion as major source of band broadening in field-amplified sample stacking under negligible electroosmotic flow velocity conditions. J Chromatogr A 1217 (26):4476-4486.
263. Haselberg R, Ratnayake CK, de Jong GJ, Somsen GW (2010) Performance of a sheathless porous tip sprayer for capillary electrophoresis-electrospray ionization-mass spectrometry of intact proteins. J Chromatogr A 1217 (48):7605-7611.
264. Douglas KT, Sharma RK, Walmsley JF, Hider RC (1983) Ionization processes of some harmala alkaloids. Mol Pharmacol 23 (3):614-618.
265. Popovych O, Tomkins RPT (1981) Nonaqueous solution chemistry. R.E. Krieger.
266. Bowser MT, Kranack AR, Chen DDY (1998) Analyte-additive interactions in nonaqueous capillary electrophoresis: a critical review. TrAC, Trends Anal Chem 17 (7):424-434.
267. Carabias-Martínez R, Rodríguez-Gonzalo E, Hernández-Méndez J, Cruz EM, Domínguez-Álvarez J (2006) Ion-pair association and acid-base equilibria in nonaqueous capillary electrophoresis of weakly basic compounds. Electrophoresis 27 (2):423-432.
268. Rittgen J, Pütz M, Zimmermann R (2011) Identification of fentanyl derivates at trace levels with non-aqueous capillary electrophoresis - electrospray - tandem mass spectrometry (MS^n, n = 2,3): Analytical method and forensic applications. Electrophoresis (submitted).
269. Thormann W, Wey AB, Lurie IS, Gerber H, Byland C, Malik N, Hochmeister M, Gehrig C (1999) Capillary electrophoresis in clinical and forensic analysis: Recent advances and breakthrough to routine applications. Electrophoresis 20 (15-16):3203-3236.
270. Rätsch C (ed) (1998) Enzyklopädie der psychoaktiven Pflanzen, vol 6. 6 edn. AT Verlag, Stuttgart.
271. Schwarz MJ, Houghton PJ, Rose S, Jenner P, Lees AD (2003) Activities of extract and constituents of Banisteriopsis caapi relevant to parkinsonism. Pharmacol Biochem Behav 75 (3):627-633.

272. Frison G, Favretto D, Zancanaro F, Fazzin G, Ferrara SD (2008) A case of β-carboline alkaloid intoxication following ingestion of Peganum harmala seed extract. Forensic Sci Int 179 (2-3):e37-e43.
273. Takayama H, Kurihara M, Kitajima M, Said IM, Aimi N (1998) New indole alkaloids from the leaves of Malaysian *Mitragyna speciosa*. Tetrahedron 54:8433-8440.
274. Cherkaoui S, Mateus L, Christen P, Veuthey J (1997) Micellar electrokinetic capillary chromatography for selected tropane alkaloid analysis in plant extract. Chromatographia 46 (7):351-357.
275. Mateus L, Cherkaoui S, Christen P, Veuthey J-L (1999) Capillary electrophoresis-diode array detection - electrospray mass spectrometry for the analysis of selected tropane alkaloids in plant extracts. Electrophoresis 20 (17):3402-3409.
276. Kursinszki L, Hank H, László I, Szőke É (2005) Simultaneous analysis of hyoscyamine, scopolamine, 6β-hydroxyhyoscyamine and apoatropine in Solanaceous hairy roots by reversed-phase high-performance liquid chromatography. J Chromatogr A 1091 (1-2):32-39.
277. Kikura-Hanajiri R, Kawamura M, Maruyama T, Kitajima M, Takayama H, Goda Y (2009) Simultaneous analysis of mitragynine, 7-hydroxymitragynine, and other alkaloids in the psychotropic plant “kratom” (<i>Mitragyna speciosa</i>) by LC-ESI-MS. Forensic Toxicology 27 (2):67-74.
278. Kartal M, Altun ML, Kurucu S (2003) HPLC method for the analysis of harmol, harmalol, harmine and harmaline in the seeds of Peganum harmala L. J Pharm Biomed Anal 31 (2):263-269.
279. Ferreres F, Llorach R, Gil-Izquierdo A (2004) Characterization of the interglycosidic linkage in di-, tri-, tetra- and pentaglycosylated flavonoids and differentiation of positional isomers by liquid chromatography/electrospray ionization tandem mass spectrometry. J Mass Spectrom 39 (3):312-321.
280. Schmidt MM, Sharma A, Schifano F, Feinmann C (2011) “Legal highs” on the net—Evaluation of UK-based Websites, products and product information. Forensic Sci Int 206 (1-3):92-97.
281. Arndt T, Claussen U, Güssregen B, Schröfel S, Stürzer B, Werle A, Wolf G (2010) "Krypton"-Konsum als Ursache eines positiven O-Desmethyltramadol-Nachweises im Urin einer Opiatabhängigen in der Entwöhnungstherapie. Toxichem Krimtech 77:1-23.
282. Kubiatowicz (2008) Danger! One prick can cause instant death. Collections: A Journal for Museum and Archives Professionals 4 (4):281-294.
283. Vepsäläinen JJ, Auriola S, Tukiainen M, Ropponen N, Callaway JC (2005) Isolation and Characterization of Yuremamine, a New Phytoindole. Planta Med 71 (EFirst):1053,1057.
284. Lo M, Matsumoto K, Iwai M, Tashima K, Kitajima M, Horie S, Takayama H (2011) Inhibitory effect of Iboga-type indole alkaloids on capsaicin-induced contraction in isolated mouse rectum. Journal of Natural Medicines 65 (1):157-165.
285. Kombian SB, Saleh TM, Fiagbe NIY, Chen X, Akabutu JJ, Buolamwini JK, Pittman QJ (1997) Ibogaine and a Total Alkaloidal Extract of Voacanga africana Modulate Neuronal Excitability and Synaptic Transmission in the Rat Parabrachial Nucleus In Vitro. Brain Res Bull 44 (5):603-610.
286. Witte L, Müller K, Arfmann HA (1987) Investigation of the Alkaloid Pattern of Datura innoxia Plants by Capillary Gas-Liquid-Chromatography-Mass Spectrometry. Planta Med 53 (02):192,197.

287. Fliniaux M-A, Manceau F, Jacquin-Dubreuil A (1993) Simultaneous analysis of l-hyoscyamine, l-scopolamine and dl-tropic acid in plant material by reversed-phase high-performance liquid chromatography. J Chromatogr A 644 (1):193-197.
288. Boros B, Farkas Á, Jakabová S, Bacskay I, Kilár F, Felinger A (2010) LC-MS Quantitative Determination of Atropine and Scopolamine in the Floral Nectar of <i>Datura</i> Species. Chromatographia 71 (0):43-49.
289. Yuan B, Zheng C, Teng H, You T (2010) Simultaneous determination of atropine, anisodamine, and scopolamine in plant extract by nonaqueous capillary electrophoresis coupled with electrochemiluminescence and electrochemistry dual detection. J Chromatogr A 1217 (1):171-174.
290. Callaway JC, Raymon LP, Hearn WL, McKenna DJ, Grob CS, Brito GS, Mash DC (1996) Quantitation of N,N-Dimethyltryptamine and Harmala Alkaloids in Human Plasma after Oral Dosing with Ayahuasca. J Anal Toxicol 20 (6):492-497.
291. Kikura-Hanajiri R, Hayashi M, Saisho K, Goda Y (2005) Simultaneous determination of nineteen hallucinogenic tryptamines/β-calbolines and phenethylamines using gas chromatography–mass spectrometry and liquid chromatography–electrospray ionisation-mass spectrometry. J Chromatogr B 825 (1):29-37.
292. Pires APS, De Oliveira CDR, Moura S, Dörr FA, Silva WAE, Yonamine M (2009) Gas chromatographic analysis of dimethyltryptamine and β-carboline alkaloids in ayahuasca, an amazonian psychoactive plant beverage. Phytochem Anal 20 (2):149-153.
293. McIlhenny EH, Pipkin KE, Standish LJ, Wechkin HA, Strassman R, Barker SA (2009) Direct analysis of psychoactive tryptamine and harmala alkaloids in the Amazonian botanical medicine ayahuasca by liquid chromatography–electrospray ionization-tandem mass spectrometry. J Chromatogr A 1216 (51):8960-8968.
294. Gambelunghe C, Aroni K, Rossi R, Moretti L, Bacci M (2008) Identification of N,N-dimethyltryptamine and β-carbolines in psychotropic ayahuasca beverage. Biomed Chromatogr 22 (10):1056-1059.
295. Stockigt J, Sheludko Y, Unger M, Gerasimenko I, Warzecha H, Stockigt D (2002) High-performance liquid chromatographic, capillary electrophoretic and capillary electrophoretic-electrospray ionisation mass spectrometric analysis of selected alkaloid groups. J Chromatogr A 967 (1):85-113.
296. Gerasimenko I, Sheludko Y, Unger M, Stöckigt J (2001) Development of an efficient system for the separation of indole alkaloids by high performance liquid chromatography and its applications. Phytochem Anal 12 (2):96-103.
297. Nolte T, Posch TN, Huhn C, Andersson JT (2012) Desulfurized Fuels from Athabasca Bitumen and Their Polycyclic Aromatic Sulfur Heterocycles. Analysis Based on Capillary Electrophoresis Coupled with TOF MS. Energy & Fuels.
298. Esseiva P, Ioset S, Anglada F, Gasté L, Ribaux O, Margot P, Gallusser A, Biedermann A, Specht Y, Ottinger E (2007) Forensic drug Intelligence: An important tool in law enforcement. Forensic Sci Int 167 (2–3):247-254.
299. Section UNIDCPS (2001) Drug Characterization/impurity Profiling: Background and Concepts; Manual for Use by National Laboratories and Drug Testing Laboratories. United Nations Publications.
300. Chawla S, Pietschmann T, Le Pichon T, Thomas J, Tullis M (2003) The opium economy in Afghanistan: An international problem. United Nations, Office on Drug and Crime, New York.
301. Gröger T, Schäffer M, Pütz M, Ahrens B, Drew K, Eschner M, Zimmermann R (2008) Application of two-dimensional gas chromatography combined with pixel-

based chemometric processing for the chemical profiling of illicit drug samples. J Chromatogr A 1200 (1):8-16.
302. Cruces-Blanco C, Airado-Rodríguez D, García-Campaña A (2013) Methods of Analysis of Opium Alkaloids. In: Ramawat KG, Mérillon J-M (eds) Natural Products. Springer Berlin Heidelberg, pp 1069-1120.
303. Wu C-Y, Siggia S, Robinson T, Waskiewicz RD (1973) Separation of cinchona alkaloids, opium alkaloids, heroin, and related narcotics by dynamic-coating high-speed liquid chromatography. Anal Chim Acta 63 (2):393-402.
304. Schwartz RS, David KO (1985) Liquid chromatography of opium alkaloids, heroin, cocaine, and related compounds using electrochemical detection. Anal Chem 57 (7):1362-1366.
305. Baker PB, Gough TA (1981) The Separation and Quantitation of the Narcotic Components of Illicit Heroin Using Reversed-Phase High Performance Liquid Chromatography. J Chromatogr Sci 19 (10):483-489.
306. Desage M, Guilluy R, Brazier JL, Chaudron H, Girard J, Cherpin H, Jumeau J (1991) Gas chromatography with mass spectrometry or isotope-ratio mass spectrometry in studying the geographical origin of heroin. Anal Chim Acta 247 (2):249-254.
307. Neumann H, Gloger M (1982) Profiling of illicit heroin samples by high-resolution capillary gas chromatography for forensic application. Chromatographia 16 (1):261-264.
308. Neumann H (1994) Comparison of heroin by capillary gas chromatography in Germany. Forensic Sci Int 69 (1):7-16.
309. Moore JM, Allen AC, Cooper DA (1984) Determination of manufacturing impurities in heroin by capillary gas chromatography with electron capture detection after derivatization with heptafluorobutyric anhydride. Anal Chem 56 (4):642-646.
310. Chiarotti M, Fucci N, Furnari C (1991) Comparative analysis of illicit heroin samples. Forensic Sci Int 50 (1):47-56.
311. Neumann H (1984) Analysis of opium and crude morphine samples by capillary gas chromatography : Comparison of impurity profiles. J Chromatogr A 315 (0):404-411.
312. Mitsui T, Hida M, Fujimura Y (1995) Determination of the total amount of morphine alkaloids in opium by pyrolysis-gas chromatography using principal component analysis. J Anal Appl Pyrolysis 32 (0):205-212.
313. Furmanec D (1974) Quantitative gas chromatographic determination of the major alkaloids in gum opium. J Chromatogr A 89 (1):76-79.
314. Simó C, García-Cañas V, Cifuentes A (2010) Chiral CE-MS. ELECTROPHORESIS 31 (9):1442-1456.
315. Akamatsu S, Mitsuhashi T (2014) MEKC–MS/MS method using a volatile surfactant for the simultaneous determination of 12 synthetic cannabinoids. J Sep Sci 37 (3):304-307.
316. Balayssac S, Retailleau E, Bertrand G, Escot M-P, Martino R, Malet-Martino M, Gilard V (2014) Characterization of heroin samples by 1H NMR and 2D DOSY 1H NMR. Forensic Sci Int 234 (0):29-38.
317. Philipp AA, Wissenbach DK, Zoerntlein SW, Klein O, Kanogsunthornrat J, Maurer HH (2009) Studies on the metabloism of mitragynine, the main alkaloid of the herbal drug Kratom, in rat and human urine using liquid chromatography-linear ion trap mass spectrometry. J Mass Spectrom 44:1249-1261.
318. Phillip AA, Wissenbach DK, Weber AA, Zapp J, Zoerntlein SW, Kanogsunthornrat j, Maurer HH (2010) Us of liquid chromatography coupled to low- and high resolution-linear ion trap mass spectrometry for studying the metabolsim of

paynantheine, an alkaloid of the herbal drug *Kratom* in rat and human urine. Anal Bioanal Chem 396:2379-2391.
319. Morales-Cid G, Cárdenas S, Simonet BM, Valcárcel M (2009) Direct automatic determination of free and total anesthetic drugs in human plasma by use of a dual (microdialysis-microextraction by packed sorbent) sample treatment coupled at-line to NACE-MS. Electrophoresis 30 (10):1684-1691.
320. Chambers MC, Maclean B, Burke R, Amodei D, Ruderman DL, Neumann S, Gatto L, Fischer B, Pratt B, Egertson J, Hoff K, Kessner D, Tasman N, Shulman N, Frewen B, Baker TA, Brusniak M-Y, Paulse C, Creasy D, Flashner L, Kani K, Moulding C, Seymour SL, Nuwaysir LM, Lefebvre B, Kuhlmann F, Roark J, Rainer P, Detlev S, Hemenway T, Huhmer A, Langridge J, Connolly B, Chadick T, Holly K, Eckels J, Deutsch EW, Moritz RL, Katz JE, Agus DB, MacCoss M, Tabb DL, Mallick P (2012) A cross-platform toolkit for mass spectrometry and proteomics. Nat Biotech 30 (10):918-920.
321. Kessler W (2006) Hauptkomponentenanalyse. In: Multivariate Datenanalyse. Wiley-VCH Verlag GmbH & Co. KGaA, pp 21-88.
322. Dane AD, Hendriks MMWB, Reijmers TH, Harms AC, Troost J, Vreeken RJ, Boomsma DI, van Duijn CM, Slagboom EP, Hankemeier T (2014) Integrating Metabolomics Profiling Measurements Across Multiple Biobanks. Anal Chem 86 (9):4110-4114.
323. Philipp AA, Wissenbach DK, Weber AA, Zapp J, Maurer HH (2010) Phase I and II metabolites of speciogynine, a diastereomer of the main Kratom alkaloid mitragynine, identified in rat and human urine by liquid chromatography coupled to low- and high-resolution linear ion trap mass spectrometry. J Mass Spectrom 45 (11):1344-1357.
324. Philipp AA, Wissenbach DK, Weber AA, Zapp J, Maurer HH (2011) Metabolism studies of the Kratom alkaloids mitraciliatine and isopaynantheine, diastereomers of the main alkaloids mitragynine and paynantheine, in rat and human urine using liquid chromatography–linear ion trap-mass spectrometry. J Chromatogr B 879 (15–16):1049-1055.
325. Philipp A, Wissenbach D, Weber A, Zapp J, Maurer H (2011) Metabolism studies of the Kratom alkaloid speciociliatine, a diastereomer of the main alkaloid mitragynine, in rat and human urine using liquid chromatography-linear ion trap mass spectrometry. Anal Bioanal Chem 399 (8):2747-2753.
326. Roscher J, Posch TN, Pütz M, Huhn C (2012) Forensic analysis of mesembrine alkaloids in Sceletium tortuosum by nonaqueous capillary electrophoresis mass spectrometry. ELECTROPHORESIS 33 (11):1567-1570.
327. Maruyama T, Kawamura M, Kikura-Hanajiri R, Takayama H, Goda Y (2009) The botanical origin of kratom (Mitragyna speciosa; Rubiaceae) available as abused drugs in the Japanese markets. Journal of Natural Medicines 63 (3):340-344.
328. Phillipson JD, Hemingway SR, Ridsdale CE (1982) The Chemotaxonomic Significance of Alkaloids in the Naucleeae S.L. (Rubiaceae). J Nat Prod 45 (2):145-162.
329. Cech NB, Enke CG (2001) Practical implications of some recent studies in electrospray ionization fundamentals. Mass Spectrom Rev 20 (6):362-387.
330. Kawamura K, Ng LL, Kaplan IR (1985) Determination of organic acids (C1-C10) in the atmosphere, motor exhausts, and engine oils. Environ Sci Technol 19 (11):1082-1086.
331. Kawamura K, Kaplan IR (1984) Capillary gas chromatography determination of volatile organic acids in rain and fog samples. Anal Chem 56 (9):1616-1620.

332. Kvitvang HFN, Andreassen T, Adam T, Villas-Bôas SG, Bruheim P (2011) Highly Sensitive GC/MS/MS Method for Quantitation of Amino and Nonamino Organic Acids. Anal Chem 83 (7):2705-2711.
333. Soga T, Igarashi K, Ito C, Mizobuchi K, Zimmermann H-P, Tomita M (2009) Metabolomic Profiling of Anionic Metabolites by Capillary Electrophoresis Mass Spectrometry. Anal Chem 81 (15):6165-6174.
334. Monton MRN, Soga T (2007) Metabolome analysis by capillary electrophoresis-mass spectrometry. J Chromatogr A 1168 (1-2):237-246.
335. Sato S, Soga T, Nishioka T, Tomita M (2004) Simultaneous determination of the main metabolites in rice leaves using capillary electrophoresis mass spectrometry and capillary electrophoresis diode array detection. The Plant Journal 40:151-163.
336. Soga T, Ohashi Y, Ueno Y, Naraoka H, Tomita M, Nishioka T (2003) Quantitative Metabolome Analysis Using Capillary Electrophoresis Mass Spectrometry. Journal of Proteome Research 2 (5):488-494.
337. Rodríguez-Gonzalo E, Domínguez-Álvarez J, García-Gómez D, García-Jiménez M-G, Carabias-Martínez R (2010) Determination of endocrine disruptors in honey by CZE-MS using restricted access materials for matrix cleanup. Electrophoresis 31 (13):2279-2288.
338. Pantůčková P, Gebauer P, Boček P, Křivánková L (2011) Recent advances in CE-MS: Synergy of wet chemistry and instrumentation innovations. Electrophoresis 32 (1):43-51.
339. Masár M, Kaniansky D, Bodor R, Jöhnck M, Stanislawski B (2001) Determination of organic acids and inorganic anions in wine by isotachophoresis on a planar chip. J Chromatogr A 916 (1–2):167-174.
340. Oefner P, Hafele R, Bartsch G, Bonn G (1990) Isotachophoretic separation of organic acids in biological fluids. J Chromatogr A 516 (1):251-262.
341. Smith RD, Loo JA, Barinaga CJ, Edmonds CD, Udseth HR (1989) Capillary zone electrophoresis and isotachophoresis-- mass spectrometry of polypeptides and proteins based upon an electrospray ionization interface. J Chromatogr A 480:211-232.
342. Udseth HR, Loo JA, Smith RD (1989) Capillary isotachophoresis/mass spectrometry. Anal Chem 61 (3):228-232.
343. Peterson ZD, Bowerbank CR, Collins DC, Graves SW, Lee ML (2003) Advantages and limitations of coupling isotachophoresis and comprehensive isotachophoresis-capillary electrophoresis to time-of-flight mass spectrometry. J Chromatogr A 992 (1-2):169-179.
344. Rubin S, Schwartz O, Bercovici M (2014) Sample distribution in peak mode isotachophoresis. Physics of Fluids (1994-present) 26 (1):-.
345. Bahga SS, Santiago JG (2013) Coupling isotachophoresis and capillary electrophoresis: a review and comparison of methods. Analyst 138 (3):735-754.
346. Malá Z, Gebauer P, Boček P (2013) Electrolyte system strategies for anionic isotachophoresis with electrospray-ionization mass-spectrometric detection. 1. Regular isotachophoresis and free-acid isotachophoresis. ELECTROPHORESIS 34 (20-21):3072-3078.
347. Gebauer P, Malá Z, Boček P (2013) Electrolyte system strategies for anionic isotachophoresis with electrospray-ionization mass-spectrometric detection. 2. Isotachophoresis in moving-boundary systems. ELECTROPHORESIS 34 (24):3245-3251.
348. Gebauer P, Malá Z, Boček P (2014) Electrolyte system strategies for anionic ITP with ESI-MS detection. 3. The ITP spacer technique in moving-boundary

systems and configurations with two self-maintained ITP subsystems. ELECTROPHORESIS 35 (5):746-754.
349. Holloway CJ, Pingoud V (1981) The analysis of amino acids and peptides by isotachophoresis. Electrophoresis 2 (3):127-134.
350. Bocek P, Gebauer P, Deml M (1981) Concept of the effective mobility of the hydrogen ion and its use in cationic isotachophoresis. J Chromatogr A 219 (1):21-28.
351. Caslavska J, Thormann W (1997) Bidirectional isotachophoresis in open-tubular, untreated fused-silica capillaries. J Chromatogr A 772 (1–2):3-17.
352. Bahga SS, Chambers RD, Santiago JG (2011) Coupled Isotachophoretic Preconcentration and Electrophoretic Separation Using Bidirectional Isotachophoresis. Anal Chem 83 (16):6154-6162.
353. Saito RM, Alves Brito-Neto JG, Lopes FS, Blanes L, da Costa ET, Rajh Vidal DT, Hotta GM, do Lago CL (2010) Ionic mobility of the solvated proton and acid-base titration in a four-compartment capillary electrophoresis system. Analytical Methods 2 (2):164-170.
354. Gileadi E, Kirowa-Eisner E (2006) Electrolytic conductivity—the hopping mechanism of the proton and beyond. Electrochim Acta 51 (27):6003-6011.
355. Izutsu K (2009) Electrochemistry in nonaqueous solutions. John Wiley & Sons.
356. Herrmann KM, Weaver LM (1999) THE SHIKIMATE PATHWAY. Annu Rev Plant Physiol Plant Mol Biol 50 (1):473-503.
357. Toya Y, Ishii N, Hirasawa T, Naba M, Hirai K, Sugawara K, Igarashi S, Shimizu K, Tomita M, Soga T (2007) Direct measurement of isotopomer of intracellular metabolites using capillary electrophoresis time-of-flight mass spectrometry for efficient metabolic flux analysis. J Chromatogr A 1159 (1-2):134-141.
358. Duke CC, MacLeod JK, Williams JF (1981) Nuclear magnetic resonance studies of d-erythrose 4-phosphate in aqueous solution. Structures of the major contributing monomeric and dimeric forms. Carbohydr Res 95 (1):1-26.

10. CURRICULUM VITAE

Curriculum Vitae	
Name	Tjorben Nils Posch
Date of birth	4th of October, 1984
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Education	
June 2014- present	Scientist at the Institute of Energy and Environmental Technology e.V.
January 2011-December 2013	PhD-Student at the Forschungszentrum Jülich in the Helmholtz-Hochschul-Nachwuchsgruppe NoStraBiS (Prof. Dr. Carolin Huhn)
March-May 2013	Research stay at the University of British Columbia, Vancouver, Canada, (Prof. Dr. D.D. Chen)
May 2010-November 2010	Master thesis at the Forschungszentrum Jülich, Helmholtz-Hochschul-Nachwuchsgruppe NoStraBiS, (Prof. Dr. Carolin Huhn)
2008-2010	Student assistant at the University of Duisburg–Essen, Instrumentelle Analytische Chemie (Prof. Dr. Torsten Schmidt)
2008-2010	Masterstudies “Water Science“, University of Duisburg-Essen
March 2008-June 2008	Bachelor thesis at the University of Waikato, Hamilton, New Zealand
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2004	Community service, Jugendhaus Schmachtendorf, Oberhausen
1997-2004	Abitur at the Freiherr-vom-Stein-Gymnasium, Oberhausen,
1995-1997	Elly-Heuss-Knapp-Gymnasium, Duisburg

10.1. LIST OF PUBLICATIONS

Posch, T.N., Pütz, M., Huhn, C. *"Electromigrative Separation Techniques in Forensic Science: Combining Selectivity, Sensitivity and Robustness"*, submitted for publication in Anal. Bioanal. Chem.

Woltmann, E., Meyer, H., Weigel, D., Pritzke, H., Posch, T. N., Kler, P., Schürmann, K., Roscher, J., Huhn, C. *"Applicability of UV laser-induced solid-state fluorescence spectroscopy for the characterization of solid dosage forms",* **2014,** *Anal. Bioanal. Chem.,* 1-16, DOI: 10.1007/s00216-014-8074-3

Beyreiss, R., Geißler, D., Ohla, S. Nagel, S., Posch, T.N., Belder, D. "*Label-Free Fluorescence Detection of Aromatic Compounds in Chip Electrophoresis Applying Two-Photon Excitation and Time-Correlated Single-Photon Counting",* **2013**, *Anal. Chem.*, 85, 8150-8157*,* DOI**:** 10.1021/ac4010937

Kler, P., Posch, T.N., Pattky M., Tiggelaar, R.M., Huhn C. "*Column coupling isotachophoresis–capillary electrophoresis with mass spectrometric detection: Characterization and optimization of microfluidic interfaces",* **2013***, J. Chromatogr. A*, 1297, 204-212**,** DOI: 10.1016/j.chroma.2013.04.046

Nolte, T., Posch, T.N., Huhn, C., Andersson, J.T. *"Desulfurized Fuels from Athabasca Bitumen and Their Polycyclic Aromatic Sulfur Heterocycles: Analysis Based on Capillary Electrophoresis Coupled with TOF MS"* **2013** *Energy Fuels*, 27, 97-107, DOI: 10.1021/ef301424d

Roscher, J., Posch, T.N., Pütz, M., Huhn, C., *"Forensic analysis of mesembrine alkaloids in sceletium tortuosum by nonaqueous capillary electrophoresis mass spectrometry",* **2012**, *Electrophoresis*, 33, 1567-1570, DOI: 10.1002/elps.201100683

Posch, T.N., Martin, N., Pütz, M., Huhn, C. "*Non-aqueous capillary electrophoresis-mass spectrometry: A versatile, straightforward tool for the analysis of alkaloids from psychoactive plant extracts",* **2012**, Electrophoresis, 33, 1557-1566, DOI: 10.1002/elps.201100682

Posch, T.N., Müller, A., Schulz, W., Pütz, M., Huhn, C. "*Implementation of a Design of Experiments to Study the Influence of the Background Electrolyte on Separation and Detection in Non-Aqueous Capillary Electrophoresis-Mass spectrometry"* **2012** *Electrophoresis*, 33, 4, 583–598*,* DOI: 10.1002/elps.201100381

Qian, Y., Posch, T., Schmidt, T.C., "*Sorption of polycyclic aromatic hydrocarbons (PAHs) on glass surfaces",* **2011***, Chemosphere* 2011, 82, 859-865, DOI: 10.1016/j.chemosphere.2010.11.002

10.2. LIST OF ORAL PRESENTATIONS

"Screening for Plant Metabolites with Capillary Electromigrative Separation Techniques"-23. Doktorandenseminar des AK Separation Science, **2013**, Hohenroda, Germany

"Non-Aqueous Capillary Electrophoresis-Mass Spectrometry as a Straightforward Tool for the Forensic Analysis of Biogenic Drugs from Mitragyna speciosa"-50th TIAFT meeting **2012**, Hamamatsu, Japan

"The Separation Mechanism and the influence of the background electrolytes on detection in non-aqueous capillary electrophoresis-mass spectrometry"-CE Forum Regensburg, **2011,** Regensburg, Germany

"Forensic Profiling of Heroin Side Compounds by capillary electrophoresis"-51st TIAFT meeting **2013**, Funchal, Portugal

10.3. LIST OF POSTER PRESENTATIONS

CIA 2013, Berlin, Germany

CE-Forum 2013, Jena, Germany

ANAKON 2013, Essen, Germany

TIAFT 2012, Hamamatsu, Japan

MSB 2012, Geneva, Switzerland

CE-Forum 2012, Aalen, Germany

Langenauer Wasserforum 2011, Langenau, Germany

International AOAC Symposium 2011, Erlangen, Germany

ITP 2011, Tiflis, Georgia

GDCh-Wissenschaftsforum 2011, Bremen, Germany

Symposium der GTFCh 2011, Mosbach, Germany

ANAKON 2011, Zürich, Switzerland

Langenauer Wasserforum 2011, Langenau, Germany

CE-Forum 2010, Jülich, Germany

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