Selectivity enhancement in capillary electrophoresis – development of a two-dimensional separation and a dual detection system

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An dieser Stelle möchte ich mich bei allen bedanken, die mich auf dem Wege zur Promotion unterstützt und begleitet haben.

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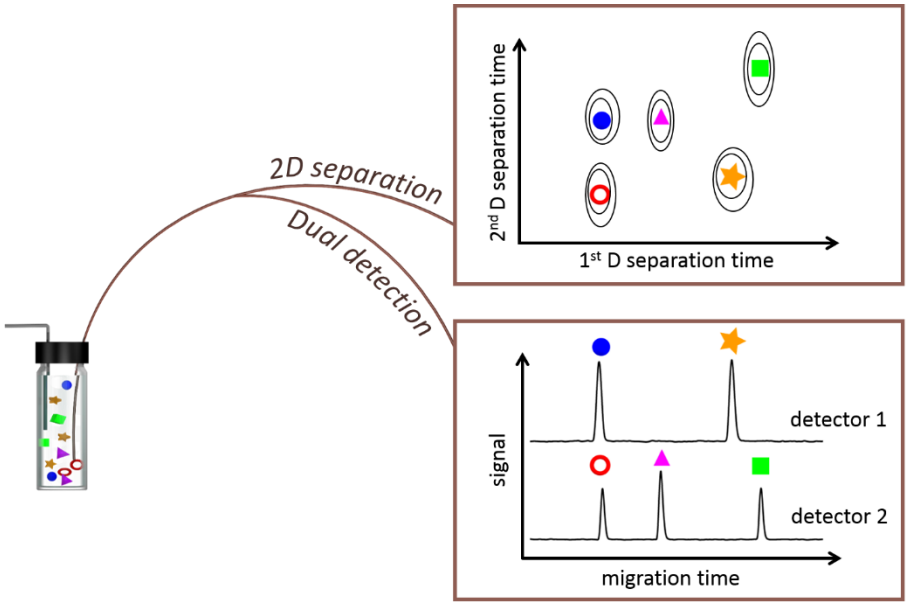
1.1. Abstract



For the identification and quantification of analytes in complex samples highly selective analytical strategies are required. The selectivity of single separation techniques such as gas chromatography (GC), liquid chromatography (LC), or capillary electrophoresis (CE) with common detection principles can be enhanced hyphenating orthogonal separation techniques but also complementary detection systems. In this review, two-dimensional systems containing CE in at least one dimension are described, namely LC-CE or 2D CE systems. Particular attention is paid to the selectivity enhancement due to the orthogonality of the different separation mechanisms. As an alternative concept, dual detection approaches are reviewed using the common detectors of CE such as UV/VIS, laser-induced fluorescence, capacitively coupled contactless conductivity (C^4D), electrochemical detection, and mass spectrometry. Special emphasis is given to dual

detection systems containing C^4D as detection concept. Selectivity enhancement is achieved due to the complementarity of the different techniques.

This chapter has been submitted as review article.

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Author contributions

AB wrote the manuscript and did the literature research. Both authors revised the manuscript. *FMM* is the corresponding author.

1.2. Introduction

Separation and determination of analytes in complex samples is a challenging task in analytical chemistry. Especially biological and environmental samples consist of a complex matrix and a variety of different analytes rendering their analytical determination difficult. Thus, high selectivity is needed to identify and quantify all components. In common analytical strategies, such samples are separated by a chromatographic or an electrophoretic technique before detection. However, using single separation techniques such as gas chromatography (GC), liquid chromatography (LC), or capillary electrophoresis (CE) the peak capacity is often too low resulting in coelution or comigration. Therefore, multidimensional separation techniques are used to enhance the peak capacity and consequently also the selectivity of the overall analytical process. Importantly, the hyphenated techniques should have a high degree of orthogonality, which means their separation mechanisms have to be as different as possible. Thus, coupling LC with CE is promising exploiting different chromatographic stationary phase materials and CE modes. As an example, reversed phase liquid chromatography (RPLC), size exclusion chromatography (SEC), and ion chromatography (IC) can be hyphenated to capillary zone electrophoresis (CZE), isoelectric focusing (IEF), micellar electrokinetic chromatography (MEKC), and isotachophoresis (ITP) offering a variety of combinations of separation mechanisms. Additionally, two-dimensional CE systems are described coupling different CE modes.

Two-dimensional separation systems can be classified as online or offline, heart-cutting or comprehensive techniques. Using online techniques, the transfer from one dimension to the other is performed automatically using an interfacing strategy in contrast to offline techniques where fractions are collected. As common terminology from GCxGC systems, a separation is only called “comprehensive” if the entire sample separated in the first dimension is transferred and separated in the second dimension [1]. The term was first introduced by Bushey and Jorgenson [2] to distinguish their two-dimensional LCxLC system from the former heart-cutting approaches as the whole sample was determined in both dimensions instead of few particular fractions. They also developed an HPLCxCE system and called it “comprehensive” even if they admitted to undersample the LC peaks generating just one electropherogram per minute [3]. Nowadays, in most

LCxCE systems the effluent of the LC is split for compatibility reasons and only fractions are introduced to the CE separation. They are nevertheless called “comprehensive” as the high frequency of the injections to the second dimension enables the analysis of the whole sample in contrast to heart-cutting approaches [3-6]. However, a second dimension sampling rate of at least three injections per peak of the first dimension is recommended [7].

Besides hyphenation of two separation techniques, dual detection is an alternative way to enhance selectivity. Coeluting or comigrating peaks can be selectively distinguished coupling complementary detectors. In CE, commonly used detectors are UV/VIS, mass spectrometry (MS), electrochemical detection, capacitively coupled contactless conductivity detection (C^4D), and laser-induced fluorescence (LIF). These detectors have to be chosen considering the structural and chemical characteristics of different classes of analytes. Using two or more of these detectors in one run means combining their power in terms of selectivity and sensitivity and compensating their weaknesses. The detectors can be connected in series or using the same detection space.

Several reviews have been published so far dealing with LC-CE and 2D CE. However, focus was put on interfacing and transfer strategies [8-12]. In the field of dual detection, two reviews have been published concentrating mainly on detection systems containing at least one electrochemical detector whereas emphasis was put on amperometric detection (AD) [13, 14]. In this work, both approaches towards selectivity enhancement, hyphenating either separation systems or complementary detectors, are reviewed. Electrophoresis in microfluidic chips is not covered. Two-dimensional LC-CE and 2D CE systems are described with focus on the selectivity enhancement due to the orthogonality of the different separation mechanisms. Especially online comprehensive approaches are described, but also some online heart-cutting techniques are mentioned. Furthermore, dual detection systems are reviewed using the common detectors of CE such as UV/VIS, LIF, C^4D, electrochemical detection, and MS with particular interest on dual detection approaches containing C^4D and MS.

1.3. Selectivity enhancement by two-dimensional separation

1.3.1. Hyphenation of LC and CE

Selectivity can be enhanced by the hyphenation of two separation techniques. Favorable are separation mechanisms with high orthogonality. Therefore, combination of LC and CE is considered to be very effective. Different stationary phases of the chromatographic system and different CE modes can be chosen changing the selectivity of the system. Possible combinations of methods are summarized in Table 1 considering their way of enhancing the selectivity.

Table 1: *Hyphenation of LC and CE. Overview of possible methods and their applications considering the way of selectivity enhancement.*

Method	Detector	Strategy of selectivity enhancement	Sample	Interface	Ref
RPLCxCZE	LIF	Peak capacity	Peptide standards and fluorescently labeled peptides from a tryptic digest of ovalbumin	Valve-based	[3]
	LIF	Peak capacity	Tryptic digest of horse-heart cytochrome c	Valve-based/ optically gated	[15, 16]
	ESI-MS	Peak capacity	Separation of a glycosylated peptide mixture	Transverse flow gated	[17]
	ESI-FTICR-MS	Peak capacity	Tryptic digested bovine serum albumin and human cerebrospinal fluid	Transverse flow gated	[18]
	LIF	Immunoassay	Determination of glucagon secretion from single islets of Lagerhans and their cross-reactive species	flow gated	[19]
	LIF	Peak capacity	Determination of neuropeptide Y	flow gated	[20]
	LIF	Competitive affinity probe assay	Selective determination of Fyn Src homology 2 domain	flow gated	[21]

	MALDI-TOF-TOF-MS	Peak capacity	Identification of more than 200 proteins analyzing tryptic digests of proteins in D_{20} liver cells	Hydrodynamic interface	[22]
	UV	Peak capacity	Determination of tryptic digests of proteins in human urine	Droplet-based	[23, 24]
SECxCZE	UV	Peak capacity	Separation of a standard protein mixture	Valve-based/ Transverse flow gated	[25, 26]
SEC-RPLCxCZE	LIF	Peak capacity (factor 5)	Separation of ovalbumin digest	Valve-based/ Transverse flow gated	[27]
ICxCZE	MS	Peak capacity	Separation of nucleotides and cyclic nucleotides	Modulation interface	[4]
	C^4D	Peak capacity	Separation of inorganic anions and haloacetic acids	Valve-based	[6]
IEFxRPLC	MS	Identification of proteins	*Drosophila* proteomics, identification of yeast soluble proteins	Valve-based, hydrodynamic	[28, 29]
	MS	Peak capacity	Selective detection of the test protein bovine carbonic anhydrase II in a complex protein mixture	Membrane-based	[30-32]
	MS	Identification of proteins, online digestion	Identification of Escherichia coli proteins	Membrane-based	[33]
GFC[1]xIEF	Column absorption imaging	Peak capacity	Separation of albumin and myoglobin	Membrane-based	[34]
RPLCxMEKC	UV	Peak capacity	Separation of a complex mixture of neutral components in medicine	Transverse flow gated	[35]

[1]GFC – gel filtration chromatography

1.3.1.1. Hyphenation of LC and CZE

RPLCxCZE

A common mode in CE is CZE based on electrophoretic mobility as separation mechanism. It is most often coupled to RPLC separations based on hydrophobicity using different interfacing strategies. RPLC and CZE were first coupled comprehensively by Bushey and Jorgenson [3] in 1990. They used a computer-controlled six-port valve to transfer the effluent of the LC to the CE system by switching the valve once every minute (see Figure 1).

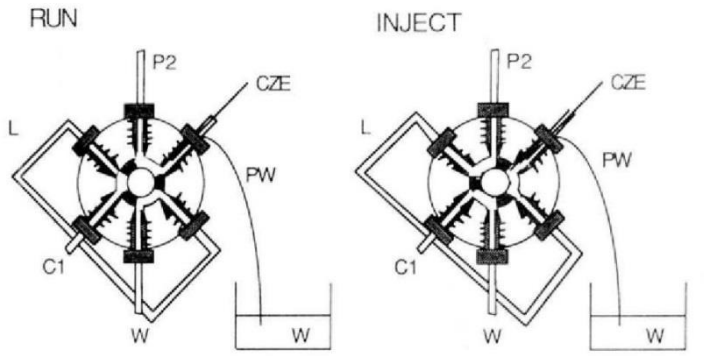

Figure 1: *Two configurations of a six-port, computer-controlled valve transferring the effluent of the RPLC (C1) to the CZE. The effluent was filled in a sample loop (L) and then flushed by a second pump (P2) over a grounded end of the CE separation capillary (CZE) and was injected electrokinetically. PW is paper wick. W is waste [3]. Figure reprinted with permission from M.M. Bushey, J.W. Jorgenson, Automated instrumentation for comprehensive two-dimensional high-performance liquid chromatography/capillary zone electrophoresis, Anal. Chem., 62 (1990) 978-984. Copyright 1990 American Chemical Society.*

The effluent, which was filled in a sample loop, was then flushed by a second pump over a grounded end of the CE separation capillary and was injected electrokinetically once per minute. The overflow went to waste. The system was used for the separation of peptide standards and fluorescently labeled peptides from a tryptic digest of ovalbumin. Even if a low LC flow rate (10 µL/min) was chosen to slow down the elution of the analytes of the first dimension, they still undersampled the LC effluent for the separation in the second dimension as the sampling frequency was too low. To enhance the sampling rate, the approach was further developed by Larmann et al. [15] replacing the six-port valve by an 8-port valve. Instead of one sample loop, two sample loops were used contra-rotating. Thus, while one sample loop was filled by the LC effluent, the content of the second sample loop was transferred to the CZE system. Again every overflow went to waste. The setup was used for the separation of tryptic digest of horse-

heart cytochrome c. As no analyte was migrating between 0 and 7.5 seconds but between 7.5 and 15 seconds, they injected overlapping, which means that always two injection plugs were separated simultaneously in the capillary. Thus, they were able to perform a CE injection every 7.5 seconds. As detector LIF was used for both approaches.

More common than valve-based approaches are gated interfaces for the coupling of RPLC and CZE. Optical and flow gated interfaces were described [15-21]. The concept of an optically gated interface is based on an “on-column” CE injection technique called optical-gating [36]. A laser beam (95% of the total laser power) photolyzed the analytes that entered the capillary. For injection, the laser beam was blocked for a short period of time. The fluorescent analytes were then separated and detected by LIF using a second laser beam (5% of the total laser power). For hyphenation to LC, the LC effluent was guided into a grounded tee connector, which split the sample. The other sides of the tee piece were connected to the waste and to a CE capillary with optical-gating, respectively [15, 16]. The use of this optical gating approach enabled fast separations due to the use of short capillaries. Separations in less than 10 minutes with CE migration times of 2.5 seconds were achieved for a sample containing fluorescein isothiocyanate-tagged tryptic digests of horse heart cytochrome c.

A major drawback of optical-gating is the limited applicability to non-fluorescent analytes. Flow gated interfaces constitute a universal alternative enabling also the coupling to other detection concepts such as MS. The concept of transverse flow gated interfacing is depicted in Figure 2 [17]. The effluent of the LC was guided into the interface by a transfer capillary, which was axially aligned to the CE separation capillary in a distance of about 70 µm. The CZE buffer was flushed vertically through the interface hindering an injection of the LC effluent into the second dimension. To inject sample into the CE capillary the flow was interrupted and an injection voltage was applied to the inlet side. Using this interface, a glycosylated peptide mixture was separated in less than 15 min by LC×CE-electrospray ionization (ESI)-MS injecting into the CE with a sampling rate of one injection every 15 seconds [17].

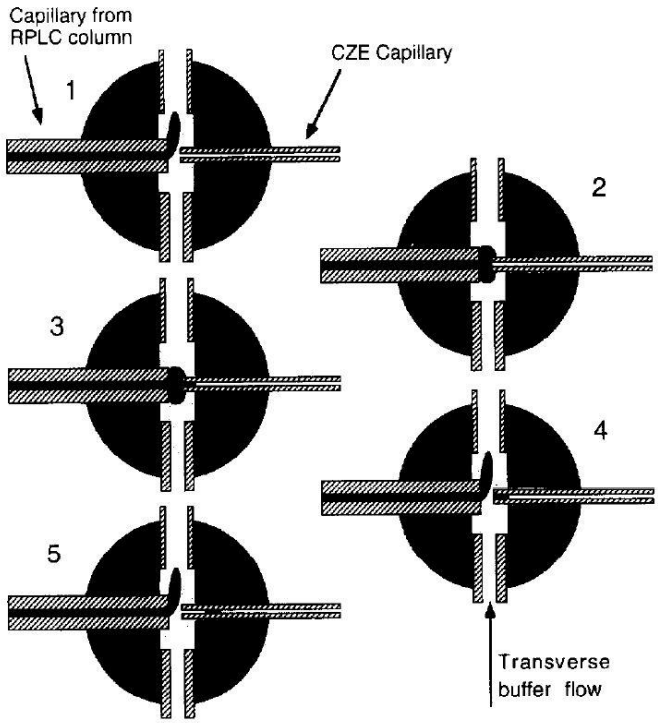

Figure 2: *Scheme of the injection principle of a flow gated interface: (1) CZE mode; flush flow on and high voltage on. (2) Preinjection mode; flush flow off and CZE voltage off. Sample diffuses across the gap. (3) Injection mode; flush flow off and CZE injection voltage on. Sample is injected electrokinetically. (4) Postinjection mode; flush flow on and CZE voltage off. Not-injected sample is flushed away. (5) CZE mode; flush flow on and CZE voltage on. CE separation takes place [17]. Figure reprinted with permission from K.C. Lewis, G.J. Opiteck, J.W. Jorgenson, D.M. Sheeley, Comprehensive on-line RPLC-CZE-MS of peptides, J. Am. Soc. Mass Spectrom., 8 (1997) 495-500. Copyright 1997 Springer Nature.*

A variation of this interface was developed by Bergström et al. [18]. In this approach the LC was coupled to the CE at a right angle. The interface was fabricated in a PDMS plate having two channels crossed at slightly different height levels, which minimized the contact area in between. The CE chamber was pressurized avoiding a leaking of the LC effluent into the CE separation channel. Injection was performed by the reduction of the pressure. High voltage for CE was applied in the pressurized chamber outside the interface minimizing the risk of bubble formation. The system was applied to samples with complex biological matrices such as tryptic digested bovine serum albumin and human cerebrospinal fluid. Detection was performed by electrospray ionization Fourier transform ion cyclotron resonance MS.

Highly selective quantitative determinations of biological macromolecules were also performed implementing an immunoassay in the two-dimensional RPLCxCZE system. After separation with RPLC the effluent was mixed on-column with the antibody reagent

via a mixing cross. Further, the sample was injected to CZE using a flow gated interface [19-21]. The preseparation with RPLC enabled the differentiation of multiple species cross-reacting with the antibody. The applicability was demonstrated using a competitive assay for glucagon with fluorescently labeled glucagon and antiglucagon as assay reagent [19]. Highly frequent injections into the CE system were performed every 1.5 seconds enabling an almost continuous monitoring of the RPLC separation. Glucagon secretion from single islets of Lagerhans and their cross-reactive species could be determined selectively at low-picomolar concentrations. The technique was further applied to a competitive immunoassay for neuropeptide Y utilizing polyclonal antisera as the immunoreagent and fluorescein-labeled neuropeptide Y as tracer [20]. A similar setup was used for a competitive affinity probe assay screening mixtures for compounds inhibiting protein-ligand interactions [21]. Mixtures containing potential binding inhibitors for a specific protein-ligand interaction were separated by RPLC. The effluent was mixed online with the protein and its selective fluorescently-labeled binding partner as affinity probe. Inhibiting compounds led to a decrease in complex formation, which was detected by LIF. As model system the Fyn Src homology 2 domain and its selective phosphopeptide binding partner was used generating electropherograms in 6 seconds intervals [21].

RPLC and CZE were further coupled using an interface called “hydrodynamic interface” [22]. The CE separation capillary was fixed on a slide bar, the inlet end immersed in a buffer vial. The LC effluent accumulated as a droplet at the LC outlet fixed in 8 cm height distance. For injection the CE capillary was immersed into the droplet moving the slide bar up leading to hydrodynamic injection due to gravity flow. Tryptic digests of proteins in D_{20} liver cancer tissue were analyzed by RPLCxCE - matrix-assisted laser desorption/ionization - time-of-flight - time-of-flight - mass spectrometry (MALDI-TOF-TOF-MS) identifying over 200 proteins in 1.5 hours. Transfer from the separation system to the detector was performed using a CE-MALDI interface directly depositing the effluent on a MALDI target at a 3 seconds time-interval.

Also droplet-based interfaces were described. In a microfluidic device, an oil as immiscible carrier fluid was added online to the LC effluent leading to spontaneous formation of nL droplets segmenting the separated analytes avoiding diffusion and remixing of already separated peaks. These droplets were then injected into the CE after

oil filtering and droplet merging [23, 24]. The interface was applied analyzing human urine [24].

SECxCZE

Besides RPLC, also SEC was used for comprehensive hyphenation separating analytes based on their size. Interfacing was again performed using valve-based and flow gated strategies. In first approaches actuated valves with 300 to 500 nL collection loops were used [15, 25]. Orthogonality of the two techniques was shown as neither SEC nor CZE could resolve a standard protein mixture in a single dimension [15]. Lemmo and Jorgenson [26] also coupled SEC to CZE comparing a valve-based and a transverse flow-gating interface. As there was no fixed sampling volume for the flow gated interface, lower flow rates could be used improving SEC resolution. The overall sensitivity could be increased 8-fold. To enhance the selectivity further, even a 3D coupling of SEC with an RPLCxCE setup was performed using a valve-based interface [27]. Thus, orthogonal separation was performed by size, hydrophobicity, and electrophoretic mobility. The addition of the third dimension led to an increase of the peak capacity by a factor 5 compared to the 2D setup. However, high sample concentrations had to be used to overcome dilution effects caused by the interface.

ICxCZE

Hyphenating IC and CE, the two most important instrumental techniques in ion analysis are combined. While the separation mechanism of IC is based on the affinity to an ion exchange column, the mechanism of CZE is dependent on electrophoretic mobility. Thus, their coupling is promising in terms of enhancement of peak capacity and selectivity. The first heart-cut combination of IC and CZE was reported by Kar and Dasgupta [37] in 1996. Almost two decades later, the first comprehensive approaches were described [4, 6]. Two major developments were essential for this coupling. The introduction of IC in capillary scale enabled the use of low flow rates down to 2-10 µL/min enhancing the compatibility to CE. The effluent of the IC usually contains high salt concentrations, which disturbs the electrophoretic separation. This drawback was overcome by the development of miniaturized ion suppressors enhancing the detection sensitivity due to the generation of pure water as effluent. An advantage of this (in an ideal case) matrix-free effluent is the decrease of any interferences in CE and the effect of sample stacking due to a lower conductivity compared to the background

electrolyte (BGE) of the CE. Ranjbar et al. [6] used a non-focusing valve-based approach. The IC was connected to a six-port valve without sample loop avoiding any loop-related peak broadening. Thus, the effluent flew directly to the waste between the injections. Switching the valve, the effluent was directed to a tee piece splitting the flow and injected into the CE capillary. The system was applied analyzing a water sample containing inorganic anions and haloacetic acids. Beutner et al. [4] developed a modulator based on a concept called capillary batch injection [38] to inject the IC effluent in the CE system. The IC effluent was directed into a modulator filled with BGE serving as CE inlet vial by a transfer capillary, which was axially aligned in close distance (<50 μm) to the CE separation capillary. The expelled effluent formed a sample cloud, which was then hydrodynamically injected into the CE capillary due to gravity flow and suction pressure of the ESI source of the MS detector. The transfer capillary was moved up and down periodically for sequential injection. Fast CE separations enabled a high sampling rate of the first dimension effluent. A model system containing nucleotides and cyclic nucleotides was chosen to show complementary separation.

1.3.1.2. Hyphenation of IEF and LC

IEFxRPLC

IEF was coupled to RPLC. The separation mechanism of IEF is based on the differences in isoelectric points. IEF is also used as first step in 2D polyacrylamide gel electrophoresis, which is widely used for protein separation, however, with limited throughput and sensitivity. Thus, the combination of IEF and RPLC as two orthogonal techniques is a promising online approach for proteomics overcoming these drawbacks and facilitating the hyphenation to MS. Chen et al. [28, 29] described a valve-based interface for the online integration of capillary IEF with capillary RPLC. In a first work, after completion of the IEF, the analytes were sequentially and hydrodynamically transferred into the RPLC until the entire sample was separated in both dimensions using a valve with internal injection loop. The system was applied for the analysis of *Drosophila* proteomics during steroid-induced programmed cell death [28]. The work was further expanded including a stacking step of the segments before the injection into the RPLC. Several trap columns were used to minimize peptide dilution and mixing during the transfer between the two dimensions and to avoid interferences of the

ampholyte (Figure 3) [29]. With this system, a larger number of yeast soluble proteins could be identified than with other methods already presented in literature with a protein loading of only 9.6 µg. Several further applications of the system to proteome analysis were published [39, 40].

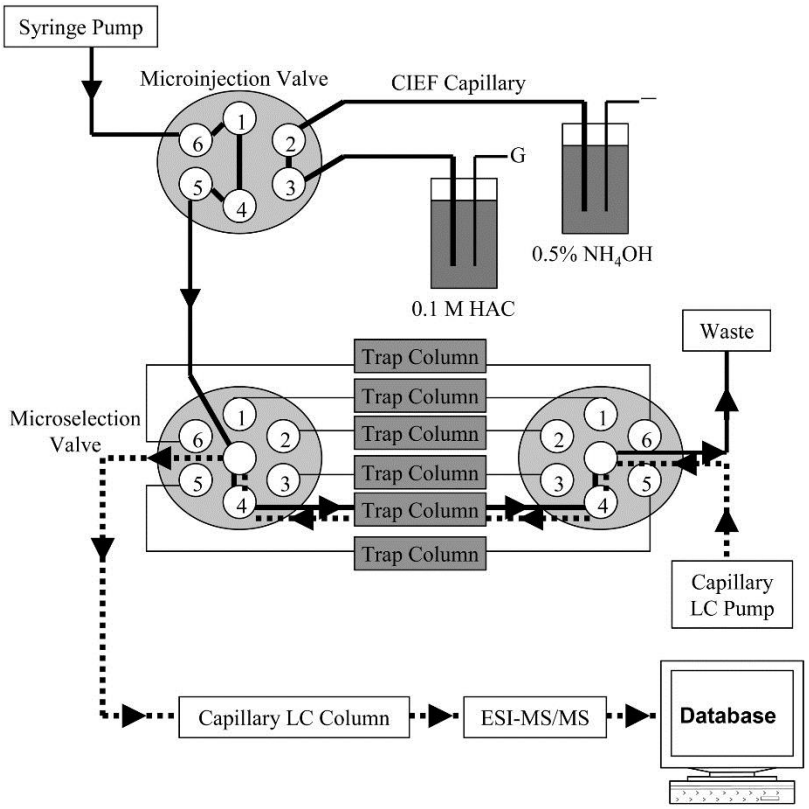

Figure 3: *Scheme of on-line integration of IEF with RPLC as a concentrating and multidimensional separation platform. Solid and dashed lines represent the flow paths for the loading of IEF fractions and the injection of fractions into a RPLC column, respectively [29]. Figure reprinted with permission from J. Chen, B.M. Balgley, D.L. DeVoe, C.S. Lee, Capillary isoelectric focusing-based multidimensional concentration/separation platform for proteome analysis, Anal. Chem., 75 (2003) 3145-3152. Copyright 2003 American Chemical Society.*

A similar approach was described by Zhou et al. [30] using a microdialysis membrane separating the IEF cathodic cell (grounded) from the separation capillary. Contrary to the catholyte, the proteins were not able to traverse the membrane. After focusing, the proteins were pushed hydrodynamically to a microselection valve collecting the fractions. This setup enabled the maintenance of a linear pH gradient in the separation capillary during the entire two-dimensional analysis as no voltage was applied on the microselection valve. The fractions collected in the valve were further trapped on a column, washed to remove the ampholyte, and injected into the RPLC-MS system using a second valve [30]. In further works, storage loops were implemented between the two

valves enabling the sampling of a greater number of fractions and the optimization of the RPLC [31]. The addition of a second sampling loop enabled the collection of IEF fractions without any sample loss [32]. The system was very selective being able to detect 100 pg (3 fmol) of bovine carbonic anhydrase II as test protein spiked into a protein complex mixture detecting around 1200 individual protein masses [32].

Further, Wang et al [33] inserted a trypsin immobilized enzyme microreactor after the IEF step digesting the focused proteins online. The digests were then trapped and desalted on two parallel trap columns and injected to RPLC-MS. The setup was based on two hollow fiber membrane interfaces. The first one was used to supply catholyte and electric contact similar to the approaches already described in this chapter. The second one was implemented right before the microreactor for the adjustment of buffer improving compatibility between protein separation and digestion. The setup enabled the identification of 101 proteins extracted from *Escherichia coli*.

Gel-filtration chromatography (GFC)xIEF

Protein analysis was also done with gel-filtration chromatography (GFC) coupled to IEF bringing the 2D polyacrylamide gel electrophoresis into a capillary format [34]. Contrary to the IEF-RPLC approaches, IEF was used as second dimension. Interfacing was performed using a microdialysis hollow fiber membrane used for desalting and carrier ampholyte mixing. After separation by GFC the effluent was split by a tee piece and injected via an 8-port injection valve into the IEF capillary passing the microdialysis interface. Detection was performed by column absorption imaging. Feasibility of the approach was shown by separation of a model mixture containing albumin and myoglobin.

1.3.1.3. Hyphenation of other LC and CE principles

RPLCxMEKC (neutral analytes)

Another CE mode is called micellar electrokinetic chromatography (MEKC). The separation mechanism is based on the partition between the hydrophobic and the hydrophilic compartments of a micelle. Thus, neutral analytes can be separated based on differences in their hydrophobicity. RPLC was hyphenated to MEKC for comprehensive two-dimensional separation of neutral species [35]. Fast MEKC separations in the second dimension were achieved by speeding up the electroosmotic

flow (EOF). A transverse flow gating interface was used for connection of the two dimensions. Separation of a complex mixture of neutral components in traditional Chinese medicines was performed.

GFC - sub-micellar SDS array CE

Skinner et al. [41] coupled GFC with sub-micellar SDS array CE for the separation of serum. An interface was fabricated into a Plexiglas plate consisting of two BGE channels and one sample channel accommodating two separation capillaries. The two capillaries were further connected to servo actuated arms enabling the retraction from the sample channel into the BGE channel to perform separation. For injection the capillaries were moved back into the sample channel. While one capillary was in injection position, the second one was immersed in the BGE channel performing separation. Thus, sampling frequency could be enhanced using two capillaries.

1.3.2. Two-dimensional CE

Besides LC coupled to CE, also two electrophoretic methods can be coupled to enhance selectivity. This is achieved by using electrophoretic methods based on different separation mechanisms such as CZE, MEKC, IEF, or ITP. Further, also 2D CZE couplings were presented using two different BGEs. There are several reviews dealing with interfacing strategies [9, 11, 42]. The challenge is the separation and isolation of the two electric circuits. In this chapter, two-dimensional approaches are classified by their separation selectivity. Only online approaches are reported with emphasis on comprehensive strategies. In Table 2, possible combinations of methods are summarized. The way of enhancing the selectivity is considered.

Table 2: *Overview of possible methods of two dimensional capillary electrophoresis and their applications considering the way of selectivity enhancement.*

Method	Detector	Selectivity enhancement	Sample	Interface	Ref
CZE-CZE	MS	Compatibility with MS	Characterization of monoclonal antibody charge variants	Valve-based	[43, 44]
CZExCZE	MS	Identification of proteins via online digestion using a microreactor	Separation of myoglobin and cytochrome c	Flow gated	[45, 46]
	LIF, MS	Online enzymatic modification	Determination of the phosphorylation status and the stoichiometry of peptides	Flow gated	[47, 48]
CZExMEKC	UV	Peak capacity	Separation of a tryptic digest of bovine serum albumin	Interface-less	[49]
	UV	Peak capacity	Determination of cationic β-blocking drugs in wastewater	Tee union interface	[50]
CZExµFFE	LIF	Peak capacity	Separation of a bovine serum albumin tryptic digest	Interface-less	[51]
cIEFxCZE	ESI-MS	Peak capacity ($P_{th.}$= 1600[1])	Separation of standard proteins, proteome analysis of the bacterium *Shewanella oneidensis*	Membrane-based	[52, 53]
	UV	Peak capacity	Separation of protein mixtures of standard proteins	Porous junction	[54, 55]
ITP-CZE	DAD	Sample preparation, preseparation	Determination of quinine and serotonin in human urine	Column coupling	[56, 57]
	MS	Sample preparation, preseparation	Determination of four human angiotensin peptides, Analysis of proteinogenic amino acids	Microfluidic interface	[58, 59]
CSExMEKC	LIF	Peak capacity	Separation of proteins and characterization of single cells	Flow gated	[60-62]
IEFxCEC	UV	Peak capacity ($P_{th.}$= 54,320[1], 168 h)	Determination of human serum proteins and standard proteins	Valve-based	[63]
	UV	Peak capacity ($P_{th.}$= 24,000[1])	Separation of bovine serum albumin digest	Valve-based	[64]
CZExGE	LIF	Peak capacity	Separation of peptide mixtures, a tryptic	Interface-less	[65]

			digest of trypsinogen, and <0.05% of an individual B2 neuron from *Aplysia californica*		
IEFxGE	LIF	Peak capacity	Separation of protein mixtures	Chip based array interface	[66]

[1]P_{th} is the maximum theoretically calculated peak capacity of the system and depends on the measurement time.

1.3.2.1. 2D CE systems using CZE for both dimensions

The general separation mode of CE is CZE separating analytes based on their electromigrative behavior. Usually different mechanisms are coupled to achieve higher orthogonality. But also two-dimensional CZE-CZE systems are known using different BGEs at different pH values. Most approaches reported are heart-cutting systems. The Neusüß group [43, 44] developed an electrically isolated valve-based interface for online 2D CZE-CZE separation with ESI-MS detection. In the first dimension any ESI incompatible BGE could be used. Some regions of interest were cut by the internal sample loop and injected into the second dimension switching the valve. In the second dimension, the BGE was MS compatible enabling the detection and selective identification of the analytes [43]. The system was used for the characterization of monoclonal antibody charge variants. They were separated using a BGE, which was not compatible with ESI. The second dimension was used to separate the interfering components from the analytes of interest enabling identification by ESI-MS [44].

In another approach, a microreactor was inserted at the distal end of the first dimension capillary [45, 46]. Proteins were separated in first dimension until the fastest compound migrated around 75% of the capillary. Then separation was stopped applying the same potential at the inlet and outlet vial of the protein separation capillary. Segments of the first dimension were sequentially moved in the microreactor altering the applied high voltages, digested and transferred to the second dimension via a flow gated interface separating the digests [45]. A scheme of the setup is depicted in Figure 4. A replaceable microreactor was constructed consisting of trypsin-modified magnetic beads which were hold in place by magnets fixed at the outside of the capillary [46].

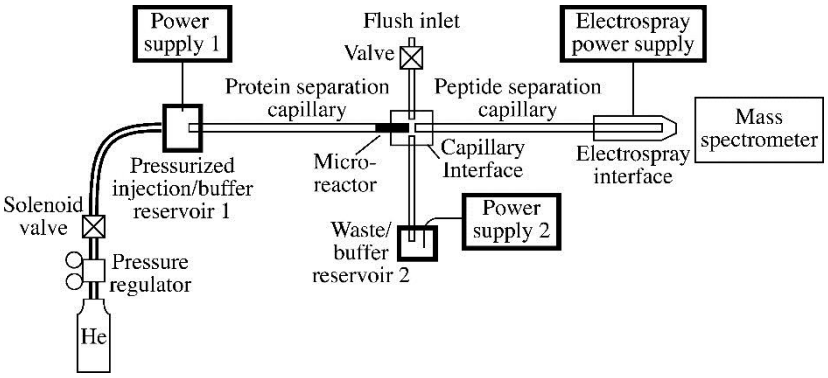

Figure 4: *Scheme of a two-dimensional CZExCZE setup with a microreactor between the two dimensions [45]. Figure reprinted with permission from R.M. Schoenherr, M. Ye, M. Vannatta, N.J. Dovichi, CE-Microreactor-CE-MS/MS for Protein Analysis, Anal. Chem., 79 (2007) 2230-2238. Copyright 2007 American Chemical Society.*

A similar setup was applied for diagonal CE using the same separation conditions in both capillaries. Thus, analytes that were not modified by an incorporated enzyme-based microreactor had the same migration times in both dimensions and were on a diagonal in a two-dimensional electropherogram. Analytes differing from the diagonal were enzymatically modified in the reactor. Thus, using immobilized alkaline phosphatase in the microreactor the phosphorylation status and stoichiometry of peptides could be monitored [47, 48].

1.3.2.2. 2D CE systems using CZE in one dimension

CZExMEKC

Enhancing the orthogonality, different CE modes depending on different separation mechanisms were combined. CZE was for example coupled to MEKC separating a tryptic digest of bovine serum albumin using a borate buffer in the first CZE dimension and a borate buffer containing sodium dodecyl sulfate in the second MEKC dimension. An interface-less fused silica system was described connecting two capillaries comprehensively (Figure 5) [49]. The two capillaries were connected by a small circular cross-section as they were not crossing each other in the same plane. Thus, they could be filled with separation media differing in concentration or pH. High voltage was applied on the first capillary inlet (Figure 5, 1) for injection and separation in the first dimension, while the outlet (Figure 5, 2) was grounded. For transferring segments into the second dimension, voltage was applied on the first capillary inlet (Figure 5, 1), but the outlet of the second capillary (Figure 5, 4) was grounded. Then HV was applied on the inlet of the second capillary (Figure 5, 3) for second dimension separation. Transfer

and separation in the second dimension was repeated periodically for a comprehensive separation.

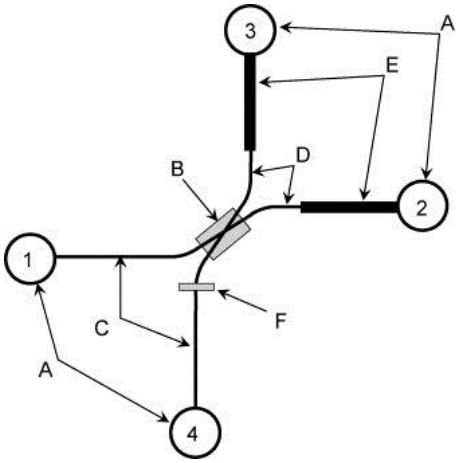

Figure 5: *Interface-less two dimensional capillary electrophoresis system. The two capillaries were connected by a small circular cross-section not crossing each other in the same plane. (A) reservoirs, (B) capillary connection, (C) fused silica capillaries (25 cm×75 µm inner diameter), (D) fused silica capillaries (5 cm×75 µm inner diameter), (E) Polyether ether ketone tubing (15 cm×0.8 mm inner diameter) and (F) detector [49]. Figure reprinted with permission from E. Sahlin, Two-dimensional capillary electrophoresis using tangentially connected capillaries, J. Chromatogr. A, 1154 (2007) 454-459. Copyright 2007 Elsevier.*

Zhang et al. [50] coupled CZE with cyclodextrin modified MEKC for the determination of cationic β-blocking drugs in wastewater. In the first dimension also a preconcentration step by cation-selective exhaustive injection and transient ITP was performed. The capillaries were coaxially aligned and coupled by a tee union interface.

CZE x micro free flow electrophoresis (µFFE)

Continuous micro free flow electrophoresis was recently coupled to CZE inserting the CE capillary directly into the µFFE separation channel by an edge on interface (Figure 6) [51]. After separation in the first dimension, the analyte peak leaving the capillary migrated directly into the µFFE separation channel without complicated fractionation strategies. In the second dimension they were deflected laterally based on their mobility in the perpendicular electric field. The migration time towards the detection zone is determined by the first dimension separation, the position of an analyte peak crossing the LIF detection zone by the second dimension µFFE separation. Peak capacities around thousand peaks per minute (1.8 min separation window) could be achieved separating fluorescently labeled small molecule bioamines.

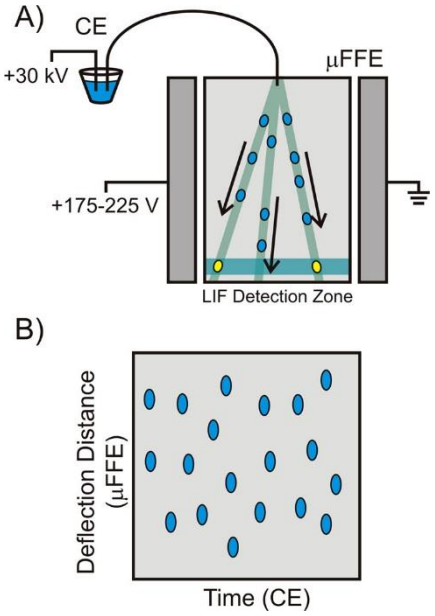

Figure 6: *(A) Mechanism of 2D CExµFFE separation. Analyte peaks migrate off the CE capillary directly into the µFFE separation channel where they are deflected laterally based on their mobility in the second dimension separation. (B) 2D Plot of CE separation time vs µFFE deflection distance [51]. Figure reprinted with permission from A.C. Johnson, M.T. Bowser, High-speed, comprehensive, two dimensional separations of peptides and small molecule biological amines using capillary electrophoresis coupled with micro free flow electrophoresis, Anal. Chem., 89 (2017) 1665-1673. Copyright 2017 American Chemical Society.*

Capillary IEF-CZE

Capillary IEF was coupled to transient ITP-CZE separating the analytes based on orthogonal mechanisms namely their isoelectric point and their electrophoretic mobility. Further, analyte concentration was performed by transient ITP. A microdialysis junction was used as interface establishing the electrical connection and providing the anolyte and BGE for the two dimensions, respectively, reaching an estimated peak capacity around 1600. Feasibility of the system coupled to ESI-MS was shown using standard proteins [52]. The system was further applied to proteome analysis of the facultative aerobic Gram-negative bacterium *Shewanella oneidensis* [53]. Instead of inserting a microdialysis membrane, interfacing of capillary IEF and CZE could also be achieved by a porous junction etching a short section of the capillary wall between the two dimensions [54]. The etched wall became a porous glass membrane allowing only small ions to pass through when high voltage was applied. This interface had low dead volume as no connection of two capillaries was necessary. However, some fragileness was observed. Thus, a partially etched interface was described improving stability [55]. Further, a monolithic immobilized pH gradient was used for capillary IEF avoiding the UV

detection interferences of carrier ampholytes. The new system was used separating a protein mixture of standard proteins extracted from milk.

ITP-CZE

Coupling ITP to CZE can enhance the selectivity as ITP provides an online sample pretreatment. Interfering matrix constituents are eliminated and analytes are preseparated and concentrated before separation with CZE. As large volumes can be injected and preconcentrated in ITP, low detection limits can be achieved for direct injection of samples with complex matrices. For example, quinine in human urine was determined by ITP-CZE-UV with a limit of detection (LOD) of 8.6 ng/mL [56]. Moreover, serotonin was selectively determined in human urine by ITP-CZE using a cyclodextrin additive in the BGE [57]. Kler et al. [58] presented a glass microfluidic chip interfacing a hybrid modular system for two-dimensional ITP-CZE separations. They performed preconcentration, separation, and identification of different angiotensin peptides comparing the transfer characteristics of different microfluidic interfaces [58]. Further, they presented a method for the analysis of the 20 proteinogenic amino acids with column-coupling ITP-CE-MS using a non-aqueous ITP method [59]. For efficient two-dimensional separation, microfluidic devices used as interfaces were characterized and optimized concerning low dead volume [58, 67].

1.3.2.3. Other 2D CE systems

CSExMEKC

Two-dimensional separation using capillary sieving electrophoresis (CSE) and MEKC is promising in terms of orthogonality for protein analysis as analytes are first separated by size and then by hydrophobicity. Dovichi's group developed a flow-gated interface for CSE-MEKC coupling with sequential injection to the second dimension performed electrokinetically. They described various applications for protein separations and single cell characterization [60-62]. The technology was applied for the separation of a protein homogenate from the bacteria *Deinococcus radiodurans* [60]. Moreover, the expression profiles of single cells and homogenates were compared using MCF-7 breast cancer cells [61]. The system was expanded for medium-throughput analysis using five separation capillaries in parallel for the analysis of expression fingerprints from a homogenate lung cancer cell line [62].

IEF - capillary electrochromatography (CEC)

Another approach for proteomics was presented by Zhang et al. [63] hyphenating IEF to capillary electrochromatography (CEC) via a nanoinjector valve. After focusing the proteins based on their isoelectric points, they were separated based on the CEC separation mechanism combining electromigration and chromatographic partitioning. A relatively high peak capacity was achieved (about 54,320) separating standard proteins using reversed-phase CEC with neutral monolithic capillary columns as the second dimension. The system was also applied to human serum proteins with good results. Wei et al. [64] used IEF coupled to pressurized CEC via a microinjection valve interface separating a bovine serum albumin digest with a theoretical peak capacity of 24,000. Again reversed phase CEC was used for the second dimension offering on-column refocusing of the effluent fractions, enhanced separation, and elution speed due to the combination of hydrodynamic flow and EOF.

Gel electrophoresis (GE) in 2D CE

Gel electrophoresis (GE) especially polyacrylamide GE is widely used in protein separation. Two-dimensional systems based on GE in capillary scale were developed. Liu et al. [65] presented a two dimensional CZE x channel GE approach. Coupling was performed without interface moving the CZE capillary outlet at a selected speed across the entrance of gel-filled channels leading to a continuous deposition of effluent. Mixtures of peptides, a tryptic digest of trypsinogen, and <0.05% of an individual B2 neuron from the marine mollusk *Aplysia californica* were separated by the system achieving theoretical plate numbers between 20,000-50,000 even if the authors admitted that the system was not completely orthogonal.

Lu et al. [66] developed a chip capillary hybrid device for 2D IEFxGE. IEF occurred in the chip whereas GE as second dimension was performed in an array of capillaries (Figure 7). The device consisted of three chips buttered together whereas the middle part was movable. After IEF in the channel of the chip (marked in red, from A1 to C2), the middle part was moved leading to subdivisions of the chip channel. The channels were now connected to the capillaries in the array and gel electrophoresis was performed.

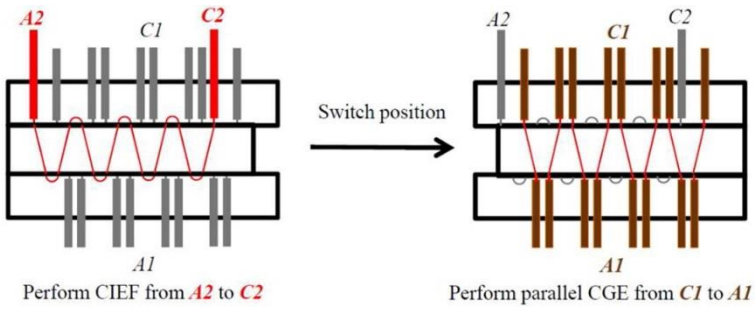

Figure 7: *Chip capillary hybrid device for two-dimensional capillary IEF - capillary GE. Left: hybrid device at the first dimension separation position. After IEF in the channel of the chip (marked in red, from A1 to C2), the middle part was moved leading to subdivisions of the chip channel. Right: hybrid device at the second dimension separation position. The channels were connected to the capillaries in the array performing gel electrophoresis [66]. Figure reprinted with permission from J.J. Lu, S. Wang, G. Li, W. Wang, Q. Pu, S. Liu, chip-capillary hybrid device for automated transfer of sample preseparated by capillary isoelectric focusing to parallel capillary gel electrophoresis for two-dimensional protein separation, Anal. Chem., 84 (2012) 7001-7007. Copyright 2012 American Chemical Society.*

1.4. Selectivity enhancement by dual detection concepts

Instead of hyphenation of two separation methods, dual detection can be used as an alternative concept to enhance the overall selectivity. Using complementary detectors, the selectivity and universality of different detectors is combined allowing the selective detection of a broader field of analytes of interest than with single detection strategies. Dual detection concepts have already been reviewed twice [13, 14]. However, focus was put mainly on amperometric detection concepts and C^4D has just been treated marginally. In this review, focus is put on concepts containing C^4D or MS detection. Dual detection with amperometric detection and dual optical detection concepts are summarized briefly. In Table 3, the concepts and their applications are shown pointing out the advantage concerning selectivity due to complementary detection.

Table 3: *Overview of the dual detection concepts and their applications pointing out the advantage for selectivity enhancement due to complementary detection.*

Combination	Separation method	Samples for complementary determination	Ref
UV/C^4D	CZE	Determination of a mixture of inorganic and organic ions such as vitamins	[68-70]
	CZE	Quantification of incompletely separated amino acids	[71]
	MEKC	Determination of caffeine and taurine	[72]
	CZE	Determination of inorganic ions and neutral UV absorbing substances in human urine	[73]
LEDIF/C^4D	CZE	Simultaneous detection of inorganic cations and fluorescein isothiocyanate labeled amino acids and peptides	[74]
	MEKC	Monitoring of nicotine and cotinine derivatization detecting reaction intermediates and products	[75]
LEDIF/UV/ C^4D	CZE	Determination of inorganic ions, underivatized amino acids, fluorescent and fluorophore-labeled compounds	[76]
C^4D-UV	CZE	Determination of a model mixture of 29 organic acids (partly UV absorbing) occurring in urine	[77]
UV-C^4D	CZE	Determination of the dissociation constants of the proteinogenic amino acids (partly UV absorbing)	[78]
C^4D-UV	CZE	Determination of several drugs in dietary supplements	[79]

C^4D-AD	MEKC	Determination of unconjugated aromatic acids in urine samples: electroactive biomarkers, non-electroactive species and matrix components	[80, 81]
C^4D-C^4D	CZE	Detection of inorganic model ions	[82]
	CZE	Determination of glycerol and its electrooxidation products	[83]
C^4D-MS	CZE	Determination of phenolic compounds	[84]
	CZE	Determination of sugars, biogenic amines, and carboxylic acids	[85]
	NACE	Determination of organic biomolecules and inorganic ions	[86]
UV-IM-MS	Kinetic CE	Study of the conformation and enzymatic activity of transglutaminase in presence of small molecule inhibitors	[87]
LIF-MS	CZE	Determination of β-carboline alkaloids, analysis of confiscated ayahuasca samples, ethanolic plant extracts, and labeled and non-labeled N-glycans	[88-90]
AD-AD	CZE	Determination of phenolic acids in a whiskey sample using different oxidizing potentials	[91]
AD-AD	CZE	Determination of gluthatione and gluthatione disulfide by indirect detection	[92]
LIF-DAD	CZE	Analysis of phenolic compounds in grape skin	[93]
UV-CL	CZE	Determination of labeled amino acids, peptides, and proteins	[94-97]
LIF/LS	CZE	Detection of fluorescent analytes and non-fluorescent contaminations	[98]
LEDIF-ECL	CZE	Detection of alkaloids and amino acids	[99]

1.4.1. C^4D in dual detection approaches

1.4.1.1. C^4D with optical detection

C^4D as part of dual detection is mostly coupled to optical detection such as UV/VIS but also to fluorescence detection. Optical detection methods are limited to a group of analytes fulfilling the structural requirements e.g. to absorb light with wavelengths in the UV/VIS range. Contrary, C^4D is a robust and universal detector complementing the optical detector. The dual detection approaches can be divided in two groups concerning the relative position of the two detectors. In some constructed devices the two detectors share a detection cell. Thus, the detection occurs at the same point of the capillary. This was achieved by placing the window for optical detection inside the detection gap of the C^4D detection [68, 69, 71-76]. In the second group, the detectors were placed in series leading to different effective lengths for C^4D and optical detection [77, 78, 100-103]. In these approaches, the C^4D was located inside the capillary cassette

of a commercial CE device or somewhere else along the capillary. As both methods are noninvasive and nondestructive, further coupling with MS would be possible. Conventional conductivity detection coupled to UV/VIS is not covered in this review [104, 105].

Approaches using a single detection cell

Chvojka et al. [68] first developed a combined UV/C^4D detector in 2001. For the construction of the C^4D, two ring electrodes were used consisting of a copper wire wound around the separation capillary followed by a soldering step. For UV detection, two silica fibers were fixed vertically onto the separation capillary in the 2 mm gap between the two electrodes. Stable signals were obtained determining a mixture of inorganic and organic ions. However, in this setup it was rather difficult and time-consuming to replace the separation capillary. Therefore, another modified setup was proposed based on a C^4D containing two semi-tubular electrodes embedded in a groove on a plexiglas box [69, 70]. The setup is depicted in Figure 8.

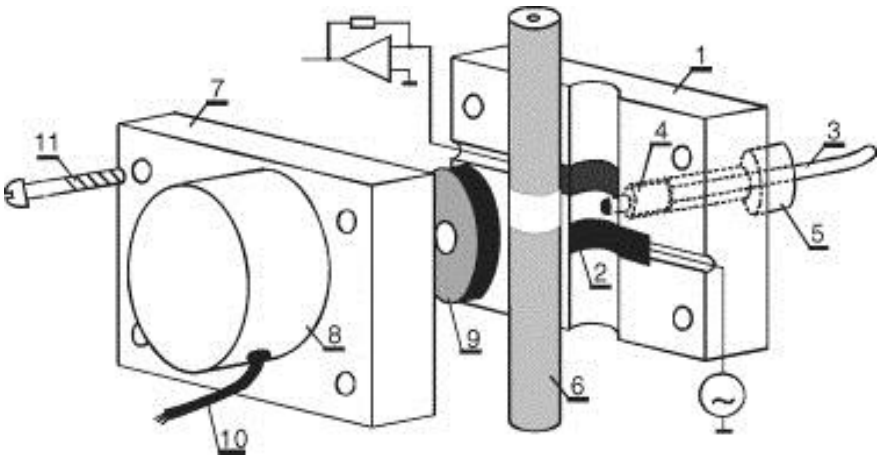

Figure 8: *Scheme of a dual UV/C^4D detector: (1) fixed plexiglas plate; (2) electrodes; (3) optical fiber bringing the radiation from the source; (4) silicone rubber ring; (5) screw pressing the silicone rubber ring to the optical fiber; (6) separation capillary; (7) movable plexiglas plate; (8) photodiode; (9) optical shielding; (10) cable connecting the photodiode with the spectrophotometric detector circuitry; (11) fixing screws [69]. Figure reprinted with permission from M. Novotný, F. Opekar, I. Jelínek, K. Štulík, Improved dual photometric-contactless conductometric detector for capillary electrophoresis, Anal. Chim. Acta, 525 (2004) 17-21. Copyright 2004 Elsevier.*

A single optical fiber was placed in the gap between the electrodes touching the capillary. Fixation was achieved by a movable plexiglas plate containing a hole to a large area photodiode. This design enabled easy exchange of the separation capillary. The

sensitivity of the C^4D was found twice as high as for the original detector. The dual detector was used for the determination of a food supplement containing several vitamins and inorganic cations [69]. It was further used for the quantification of the incompletely separated amino acids tyrosine and proline by comparing the signals of the two detectors as proline is not UV active and thus only tyrosine was detected by the UV detector [71].

Opekar and coworkers [72] developed a dual UV/C^4D detector for the use with very short capillaries (10.5 cm) enabling rapid separations. Therefore, the whole separation and detection apparatus including the separation capillary, the buffer vials, and the dual detection cell was implemented in a commercially available UV/VIS detector. The structural design of the C^4D was also based on the work of Tůma et al. [70] using two semi-tubular electrodes. For optical detection, an optical fiber transferred the radiation from the monochromator of the UV/VIS detector to the detection window located again between the two electrodes of the C^4D. The signal passing through the separation capillary was detected by a diode, which was also part of the commercial detector, on the opposite of the capillary. Complementarity of the two detectors was shown determining caffeine (UV) and taurine (C^4D) by MEKC. This dual detector approach was further used for the determination of ammonia, creatinine, uric acid, and hippuric acid in human urine using pressure-assisted injection [73].

Besides UV detection, a dual detector cell was also developed for fluorescence detection and C^4D [74]. Two tubular stainless steel electrodes were used for C^4D shielded by a copper plane to minimize the stray capacitance. The optical window was located between one electrode and the copper plane leading to slightly different effective cell volumes of the C^4D and the fluorescence detection. As excitation source, a high brightness blue light emitting diode (LED) was used. The emitted fluorescence was collected by an optical fiber fixed perpendicularly and passed through interference filters to a photo multiplier. Simultaneous detection of several inorganic cations (C^4D) and fluorescein isothiocyanate labeled amino acids and peptides (LED-induced fluorescence, LEDIF) was performed to show the complementary use.

Selectivity can be further enhanced by implementing a third detector. A three-in-one approach was described based on a standard fiber-optic adapter placed between two

tubular electrodes for C^4D [76]. It contained LEDs as light sources and two fibers collecting the photometric and the fluorescence signal (at a 45° angle to the capillary). The implementation of the fiber-optic adapter led to a comparably large detection gap of 7 mm between the electrodes. However, no additional zone broadening was found comparing a signal visible in C^4D and photometric detection. The overall complementarity of the system was shown separating model mixtures and samples containing inorganic ions, underivatized amino acids, fluorescent, and fluorophore-labeled compounds (Figure 9).

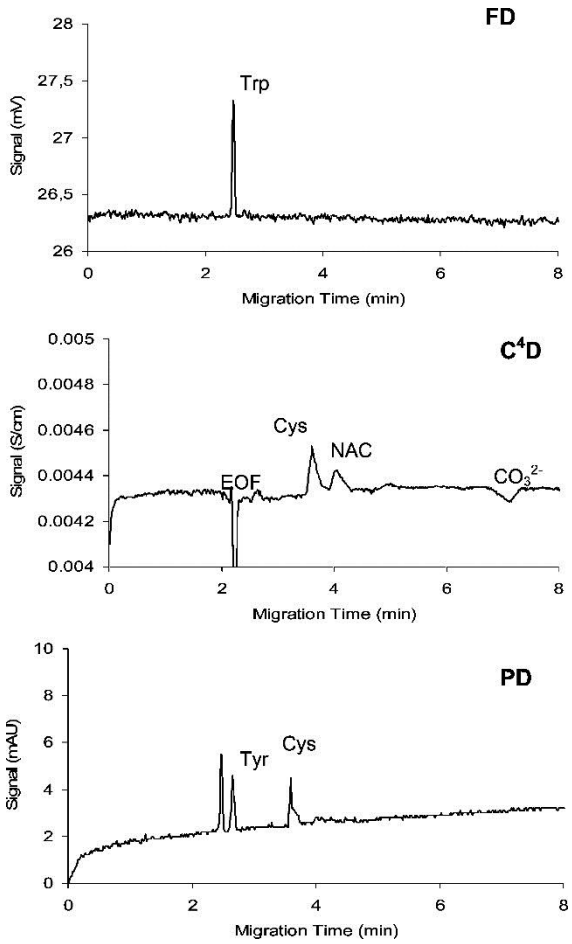

Figure 9: *Separation of a model mixture of amino acids showing complementarity of fluorescence detection (FD), photometric detection (PD), and C^4D. Conditions: Trp 0.37 mM, Tyr 0.75 mM, Cys 0.94 mM, NAC 0.56 mM separated in 20 mM N-cyclo-hexyl-2-aminoethanesulfonic acid (pH 9); LED 255 nm; frequency: 100 kHz; voltage: +15 kV; injection: 10 s, 1 kPa; capillary: ID=75 µm, l_{eff} = 31.5 cm [76]. Figure reprinted with permission from M. Ryvolová, J. Preisler, F. Foret, P.C. Hauser, P. Krásenský, B. Paull, M. Macka, Combined contactless conductometric, photometric, and fluorimetric single point detector for capillary separation methods, Anal. Chem., 82 (2010) 129-135. Copyright 2012 American Chemical Society*.

The detector setup (however without UV detection) was further employed for the monitoring of nicotine and cotinine derivatization by MEKC separating reaction

intermediates and products [75]. The C^4D enabled the determination of the EOF and the non-fluorescent products, which could be formed during the reaction.

Dual detection connected in series

Coupling of UV/VIS detection and C^4D in series is easily done implementing the C^4D into the cassette of a commercial CE instrument or placing it somewhere else along the capillary. A model mixture of 29 organic acids occurring in urine was determined by CE-C^4D-UV [77]. The dual approach enabled the detection of all analytes. C^4D was used for the small, fast migrating acids such as oxalic acid or aspartic acid whereas photometry was the detection principle of choice for the UV absorbing acids like orotic, vanillic, or hippuric acid. Moreover, also two unseparated acids could be quanitified comparing the detector signals as one was not UV absorbing and thus just visible in the C^4D [71, 77]. The complementarity of C^4D and UV detection was also used for the determination of dissociation constants of the 20 standard proteogenic amino acids [78]. The analytes lacking of chromophore such as alanine, leucine, or proline were detected by C^4D. In contrast, some amino acids were better detected using photometry as they exhibited similar conductivity to the BGE. A recent study used dual C^4D-UV detection to determine several drugs in over 100 formulations of dietary supplements sold for weight loss, fat burning, appetite reduction, and metabolite acceleration [79]. The combination of the two detectors enabled rapid screening and determination of the drugs in complex samples and gave also information on matrix interferences.

In some applications, C^4D and UV were used to compare the data or for optimization of the method instead of focusing on complementarity [100-103, 106]. A CE-C^4D-indirect UV method for the determination of γ-hydroxybutyric acid in saliva was developed and the performance of both detectors was validated [100]. Moreover, formate in blood samples could be analyzed by in-line coupling of microextractions across polymer inclusion membranes with CE-C^4D-UV [101]. In this application, the detection sensitivity of C^4D was found to be about an order of magnitude better than for UV/VIS detection. The dual detection approach was also used with ITP [102] and with transient ITP-CE for the analysis of paralytic shellfish toxins in mussel samples [103].

1.4.1.2. Electrochemical detection concepts with C^4D

Zhang et al. used a CE-C^4D-AD [81] and a MEKC-C^4D-AD [80] approach for the determination of unconjugated aromatic acids (pathological metabolites of phenylalanine) in urine samples. C^4D was used as an upstream detector, while AD was placed in the end of the capillary (end-column approach). The AD setup consisted of a three-electrode electrochemical cell using a carbon disc working electrode, a platinum auxiliary electrode and a saturated calomel electrode as the reference electrode. Complementary signals were recorded combining the universality of the C^4D and the high selectivity and sensitivity of AD. Hence, AD was used for the quantification of the electroactive biomarkers whereas C^4D was chosen for the non-electroactive species and to get background information of the chemical composition and matrix of the sample. Thus, information obtained with the two detectors complemented each other rendering the results more convincing and reliable [80, 81].

Dual C^4D was also reported [82, 83]. Stojkovic et al. [82] implemented a second detection channel into the C^4D arranged in a bridge configuration to the first one. A second capillary was inserted parallel to the separation capillary using the same inlet and outlet vial. This led to the generation of a reference signal enabling the compensation of baseline drifts due to changes in temperature or buffer composition. The reference signal could also be generated looping the separation capillary back into the second detection channel. Feasibility of the system was demonstrated detecting inorganic model cations. Recently, dual C^4D was used for the determination of glycerol and its electrooxidation products (neutral diols and carboxylates) [83]. The two detectors were placed in 10 cm and 50 cm effective length of the separation capillary. The fast migrating compounds were detected at the second detector while the slow migrating ions were detected at the first detector reducing the overall analysis time.

1.4.2. MS in dual detection approaches

1.4.2.1. C^4D with MS detection

The coupling of CE and MS was first introduced by Smith et al. [107] and has evolved enormously in the last decades. Nowadays, MS can be considered as the ultimate detector providing high selectivity and enabling the identification of analytes giving

molecular weight and structural information [108-110]. In earlier studies using dual C^4D-MS detection, the universal C^4D was not used as full detector for quantitative analysis complementary to the MS. Rather it was necessary for the optimization of the timing of a voltage switching step for a two-dimensional ITP-CE-MS separation [58] or for an interface-free CE-MS method development [111]. Recently, however, two dual C^4D-MS approaches were described demonstrating the complementarity of the two detectors enhancing the overall selectivity [84, 85]. The challenge in these approaches lied in the opposing requirements of the two detectors. For ESI-TOF-MS, volatile BGEs are essential whereas for C^4D, BGEs with rather low conductivity and high ionic strength are advantageous. Beutner et al. [84] used C^4D with ESI-TOF-MS for the determination of various phenolic compounds showing complementary response behavior for the different analytes. Moreover, the C^4D was used to monitor the EOF. Parameter selection was adjusted to the separation requirements and the compatibility of both detectors. An NH_4Ac based BGE was taken as compromise ensuring good compatibility with the MS and an acceptable signal-to-noise ratio of the C^4D. Detector-induced band broadening could be neglected in the CE-C^4D-MS system.

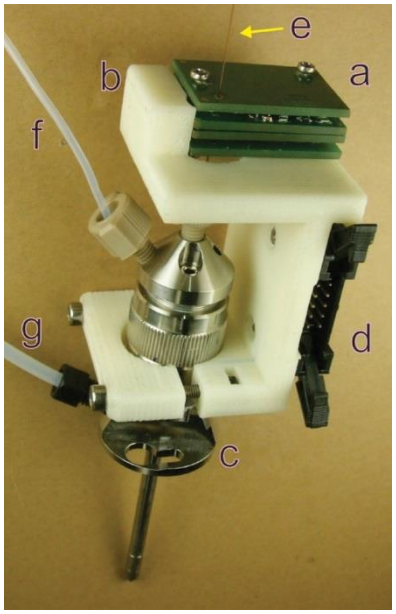

Figure 10: *Photograph of a dual C^4D-ESI arrangement: (a) C^4D immobilized on a (b) 3D-printed support, (c) ESI interface, (d) electrical connections, (e) separation capillary, (f) sheath liquid, and (g) nebulizer gas [85]. Figure reprinted with permission from K.J.M. Francisco, C.L. do Lago, A capillary electrophoresis system with dual capacitively coupled contactless conductivity detection and electrospray ionization tandem mass spectrometry, Electrophoresis, 37 (2016) 1718-1724. Copyright 2016 John Wiley and Sons.*

Francisco et al. [85] used a commercial CE system coupled to dual C^4D-ESI-quadrupol MS. An image of the C^4D-ESI setup is depicted in Figure 10. Different examples for applications were shown covering a wide range of different pH values of the BGEs. Thus, selection of suitable BGEs was performed determining sugars at high pH, biogenic amines at low pH, and carboxylic acids at neutral medium with BGEs based on triethylamine or acetic acid. Recently, the proposed setup was developed further embedding the whole system in a 3D-printed cartridge improving thermal control of the CZE and avoiding excessive Joule-heating [112].

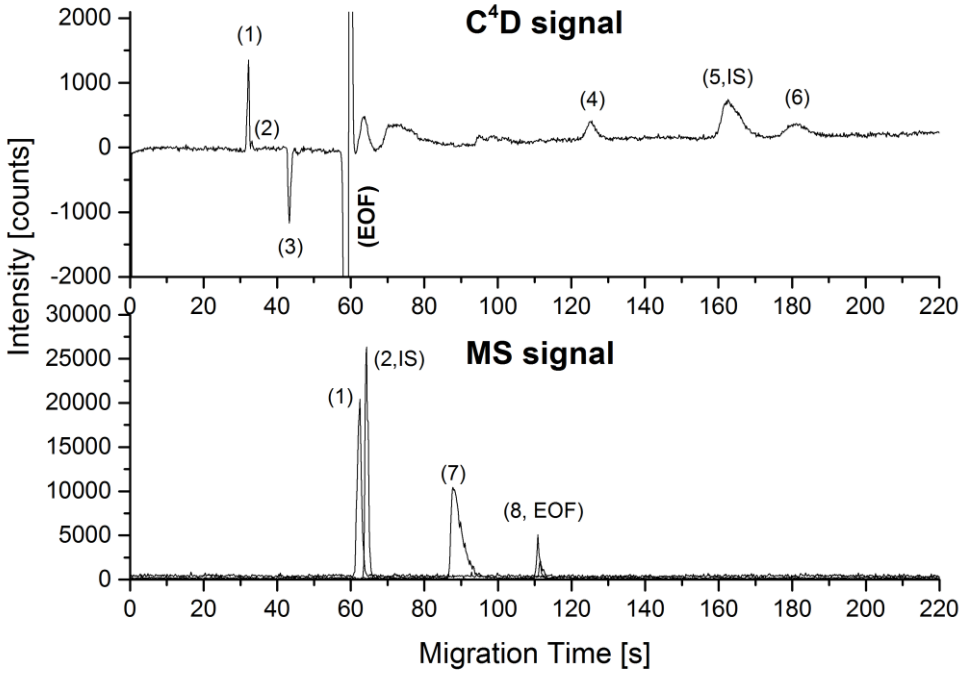

Figure 11: *Electropherograms of an extract of a food supplement obtained by NACE with dual C^4D-MS detection showing complementary information. The sample contained choline (1), acetylcholine (2, IS), Na^+ (3), chloride (4), bromide (5, IS), nitrate (6), thiamine (7), and nicotinamide (8, EOF). The length of the separation capillary (ID= 50 µm) was 30 cm, which was the effective length to the MS. The effective length to the C^4D was 15.2 cm. Injection time was 0.1 s. Separation was performed in a 2 M HAc/ACN BGE containing 4 mM NH_4Ac applying a HV of 10 kV [86]. Figure reprinted with permission from A. Beutner, B. Scherer, F.-M. Matysik, Dual detection for non-aqueous capillary electrophoresis combining contactless conductivity detection and mass spectrometry, Talanta, 183 (2018) 33-38. Copyright 2018 Elsevier.*

Another approach used non-aqueous BGEs as they are highly compatible with ESI due to their volatility. Further, they exhibit low background conductivity being promising for the use with C^4D [86]. A non-aqueous capillary electrophoresis (NACE) method using an acetonitrile (ACN) based BGE was developed. Additionally, the influence of the ID of the separation capillary was investigated considering both detectors. A capillary with ID 50

µm was found to be best suited for the dual approach. The method was applied to a food supplement quantifying the content of organic biomolecules with MS as well as inorganic ions with C^4D (Figure 11).

In summary, even if the MS is more sensitive and selective for most of the analytes, the hyphenation to a low-selectivity detector such as C^4D can enhance the gained information. The ionization efficiency of the ESI-MS is analyte-dependent and most TOF-MS systems have limitations determining analytes with low m/z. Thus, for some analytes such as inorganic ions the C^4D can be more sensitive. Furthermore, the EOF can be monitored without the requirement of an EOF marker in the sample. The C^4D can also provide information about unexpected features of the sample.

1.4.2.2. UV and fluorescence detection with MS detection

Coupling MS with UV detection is often easily performed connecting commercial equipment in series. However, the sensitivity of the MS detection is much higher compared to UV detection limited by the length of the optical pathway. Furthermore, only analytes with special structural requirements are detectable by UV. Consequently, dual detection with UV and MS was rarely used for complementary quantitative analysis. The MS was rather used for identification of UV detected peaks [87] or the UV detector was used separately for optimization reasons [106]. Mironov et al. [87] developed a method based on kinetic CE coupled on-line with UV detection and ion mobility mass spectrometry (CE-UV-IM-MS) both detectors giving complementary information. This approach was applied to study the effect of small-molecule inhibitors on the conformational distribution and the enzymatic activity of a human tissue transglutaminase. UV detection was used to quantify the interconversion dynamics of separated protein conformers whereas conformer sizes, molecular weights, and structures were identified by IM-MS.

A problem often stated is the huge distance between the detection sites of the UV detector and the MS rendering a direct comparison of the two recordings difficult. Overcoming this drawback, Foret et al. [113] developed a miniaturized, integrated CE-UV-ESI-MS interface. It incorporated a fiber optic detection cell monitoring the UV-active compounds just prior their admittance into the MS allowing also the use of short separation capillaries.

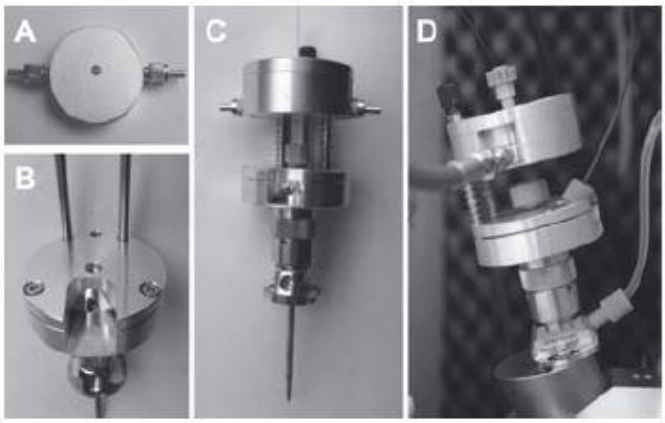

Figure 12: *Pictures of a CE-LIF-MS setup. (A) LIF detection cell; (B) the lower platform mounted on the CE-MS sprayer; (C) full setup with sprayer, lower and upper platform (with detection cell) and capillary; (D) CE-LIF-MS setup mounted on top of the MS inlet with attached optical fibers, sheath liquid tubing and nebulizer tubing [89]. Figure reprinted with permission from C. Huhn, L.R. Ruhaak, J. Mannhardt, M. Wuhrer, C. Neusüß, A.M. Deelder, H. Meyer, Alignment of laser-induced fluorescence and mass spectrometric detection traces using electrophoretic mobility scaling in CE-LIF-MS of labeled N-glycans, Electrophoresis, 33 (2012) 563-566. Copyright 2012 John Wiley and Sons.*

LIF detection is a very sensitive optical detection technique for CE offering extremely low detection limits and precise quantification for many analytes of interest having a sufficiently high fluorescence quantum yield. A CE-LIF-MS setup was developed placing the optical cell directly in front of the electrospray interface minimizing the difference in effective separation length between the two detection sites [88, 89] (Figure 12). Methods for the determination of β-carboline alkaloids were developed requiring low volatile BGEs, which have no fluorescence quenching constituents. The two detectors showed complementary selectivity analyzing confiscated ayahuasca samples and ethanolic plant extracts [88] as well as labeled and non-labeled N-glycans [89, 90].

1.4.3. Amperometry in further dual detection approaches

Electrochemical detectors are highly sensitive, easy to miniaturize and exhibit considerable selectivity. The selectivity of AD systems is highly variable as changing the potential, analytes with different redox activity are detected. Dual detection using C^4D and AD has already been described in chapter 3.1.2. Further dual detection approaches dealing with electrochemical detection, especially amperometric systems, were reviewed in detail by Opekar et al. [13]. Therefore, this topic is just summarized briefly

mentioning some recent publications. The most common approach is the dual-electrode amperometric detection using two working electrodes. Direct and indirect detection modes are used. In direct mode, the electrodes can be placed in series or used in parallel. In series, the first electrode converts an analyte into a product detected at the second electrode enhancing sensitivity, but also selectivity for mixtures of electroactive and inactive species. Placing them in parallel, usually different working potentials are applied resulting in different selectivity for the two electrodes. A dual-electrode detector in parallel electrode configuration was developed inserting dual carbon fiber electrodes directly into a funnel-etched separation capillary [91]. The detector could be operated in two different direct detection modes. In one mode, an oxidizing potential was applied to one working electrode whereas a reducing potential was applied to the second electrode. This resulted in an increase of sensitivity compared to single electrode detection as the analytes switched continuously between their oxidized and their reduced form. In the second mode, different oxidizing working potentials were applied to the electrodes. The setup was applied to a whiskey sample selectively identifying several phenolic acids.

In indirect detection mode, the electrodes are placed in series whereas the upstream electrode converts an auxiliary substance present in the separation media into a species reacting with the analyte. The second electrode detects the amount of unreacted species. Recently, a detector was designed using a coaxial postcolumn reactor (Figure 13) [92]. Bromide reagent was introduced hydrodynamically and converted to bromine by a Pt film sputtered onto the outer surface of the separation capillary at the outlet. Analytes that reacted with bromine could be detected determining the difference in bromine concentration at a platinum microdisk electrode located at the end of the reactor. The detector was characterized determining glutathione and glutathione disulfide.

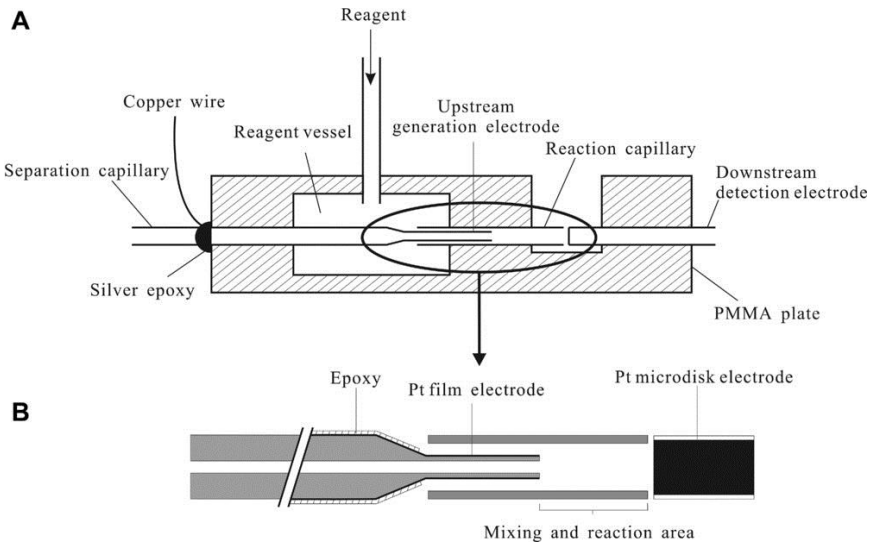

Figure 13: *Dual amperometric detection setup. (A) Scheme showing the serial dual electrode detection system based on a coaxial postcolumn reactor. (B) Enlarged view on the serial dual-electrode configuration [92]. Figure reprinted with permission from F. Du, S. Cao, Y.-S. Fung, A serial dual-electrode detector based on electrogenerated bromine for capillary electrophoresis, Electrophoresis, 35 (2014) 3556-3563. Copyright 2014 John Wiley and Sons.*

However, one can argue that only the direct parallel dual-electrode configuration using two different potentials can be seen as real dual detection approach as both electrodes were used for complementary quantitative determinations. In the indirect detection mode as well as in the direct detection mode in series, just one electrode was used for final detection whereas the other electrode was necessary to generate the detected species. Nevertheless, this also enhanced sensitivity and selectivity especially in complex matrices.

Amperometric detection was further used together with ECL and UV detection. The use of dual UV/AD detection enabled the simultaneous detection of analytes having either UV absorbing or electroactive properties. Electrochemiluminescence (ECL) is mostly used to detect a great variety of amine-containing analytes using $[Ru(bpy)_3]^{2+}$ as luminescence agent. Both combinations were described in detail elsewhere [13].

1.4.4. Dual optical detection approaches

Further, two optical detection techniques were used as dual detection approach for CE. Besides UV coupled to LIF [93], UV and LIF were coupled to chemiluminescence (CL) [94-97], light scattering (LS) [98], and ECL detection [99]. A method for the analysis of

phenolic compounds in grape skin by CE combined with dual fluorescence and diode array absorption detection (DAD) was described [93]. Both detectors were used for quantitative complementary determination. Tsukagoshi et al. [94-97] introduced a dual UV/CL detection approach. The UV detection was carried out on-capillary whereas the CL detection was performed end-capillary in a CL detection cell. The cell was placed in a small light-tight box together with a photomultiplier. Interestingly, the CL detection cell was simultaneously the outlet reservoir for electrophoretic separation grounded via a platinum wire. The CL reaction was based on a luminol-hydrogen peroxide. The running buffer contained 4 µM microperoxidase whereas the outlet reservoir was filled with the same electrolyte containing hydrogen peroxide instead of the enzyme leading to the CL reaction of the luminol labeled compounds at the outlet of the separation capillary [96]. The system was applied to selectively detect UV-active analytes and compounds with CL properties [94, 97]. Moreover, LIF was used simultaneously with LS for the detection of individual particles separated by CE [98]. A sheath flow cuvette at the end of the CE capillary was used. The outflow was excited by a laser and fluorescence and scattering signals were collected in an angle of 90°. Scattering alone was not sufficient for the identification of the analyte particles as it is a rather unselective detection technique. Thus, the fluorescent signals were used for identification. However, the scattering signals could detect non-fluorescent contaminations. Chang et al. [99] combined sequential LEDIF and ECL for the dual detection of alkaloids and amino acids. CE-LEDIF was placed online for the determination of compounds possessing primary amino groups and ECL located at the outlet of the capillary was suitable for secondary and tertiary amines using different derivatizing agents.

1.5. Conclusion

The review showed that selectivity enhancement in CE can be achieved by two-dimensional hyphenation of separation techniques or by coupling different detection principles. In particular, the diversity of the combined techniques, namely the orthogonality for 2D separation systems or the complementarity for dual detection systems is of importance. Exploiting the different chromatographic materials and CE modes, high orthogonality can be achieved resulting in high peak capacities being necessary for several applications for example in proteomics. Besides this, the way and the frequency of transferring the analytes from the first to the second dimension plays an important role for the selectivity. Several reviews have already summarized the topic of 2D separation techniques with focus on interfacing strategies [8-12]. Using complementary detection principles as an alternative concept, a variety of analytes could be determined as the selectivity of the two detectors is combined resulting in less restrictions due to structural or chemical requirements of the analytes. Usually the separation technique is considered to be responsible for the overall selectivity of the system. The detection principle is often somewhat underestimated. However, every detector has its own selectivity being enhanced by bringing complementary principles together. Especially, using MS as detector results in highly selective determinations. Further, using AD the selectivity of the single detector can be controlled by choosing an appropriate redox potential. Combinations with universal detectors such as UV/VIS and C^4D are advantageous expanding the applicability to a broad field of analytes. The topic of dual detection was reviewed twice [13, 14]. However, C^4D was rarely covered in these publications.

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2.Scope of this thesis

Selectivity enhancement can be achieved by hyphenation of two (or more) orthogonal separation techniques or as alternative concept by coupling of two complementary detectors. In this work, both concepts are presented. A modulator was developed hyphenating the two most important techniques in ion analysis, namely ion chromatography (IC) and capillary electrophoresis (CE). Proof-of-concept measurements were performed using a model system consisting of nucleotides and cyclic nucleotides.[1] Moreover, a configuration is presented enabling the simultaneous ICxCE analysis of anions and cations and methodical studies were performed taking into account the complex transport situation.

As alternative concept, capacitively coupled contactless conductivity detection (C^4D) and electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS), two important detectors for CE, were coupled to a dual detection system. The experimental protocol took into account the requirements of separation and compatibility aspects. In a first study, the system was used with aqueous background electrolytes (BGE) determining several phenolic compounds. Further, non-aqueous BGEs were used. The method was applied to an extract of a food supplement containing small inorganic ions and organic biomolecules.

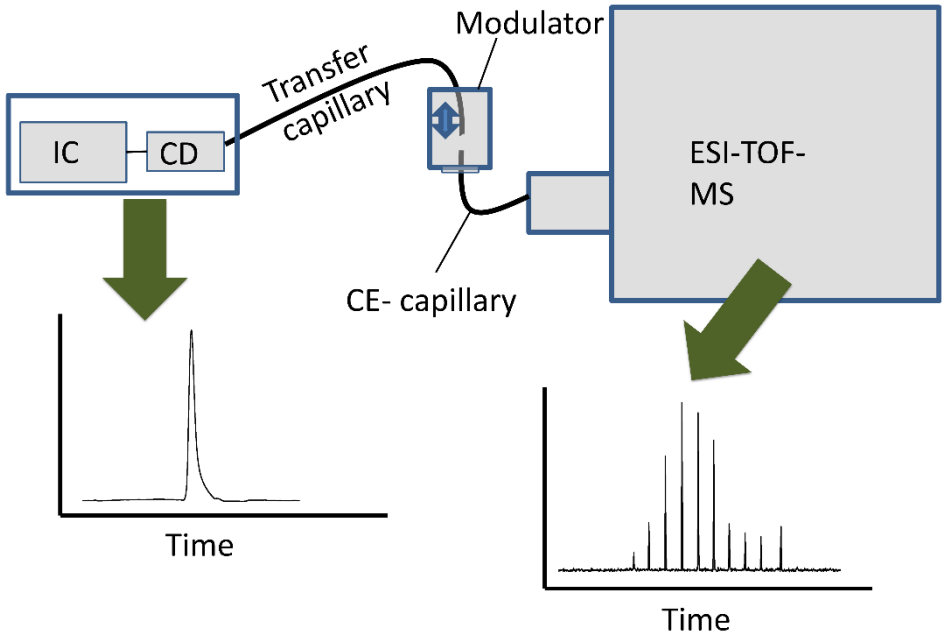
Selectivity enhancement is also achieved using a highly selective detector such as MS. However, not every BGE used in CE is compatible with ESI-TOF-MS. Sodium dodecyl sulfate (SDS)-based BGEs used in microemulsion electrokinetic chromatography (MEEKC) are an example for incompatible BGEs. The application of surfactant-free microemulsions (SFME) for MEEKC with UV/Vis and MS detection was investigated using a sample containing several vitamins.

[1] This work was part of a previous study:
A. Beutner, Advanced applications of fast capillary electrophoresis, Master thesis (2014), pp. 23-29 and 40-51.
J. Mark, Analytical approaches to the analysis of small samples and hyphenation of fast capillary electrophoresis to other instrumental techniques, PhD thesis (2014), pp. 94-100.

3. Comprehensive hyphenation of capillary ion chromatography and capillary electrophoresis

3.1. Two-dimensional separation of ionic species by hyphenation of capillary ion chromatography × capillary electrophoresis - mass spectrometry

3.1.1. Abstract



The separation of complex mixtures such as biological or environmental samples requires high peak capacities, which cannot be established with a single separation technique. Therefore, multidimensional systems are in demand. In this work, we present the hyphenation of the two most important (orthogonal) techniques in ion analysis, namely, ion chromatography (IC) and capillary electrophoresis (CE), in combination with mass spectrometry. A modulator was developed ensuring a well-controlled coupling of IC and CE separations. Proof-of-concept measurements were performed using a model

system consisting of nucleotides and cyclic nucleotides. The data are presented in a multidimensional contour plot. Analyte stacking in the CE separation could be exploited on the basis of the fact that the suppressed IC effluent is pure water.

This chapter has been published.

Andrea Beutner, Sven Kochmann, Jonas Josef Peter Mark, Frank-Michael Matysik, Two-dimensional separation of ionic species by hyphenation of capillary ion chromatography × capillary electrophoresis - mass spectrometry, Anal. Chem., 87 (2015) 3134-3138 (DOI: 10.1021/ac504800d).

This chapter was part of a previous study.

Andrea Beutner, Advanced applications of fast capillary electrophoresis, Master thesis (2014), pp. 23-29 and 40-51.

Jonas Mark, Analytical approaches to the analysis of small samples and hyphenation of fast capillary electrophoresis to other instrumental techniques, PhD thesis (2014), pp. 94-100.

This chapter is part of a patent.

Frank-Michael Maytsik, Andrea Beutner, Jonas Josef Peter Mark, Sven Kochmann, Method and device for two-dimensional separation of ionic species, international patent, WO 2015/162219 A1.

Author contributions

AB and *SK* did the experimental work for the coupling of ICxCE-MS presented in this chapter whereas *SK* was responsible for the control of IC and *AB* for the control of CE-MS. *JJPM* and *SK* did pre-experiments and technical groundwork on less complex mixtures for the ICxCE-MS coupling and trouble-shooting in the initial stages. *AB* did the experimental work and data evaluation for CE-MS presented in this chapter. *SK* did the data evalutation for IC measurements. *AB* did the data evaluation for the ICxCE-MS measurements. The results were discussed by all authors. *AB* wrote the manuscript presented in this chapter. *FMM* is the corresponding author.

3.1.2. Introduction

The separation of complex environmental and biological samples is a challenging task in analytical chemistry. Common liquid phase separation techniques are chromatography high performance liquid chromatography (LC), ion chromatography (IC), or capillary electrophoresis (CE). However, these one-dimensional methods offer rather low peak capacities [1, 2]. Hence, separation of complex samples is challenging or is not possible at all [3, 4]. In contrast, multidimensional separation systems possess a much higher peak capacity [5, 6]. Due to this, they are more suitable to study complex samples [1, 7, 8]. In recent years, much attention was attracted by multidimensional separation techniques [1, 5, 7, 9]. In this context, the comprehensive hyphenation of two gas chromatographic separations (GC×GC) played a pioneering role [10]. Multidimensional separation systems with liquid mobile phases were described, such as two-dimensional liquid chromatography (LC×LC) [11] and ion chromatography-reversed phase liquid chromatography [9, 12]. Also, hyphenation to electrophoretic techniques was described [10]. LC was first coupled to CE by Jorgenson and co-workers in 1990 [13]. Furthermore, they developed a flow-gated interface for two-dimensional separation [14, 15]. Recently, Ramsey and co-workers [5, 16] presented the hyphenation of LC and chip-based electrophoresis with mass spectrometry (MS). Using LC-CE-MS, they could realize the separation of a complex peptide mixture in less time and with less method development than using conventional LC-MS [17]. Moreover, electrochromatography was coupled to CE [18]. Huhn and co-workers [7] developed a system to couple isotachophoresis to capillary electrophoresis. In the past decade, considerable progress was achieved in the fields of IC and fast CE [19-24]. Instrumental IC on the capillary scale was developed [19]. Capillary high performance ion chromatography (cHPIC) is now able to separate ions with low flow rates down to 2-10 µL/min and can be hyphenated to MS [20]. In addition, the sensitivity of conductivity detection is enhanced by efficient miniaturized ion suppressors generating pure water as the final effluent [21]. On the other hand, CE separations based on short capillaries have been demonstrated with migration times in the range of seconds [22-25]. These recent developments in cHPIC and fast CE lead to an enhanced compatibility of the two dominating techniques for ion separations in terms of hyphenation. In this paper, we present the comprehensive hyphenation of cHPIC and CE. An appropriate modulator was developed ensuring a well-

controlled coupling of the two separation systems. For this, a variation of the previously described concept of capillary batch injection (CBI) was used [26, 27]. Briefly, with CBI, it is possible to inject small discrete sample volumes into the separation capillary using an injection capillary. In the present work, the CBI approach is adapted to the coupling of cHPIC and CE. In contrast to the previously described CBI concept in the present modulator system, there is a continuous flow through the transfer capillary, which represents the analogue of the injection capillary in conventional CBI.

3.1.3. Experimental

The overall experimental setup is depicted in Figure 1. It comprises a capillary high performance ion chromatograph, a modulator, and a laboratory built capillary electrophoresis device. An ICS-5000 (Dionex, Thermo Scientific) ion chromatograph was used for capillary-scale IC separations. It consisted of a dual pump module with both capillary (pump 1) and analytical pump (pump 2), an eluent generator module (EG KOH 300 with subsequent trap column), and a detector/ chromatography module.

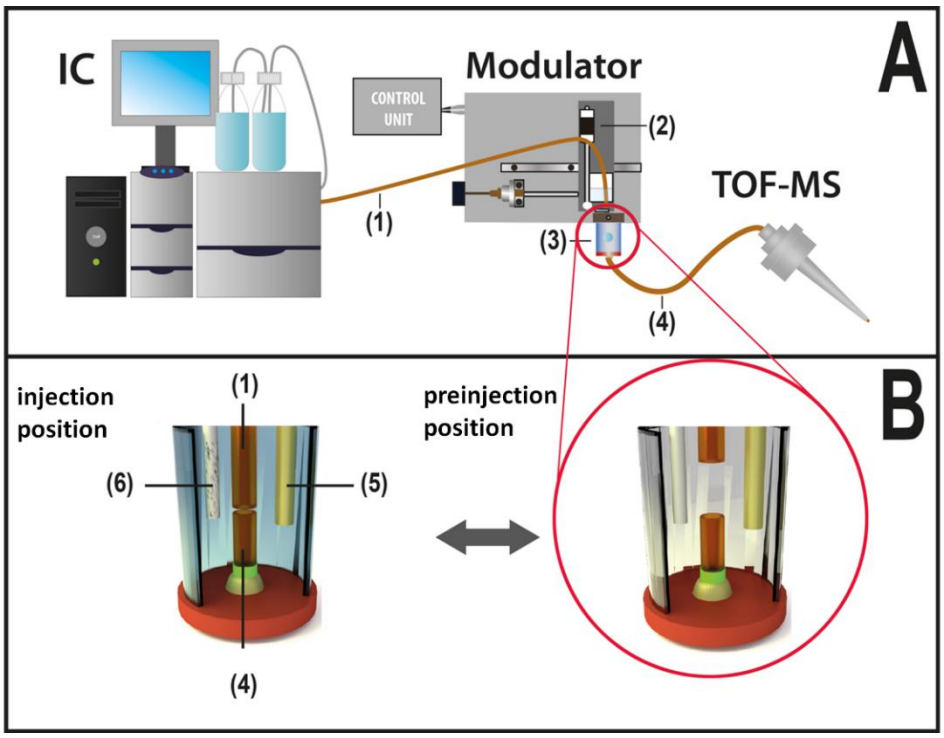


Figure 1: *(A) Setup of the IC×CE-MS system comprising a cHPIC, a modulator, a CE, and a TOF-MS. (1) Transfer capillary, (2) positioning unit with stepper motor, (3) CE inlet cell, and (4) separation capillary. (B) Scheme of the CE cell. On the left side, the transfer capillary (1) is in injection position; on the right side, it is in preinjection position. Furthermore, (5) the high voltage electrode and (6) the stirrer are depicted.*

The latter module comprises an inline eluent degasser, a four-port injection valve (injection volume, 0.4 µL), a column oven, an anion capillary eluent suppressor, and a conductivity detector. The modulator controls the transfer of the cHPIC effluent by a movable transfer capillary (ID=75 µm, l=60 cm) (Figure 1A (1)) into the CE separation capillary (ID=25 µm, l=20.5 cm) (Figure 1A (4)). A modified capillary batch injection (CBI) device was used as modulator. The setup of the CBI is described elsewhere [26]. Briefly, it consists of a vertical positioning unit (Figure 1A (2)) moving the transfer capillary, which is fixed on a holder, up and down in the Z-direction. This is achieved by a 1.8° stepper motor with leadscrew which reaches a positioning precision of 1 µm/step. The end of the transfer capillary is guided through a 0.38 mm ID glass guide (Hilgenberg, Malsfeld, Germany), fixed in a purpose-built manual x,y-positioner, into the electrophoretic cell (Figure 1A (3)). With the help of the positioning units, the transfer capillary is aligned with the separation capillary, which is located in the axial direction on the bottom of the electrophoretic cell (Figure 1B). Positioning is controlled using a laboratory-modified microscopic video camera (DigiMicro 1.3, dnt, Dietzenbach, Germany). The CE cell is further equipped with a stirrer. A micrOTOF-MS (Bruker Daltonik, Massachusetts, USA) with a coaxial sheath liquid electrospray interface (Agilent Technologies, California, USA) was used for detection. A mixture of 2-propanol, water, and ammonia (49.9:49.9:0.2, v/v/v) was used as sheath liquid at a flow rate of 8 µL/min. Nebulizer gas pressure was set to 1 bar. The electrospray voltage was 4 kV. Further parameters are listed elsewhere [28].

3.1.4. Results and discussion

During the measurement, a constant flow of analytes dissolved in pure water was delivered from the cHPIC. A permanent flow of pure water from the transfer capillary toward the inlet of the CE capillary would lead to a current breakdown. A protocol of periodical axial movement of the transfer capillary was chosen to overcome this problem. This modulation enabled a periodical injection of small sample plugs into the separation capillary. Sample is only injected into the CE separation capillary when transfer capillary and separation capillary are in a distance of about 30-50 µm. This position is called “injection position” (Figure 1B). Between two injections, the capillary is moved about 3-5 mm upward. This position is called the “preinjection position”

(Figure 1B). One interval of injection was comprising the injection time t_{inj}, the preinjection time t_{preinj}, and the time the stepper motor needed to drive from injection position to preinjection position and backward (see protocol according to Figure 3). In order to generate comprehensive analytical information, it is desirable to keep the interval between two successive injections as short as possible. However, if it was shorter than the migration time of the species, more than one separation was taking place in the capillary at the same time influencing the separation performance. Obviously, this depends on the nature of the CE system and the corresponding migration times of the sample components. The faster the analytes migrate through the capillary, the faster they leave the capillary again and the smaller the interval of subsequent injections can be set.

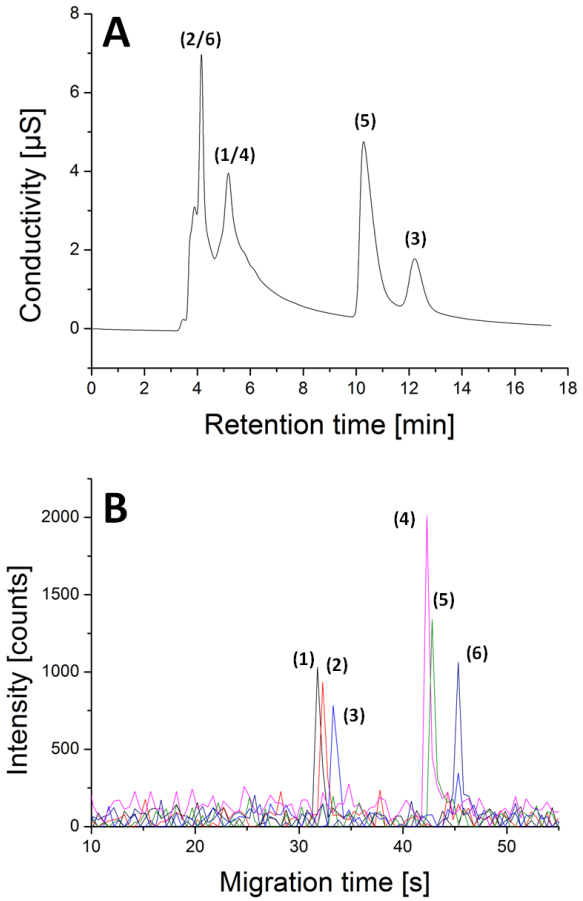


Figure 2: *Separation of the model system using cHPIC (A) and CE (B) at 22.5 kV. Concentrations A: cAMP (1), cCMP (2), and cGMP (3), 100 µM each; AMP (4), GMP (5), and CMP (6), 300 µM each; B: cAMP (1), cCMP (2), and cGMP (3), 50 µM each; AMP (4), GMP (5), and CMP (6), 150 µM each (extracted ion traces are shown).*

A model system comprising a mixture of nucleotides (AMP, GMP, CMP) and cyclic nucleotides (cAMP, cGMP, cCMP) was used as sample as neither cHPIC nor CE could

separate the model analytes. Figure 2 shows the separation results using either the cHPIC or CE as single separation techniques. Both techniques are associated with several coelutions/comigrations under the applied conditions.

Thus, this sample mixture was chosen to study the enhanced performance of the cHPICxCEMS system. Modulation times of the cHPICxCE-MS measurements of this model system were studied and optimized as illustrated in Figure 3.

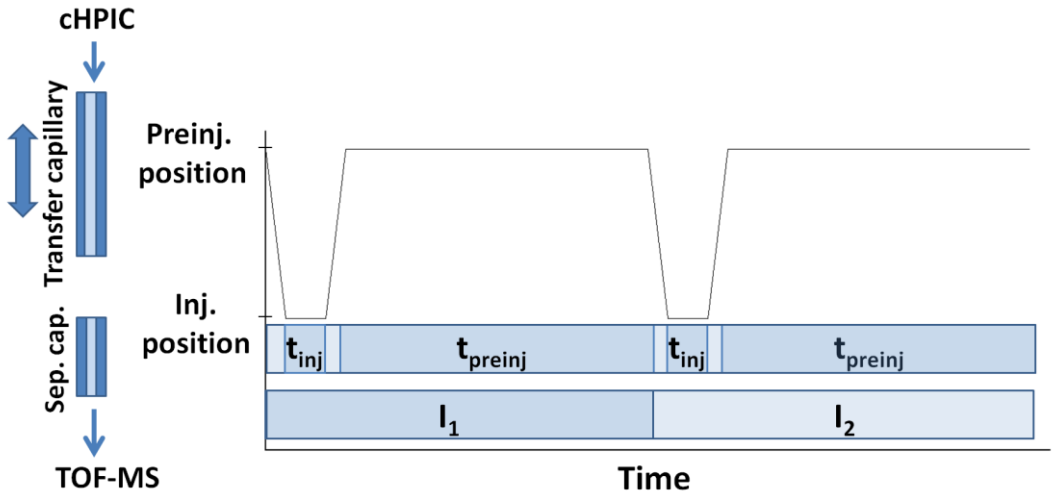


Figure 3: *Scheme for illustration of the modulation process (movement of the transfer capillary toward the separation capillary). One injection interval (I) comprises injection time t_{inj}, preinjection time t_{preinj}, and the time the stepper motor needs to switch between them.*

These experiments were performed without the cHPIC. Instead, a syringe pump (UMP3, WPI, Florida, USA), a microliter syringe (1000 µL Nanofil syringe, WPI), and a fused silica injection capillary (ID = 75 µm) were used. This setup of the CBI was described in detail in previous works [26]. The injection capillary was axially aligned with the separation capillary and moved periodically up and down from inject to preinjection positions while sample was expelled continuously with a flow rate of 5 µL/min. The syringe was filled with a model solution containing 150 µM AMP and 50 µM cAMP in pure water. The CE high voltage source was permanently set to 22.5 kV. For optimization of the injection times, measurements were performed with injection times of 1, 2, 4, and 8 s whereas the preinjection time was kept at 15 s. Furthermore, the preinjection time was varied (60, 30, 20, 15, 10, 6, and 2 s) using an injection time of 2 s in order to optimize the preinjection time. The shortest preinjection time possible without overloading the capillary was 15 s. A further reduction of preinjection times resulted in double peaks,

and separation was not accomplished anymore. Two seconds was chosen as the smallest injection interval possible for this model system, being a compromise between small injection intervals and still having sufficient signal intensities. It has to be pointed out that the protocol for preinjection/ injection depends on the nature of the analytes and peak characteristics of the cHPIC signals. In the case of coelectroosmotically migrating species, shorter preinjection intervals of less than 10 s should be possible [23]. For a more complex mixture of cationic and anionic species, it can be more challenging to optimize the protocol. The cHPIC flow rate was optimized by performing cHPICxCE-MS measurements (AMP, GMP, and CMP, 300 µM each; cAMP, cGMP, and cCMP, 100 µM each) at different flow rates (2, 5, and 8 µL/min). Low flow rates led to peak broadening. Higher flow rates resulted in a better IC separation efficiency. However, the flow rate is one of the determining factors for the amount of substance injected into the CE capillary. The IC effluent mainly consisted of pure water. Thus, the higher the flow rate, the more water was injected into the capillary. Each injection led to a reduction of electrophoretic current, which was recovering during the preinjection time. The drop of current increased with higher flow rates. The electrophoretic current dropped significantly (to 1-2 µA) or broke down completely with flow rates higher than 8 µL/min. This rendered any effort for separation impossible. Thus, 5 µL/min was chosen for all following measurements. At this flow rate, current fluctuations were in an acceptable range with an electrophoretic current of 5.5-7 µA. In contrast to the results achieved with the single separation techniques shown in Figure 2, the optimized cHPICxCE-MS could separate all model analytes in about 15 min. The cHPIC coeluting analytes cAMP and AMP or cCMP and CMP were baseline resolved by CE. Furthermore, cHPIC preseparated cAMP and cCMP and also AMP and GMP, which would otherwise comigrate in CE. For all peaks eluting from the cHPIC system, 5 to 7 electropherograms could be recorded in the second separation dimension. The cHPICxCE-MS measurement was plotted as a contour plot by means of a program developed within our group. The result is shown in Figure 4.

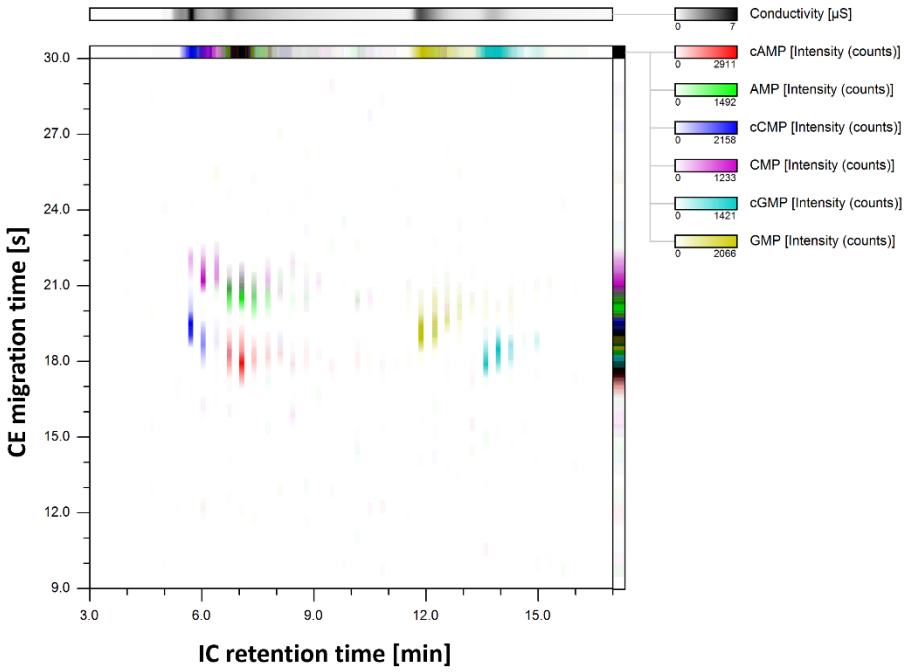


Figure 4: *Multidimensional illustration of the cHPICxCE-MS chromato-electropherogram as contour plot. The MS peak intensities (extracted ion traces) are shown by means of color gradients whereas each substance is assigned to a different color. The additional gray scale bar, which is depicted at the very top, represents the conductivity signal of the first separation dimension (cHPIC) for comparison. The lower bar is a projection of the contour plot on the x axis while the bar on the right represents the projection on the y axis.*

The time passed from cHPIC injection until CE injection is shown on the abscissa while CE migration time is depicted on the ordinate. In the upper part of Figure 4, the conductivity detection response from the first separation dimension (cHPIC) is displayed which provides complementary information to the cHPICxCE-MS data. Furthermore, the effect of sample stacking in cHPICxCE-MS measurements was investigated. Sample stacking in the CE separation capillary occurs when the specific conductivity of the background electrolyte is higher than the specific conductivity of the sample plug [29]. In the cHPICxCE-MS setup, the cHPIC effluent, which was injected into the CE, consisted of analyte zones in pure water due to the suppressor. The effect of stacking during cHPICxCE-MS measurements was examined by means of a setup comparable to cHPICxCE-MS where the IC was replaced by a microsyringe pump. Mixtures of cAMP and AMP (50 µM cAMP, 150 µM AMP) dissolved in water or in background electrolyte were used as samples. The respective sample was filled in the syringe, and the flow rate of the pump was set to 5 µL/min. For the measurements, the high voltage source was

permanently set to 22.5 kV. The optimized modulation times were used. Before the first injection, the capillary was kept for 10 s in the preinjection position to equilibrate the flow of the pump. Then, 5 intervals of injection were performed and the electropherograms were compared. The separation efficiency and peak heights of analytes were significantly enhanced in the case of the sample dissolved in water compared to the sample prepared in background electrolyte as a result of the stacking effect.

3.1.5. Conclusion

In conclusion, a modulator for the comprehensive hyphenation of cHPIC and CE was developed. The characteristics of advanced cHPIC with injection volumes of less than 1 µL and flow rates in the lower µL/min range facilitate the hyphenation with fast CE to form a comprehensive two-dimensional separation system for ionic species. Using a model mixture containing nucleotides and their cyclic derivates, it was demonstrated that the separation performance could be enhanced in the cHPICxCE-MS compared to the single techniques for ion separations. It is a special feature of this hyphenated system that the effluent of the cHPIC consists of pure water as a result of the implemented suppressor technology. This fact had to be considered for the optimization of the injection parameters but could be exploited in terms of sample stacking in the CE separation dimension.

3.1.6. Further experimental details

Fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) were prepared and conditioned as follows. Both ends of the separation capillary (l=20.5 cm, ID=25 µm) and the injection end of the transfer capillary (l=60 cm, ID=75 µm) were polished with polishing papers (32 and 12 µm grit size) at a 90° angle until the surface was smooth. The polyimide coating was removed at a length of about 5 mm. Before each use, the separation capillary was flushed 10 min with 100 mM NaOH, 10 min with ultrapure water from a Milli-Q system, and 30 min with the background electrolyte consisting of a 25 mM ammonium acetate buffer adjusted with ammonia to pH=9.15. The buffer was filtrated before use with a syringe filter (0.2 µm) (Carl-Roth, Karlsruhe, Germany). The

measurements were performed by applying a separation voltage of 22.5 kV. The cHPIC detector/chromatography module was thermally controlled at 10 °C. A Dionex Ion-Swift MAX-200 column (0.25 × 250 mm) with appropriate guard column (0.25 × 50 mm), both operated at 35 °C, was used for anionic separation. Instrument control and data acquisition were performed using Chromeleon 6.8 software. The eluent concentration (KOH) was kept constant at 40 mM hydroxide during a run.

3.1.7. References

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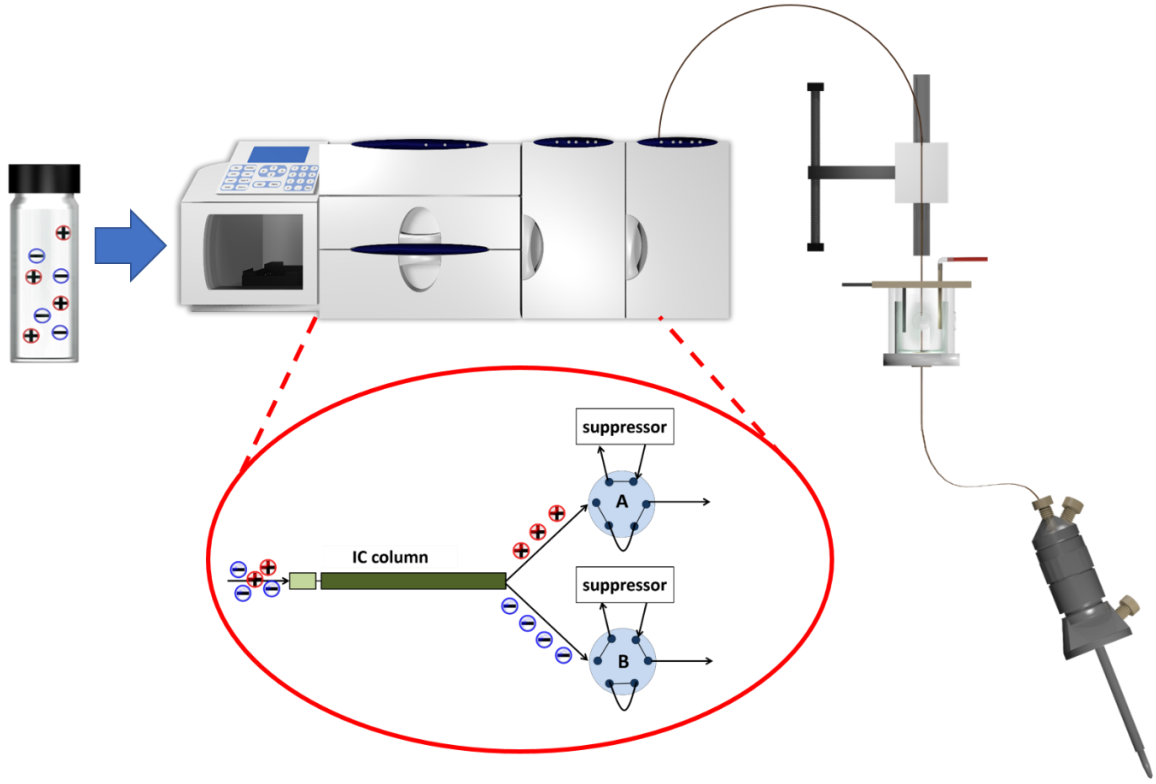
3.2. Methodical studies of the simultaneous determination of anions and cations by ICxCE-MS using arsenic species as model analytes

3.2.1. Abstract



Separating a complex sample mixture is a challenging task in analytical chemistry. Multidimensional systems are widely used to improve the peak capacity. The comprehensive hyphenation of ion chromatography (IC) and capillary electrophoresis (CE) is promising as the two most important instrumental techniques in ion analysis are combined. In this work, a new configuration for capillary anion chromatography is presented enabling the simultaneous ICxCE analysis of anions and cations using a switching valve. Electrospray ionization mass spectrometry (MS) was used for detection. As model system, a mixture of organic and inorganic arsenic species was used. The coupling of anion chromatography to CE-MS was performed via a modulator enabling periodical injection of the IC effluent to the CE. The injection parameters of the modulator were studied taking into account the complex transport situation.

This chapter has been submitted.

Andrea Beutner, Sebastian Karl Piendl, Stefan Wert, Frank-Michael Matysik, Methodical studies of the simultaneous determination of anions and cations by ICxCE-MS using arsenic species as model analytes, Anal. Bioanal. Chem, 2018 .

Author contributions

AB and *SKP* worked together on the ICxCE-MS experiments under guidance of *AB*, while *SKP* was working on the IC part and *AB* on the CE-MS part. *SW* did some of the CE-MS experiments during a research project under the supervision of *AB*. *AB* did the whole CE-MS and ICxCE-MS data evaluation presented in this work. *AB* wrote the manuscript. *FMM* is the corresponding author.

3.2.2. Introduction

The separation of samples consisting of a variety of different analytes such as biological or environmental samples is often challenging. The use of single separation strategies such as gas chromatography (GC), liquid chromatography (LC), or capillary electrophoresis (CE) does not deliver the demanded peak capacity. This drawback is overcome by hyphenation providing multidimensional systems [1-3].

A common combination is the two-dimensional comprehensive GC, which played a pioneering role in the development of multidimensional separation techniques [4, 5]. But also two-dimensional LC [6-8, 2, 9] and CE systems [10-14] are known, respectively. In GCxGC, the term “comprehensive” means that the entire sample separated in the first dimension is transferred to the second dimension [4]. The term was first used by Bushey and Jorgenson [15] to distinguish their dual LCxLC approach from the former heart-cutting techniques as the whole sample was subjected to both dimensions instead of few collected fractions. Nowadays, to distinguish them from former heart-cutting techniques, LCxCE combinations are also called comprehensive even if the LC effluent is split and only fractions are injected into the second dimension. Importantly, the injections to the second dimension have to be that frequent that again the entire sample is represented [16-19].

The combination of LC and electromigration is considered to be very effective due to its orthogonality [20, 21]. However, coupling of these two separation mechanisms is technically challenging as the electrical field of the electrophoresis system has to be isolated from the other compartments [12, 21]. Moreover, compatibility concerning the huge deviations of the flow rates and volumes of the two systems has to be considered. These challenges were solved by a variety of interfacing strategies using valve-based, gated, microfluidic chip-based, membrane-based, and droplet-based interfaces [17, 19, 22-24, 25, 26]. Wang et al. [23] developed a valve-based approach coupling capillary isoelectric focusing to nano reversed phase LC for the analysis of protein digests. An LCxCE device fully integrated on a chip was introduced by Chambers et al. [19]. Recently, Stevenson et al. [27] used a dual reservoir device to perform chromatography and electrophoresis simultaneously. The chromatographic separation runs vertically whereas electromigration was performed in horizontal direction [27].

A special form of LC is ion chromatography (IC). Ions are separated by retention time on an ion exchange column providing high orthogonality to the electrophoretic separation mechanism [28]. Besides CE, IC is one of the most important separation techniques in ion analysis. Thus, the hyphenation of IC and CE is promising. Kar and Dasgupta [29] were the first who developed an online heart-cut coupling concept for IC and CE. Since then, some developments were essential for the comprehensive hyphenation enhancing the compatibility of the two techniques. The development of the IC in capillary scale enables the separation of ions with low flow rates down to 2-10 µL/min being closer to CE flow conditions [30]. Moreover, miniaturized ion suppressors were introduced enhancing the sensitivity of the conductivity detection and generating pure water as IC effluent [31]. CE separations with migration times in the range of seconds were performed using short capillaries and high field strength enabling fast separations in the second dimension [32-35]. Recently, the first comprehensive approach hyphenating capillary IC and CE was reported by Beutner et al. [16]. A modulator was developed based on the concept of capillary batch injection [36]. The IC effluent was brought through a transfer capillary into the modulator. There, it was injected directly into the axially aligned separation capillary of the CE-MS system. To avoid an overload of the capillary the injection was performed sequentially by approaching and retracting the transfer capillary towards and from the CE separation capillary. Moreover, Ranjbar et al. [18] reported a valve-based approach for the comprehensive coupling of IC and a sequential injection-capillary electrophoresis instrument with capacitively coupled contactless conductivity detection (C^4D). Therefore, a six-port two-position valve was used as interface enabling the injection into the second dimension. However, a major drawback of the capillary suppressed IC is the limited flexibility as only anions or cations can be determined simultaneously. In anion exchange chromatography, the cations are not retarded on the column and reach the suppressor with the dead volume. The suppressors commercially available for capillary anion exchange chromatography contain cation exchange membranes. Using for example a common KOH eluent, K^+ ions are replaced by H^+ leading to an effluent consisting of pure water (and anionic counterions). Usually also cationic analytes pass the membrane. Thus, they cannot be determined further in a second dimension.

The toxicity of arsenic, its bioavailability, and transport properties are strongly dependent on its chemical form. Usually, inorganic arsenic compounds are significantly more toxic than organic compounds. Moreover, the oxidation state has an impact on the biological and chemical properties. Therefore, the determination of the total arsenic content provides only limited information about its toxicity rendering an individual speciation analysis essential for risk assessment [37, 38].

In this work, the development of a bypass system for anion chromatography is presented enabling the simultaneous analysis of anions and cations by ICxCE-MS. The ions which were not able to pass the suppressor after IC separation were guided through a bypass to the CE. A method for the determination of a mixture of organic and inorganic arsenic species with different charges serving as model system was developed. The introduction of the IC effluent into the modulator was investigated further and characterized. Detection was performed with ESI-TOF-MS in positive and negative ion mode using an elaborated switching protocol.

3.2.3. Materials and methods

3.2.3.1. Materials and chemicals

Ammonium acetate and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Isopropanol (LC-MS grade) was purchased from Carl Roth (Karlsruhe, Germany). Ultrapure water was obtained from a Milli-Q system. Arsenobetaine and arsenocholine were purchased from Wako Chemicals GmbH (Neuss, Germany). Sodium arsenate and phenylarsonic acid were purchased from Merck (Darmstadt, Germany), disodium methylarsonate and sodium dimethylarsinate were obtained from Sigma Aldrich (Hamburg, Germany). If not indicated differently, the chemicals were of analytical grade.

3.2.3.2. Capillary preparation

Fused silica capillaries purchased from Polymicro Technologies (Phoenix, AZ, USA) with IDs of 25 and 75 µm and an outer diameter of 365 µm were used. Before use, both ends of the separation capillary (ID= 25 µm, l= 22.5 cm) and one end of the transfer capillary (ID= 75 µm, l= 96 cm) were polished in a 90° angle to get a smooth surface using polishing

papers with 32 µm and 12 µm grid sizes. The polyimide coating was removed around 6 mm on the polished ends using a burner. The separation capillary was conditioned before use. Therefore, it was flushed 10 minutes with a 0.1 M sodium hydroxide solution, then rinsed with Millipore water for another 10 minutes, and equilibrated with background electrolyte for half an hour. After use, the capillary was rinsed with water.

3.2.3.3. Instrumentation

The basic ICxCE-MS setup is described elsewhere [16]. It consisted of a capillary ion chromatograph, a modulator with CE system, and a mass spectrometer. The capillary IC was connected to the modulator via a transfer capillary (ID= 75 µm, l= 96 cm) injecting the eluent coming from the IC into the separation capillary of the CE by capillary batch injection [39, 36]. Basically, that means that the transfer capillary was brought axially aligned in a close distance of about 30-50 µm to the separation capillary whereas both capillaries were located in the reservoir containing BGE. Sample was expelled and thus a sample cloud was formed, which was injected into the CE separation capillary. As the constant flow coming from the IC would overload the electrophoretic separation, a modulator was required. Therefore, the transfer capillary was moved periodically up and down by the modulator leading to sequential injection as no injection took place pulling the transfer capillary about 200 µm away from the CE.

A scheme of the overall ICxCE-MS setup with detailed description of the IC connections is depicted in Figure 1. A more detailed scheme of the modulator is depicted in Figure 2.

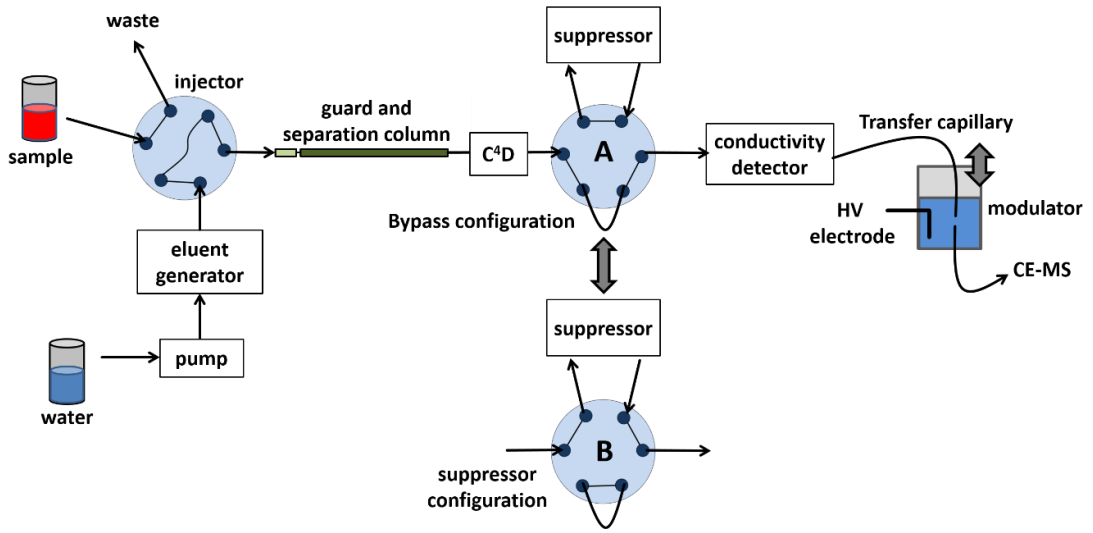


Figure 1: *Scheme of the overall IC x CE – MS setup with detailed description of the IC connections. In configuration A the suppressor is bypassed. In configuration B the suppressor pathway is used. The internal conductivity detector of the IC is not depicted.*

Ion chromatography

An ICS 5000 ion chromatograph (Dionex, Thermo Scientific) was used equipped with an anion exchange IonSwift Max-200 (0.25 x 250 mm) column with guard column (0.25 x 50 mm) in capillary scale. It consisted further of a capillary pump, an eluent generator module (EG KOH 300) containing a KCl cartridge from Dionex/Thermo Fisher Scientific, an injector with an 0.4 µL sample loop, an anion capillary eluent suppressor (ACES), and an internal conductivity detector (CD). The suppressor was regenerated electrochemically using an analytical pump, which is not depicted in the scheme for clarity reasons. The suppressor was connected to the separation column and the conductivity detector via a switching valve resulting in two configurations. Configuration B represented the generally known suppressor configuration, where K^+ ions of the eluent coming from the separation column were replaced by H^+ resulting in an effluent of analyte in pure water. Switching to configuration A, the suppressor was bypassed. Consequently, the effluent was directly pumped into the conductivity detector. In this configuration pure water was used as eluent. Thus, a gradient elution (0-20 mM KOH) was performed adapted to the switching procedure of the two configurations (see Table 1).

For the optimization of the adaption of the switching procedure to the elution gradient, a capacitively coupled contactless conductivity detector (C^4D) was placed right in front of the switching valve. This was achieved by using fused silica capillaries as connectors. Therefore, the switching from configuration A to B could be performed when the KOH

reached the switching valve avoiding KOH bypassing the suppressor. The high-resolution C^4D was constructed in the group of Prof. C. L. do Lago, Sao Paolo, Brazil. It contained a local oscillator operating at 1.1 MHz with a sine wave signal having an amplitude of 4 Vpp [40]. Further details are described elsewhere [40]. The optimized protocol is summarized in Table 1.

Table 1: *Optimized IC protocol adapting the switching procedure between the IC configurations to the elution gradient*

Time	Eluent	Configuration
0 – 7.5 min	0 mM KOH	A (bypass)
7.5 – 11 min	0 – 20 mM KOH (linear)	
11 – 12.5 min		B (suppressor)
12.5 – 20.1 min	20 mM KOH	

Further IC parameters were as follows: the flow rate was set to 5 µL/min, the injection volume was 0.4 µL, and the injection time 30 s. The column temperature was 30 °C. The suppressor ACES300 was operated at 7 mA.

Modulator and capillary electrophoresis

The effluent of the IC is pumped via a transfer capillary (ID= 75 µm; l= 96 cm) to the modulator (see Ref [16] and Figure 2).

Modulator method development and its characterization were performed using a sequential injection CE-MS setup (Figure 2). In this setup, the IC was replaced by a simple syringe pump (1) filled with sample to simulate the constant flow coming from the IC. The syringe pump constantly introduced sample via a short transfer capillary (3) (ID= 75 µm; l= 20 cm) with a flow rate of 5 µL/min into the modulation system. The transfer capillary was fixed on a stepper motor (2) moving periodically in vertical direction to perform the modulation introducing the sample sequentially into the separation capillary (7) (ID= 25 µm; l= 22.5 cm). The modulation frequency was varied and migration times and resolution were calculated. A modulation frequency of one injection (t_{inj}= 1 s) every 10 seconds was found as suitable modulation frequency and was taken for the following ICxCE-MS measurements.

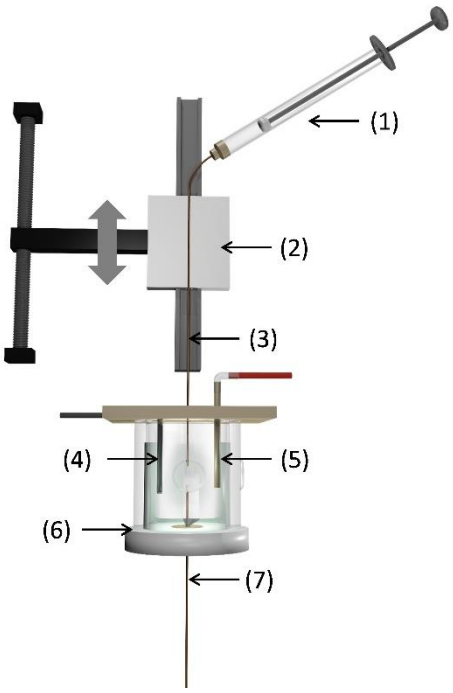


Figure 2: *Sequential injection CE-MS setup. (1) syringe pump (simulating the IC effluent), (2) stepper motor, (3) transfer capillary, (4) HV electrode, (5) stirrer, (6) electrophoretic cell containing BGE, (7) separation capillary.*

For the optimization of the CE conditions, a capillary batch injection setup was used [39]. With help of an injection capillary and a syringe pump small discrete sample volumes can be taken up and introduced into the separation capillary. A flow rate of 20 nL/s was used. The HV was set to 22.5 kV. A BGE containing 25 mM NH_4Ac was chosen and the pH was varied. A pH value of 6.9 was found as optimum and taken for the following ICxCE-MS measurements.

Electrospray ionization-mass spectrometry

For detection, a micrOTOF-MS (Bruker Daltonik, Massachusetts, USA) with a coaxial sheath liquid electrospray interface (Agilent Technologies, California, USA) was used. The following parameters were set: end plate offset: -500 V; nebulizer gas (N_2) pressure: 1.0 bar; dry gas (N_2) flow: 4.0 L/min; dry gas temperature: 190°C; spectra acquisition rate: 5 Hz. The ESI source was operated in positive and negative ion mode. For positive ion mode measurements the following parameter were adjusted: capillary voltage: -4 kV (sprayer tip grounded); capillary exit: 75.0 V; skimmer 1: 25.3 V; hexapole 1: 23.0 V; hexapole RF: 65.0 Vpp; skimmer 2: 23.0 V; lens 1 transfer: 49.0 µs; lens 1 prepulse storage: 5 µs. For negative mode measurements the ESI source was operated using the following parameters: capillary voltage: 4 kV (sprayer tip grounded); capillary

exit: -105 V; skimmer 1: -35.0 V; hexapole 1: -23.0 V; hexapole RF: 75 Vpp; skimmer 2: -23.0 V; lens 1 transfer: 40.0 µs; lens 1 prepulse storage: 10.0 µs.

Positive ion mode measurements were conducted using a sheath liquid consisting of H_2O/isopropanol/formic acid (49.9: 49.9: 0.2, v/v/v). In negative ion mode the sheath liquid contained H_2O/isopropanol/ammonia (49.9: 49.9: 0.2, v/v/v). The flow rate of the sheath liquid was 8 µL/min for both ionization modes.

As some analytes were just detectable in positive ion mode and some in negative ion mode, the polarity (and also the sheath liquid) of the ESI was switched during the ICxCE-MS measurements. Preseparated by anion IC, the positively charged and neutral analytes were eluted before the anions. Thus, the switch from the positive to the negative ion mode was performed after the detection of the cationic and neutral species after about 14 minutes.

3.2.3.4. Sample preparation

For all measurements 50 mM stock solutions of arsenocholine, arsenobetaine, dimethylarsinic acid, methylarsonic acid, arsenate, and phenylarsonic acid in ultrapure water were prepared, respectively and diluted appropriately. For IC and ICxCE-MS measurements the concentrations were as follows: arsenobetaine 250 µM, arsenocholine 50 µM, dimethylarsinic acid 500 µM, phenylarsonic acid 500 µM, methylarsonic acid 500 µM, arsenate 750 µM. For the optimization of the modulator using sequential injection CE-MS, a sample containing 31 µM arsenobetaine and 6 µM arsenocholine was used. For the pH optimization of the CE BGE, a solution containing 25 µM arsenocholine, 125 µM arsenobetaine, and 250 µM dimethylarsinic acid was prepared for the measurements in positive ion mode. The sample for measurements in negative ion mode contained 1 mM methylarsonic acid, 1 mM arsenate, and 500 µM phenylarsonic acid.

3.2.3.5. Software and data evaluation

IC control, data acquisition and evaluation was performed using the Chromeleon 6.8 software. MS control was performed using micrOTOF control 2.3 of Bruker. For data evaluation the Bruker Compass DataAnalysis 4.0 software was used.

For the control and data acquisition of the modulator, a software based on c sharp was developed. It controlled the motors, the HV, the syringe pump, and the stirrer and saved the protocol time-dependent. Moreover, it triggered the start of the TOF data acquisition enabling time synchronizing of the data. This enabled the exact determination of the motor position at any time and thus, injection points and migration times could be calculated.

3.2.4. Results and discussion

3.2.4.1. Setup development- a general discussion

In IC, simultaneous separation of anions and cations is challenging. For the separation of anions, an anion exchange column is used as separation column. Thus, the cations are not retarded and flushed through the column with the dead volume. As eluent generally strong bases such as KOH are used. They exhibit however a large background noise in conductivity detection. Furthermore, the KOH would also disturb the performance of the following CE-MS measurement. Therefore, a suppressor is implemented. The commercially available suppressor for capillary IC is equipped with a cation exchange membrane enabling the replacement of the eluent cations (K^+) by H^+ and thus converting the eluent into water. However, the exchange membrane is not selective for one specific ion and thus, cationic and small zwitterionic species can penetrate the membrane. Consequently, a setup was developed where the suppressor can be bypassed switching a valve. Thus, the separation column was then directly connected to the conductivity detector (Figure 1, configuration A). To avoid that KOH reaches the CD and the modulator, elution in configuration A took place with pure H_2O as eluent. The cations and also zwitterions were not retarded on the anion exchange column and eluted with water. The anions, however, were not eluted leading to a preseparation of anions and cations. After the cations having left the column, a linear KOH gradient was applied separating the anions. The adaption of the switching procedure and the elution gradient was monitored using a C^4D placed before the switching valve. Thus, configuration B was switched to configuration A when the background conductivity in the C^4D was increasing. For the optimized IC protocol see Table 1 in the experimental section.

After preseparation in the IC, the analytes were guided into the CE system via the modulator. There, complementary separation of all analytes was performed as CE was able to separate anions as well as cations. As detector an ESI-TOF-MS was used. One disadvantage of ESI was that some analytes are just ionizable in positive or negative ESI mode, respectively. This means that some analytes are just detectable in positive ion mode and some in negative ion mode depending on their tendency to protonation or rather deprotonation. Concerning the used instrument configuration, switching between the two ionization modes as well as changing to the respective sheath liquid took about a minute. Thus, preseparation of anions and cations in the IC enabled the switching of the two ionization modes from positive to negative (including sheath liquid) during the measurement. After the cations have been detected by the ESI-TOF-MS in positive ion mode (after about 14 minutes), the polarity was changed for the detection of anions.

3.2.4.2. ICxCE-MS method development

Ion chromatography

For the IC, a method for the determination of different inorganic arsenic species of Kochmann and Matysik [41] was adapted to the new setup. Therefore, the flow rate had to be lowered to 5 µL/min ensuring compatibility with the much lower flow rates of the CE system. Furthermore, KOH concentration of the eluent was 20 mM. Using higher concentrations the resolution of the analyte peaks increased [41]. However, using the bypass setup water was required as eluent. A high KOH concentration led to an increase of the equilibration time back to water as eluent. Consequently, 20 mM was taken as it was sufficient to perform the preseparation required to show the feasibility of the overall setup.

The setup was optimized concerning dead volume of the bypass setup. Therefore, a C^4D was placed before the switching valve. Calculating the time difference between the signals appearing in the C^4D and the internal CD placed after the switching valve, the dead volume could be determined for a known flow rate. The minimal additional dead volume of the suppressor configuration was 1.7 µL compared to the bypass configuration.

Capillary electrophoresis

CE measurements with NH_4Ac background electrolytes having different pH values (adjusted by NH_3 or acetic acid) were compared. The model analytes were divided in two groups depending on their retention behavior in IC. Thus, the first group contained arsenobetaine, arsenocholine, and dimethylarsinic acid. This group was called the positive group as the detection was performed in positive ion mode. The second sample consisted of phenylarsonic acid, monomethylarsonic acid, and arsenic acid and was measured in negative ion mode. The results are depicted in Figure 3. A BGE with pH 6.9 was chosen as optimum. In the negative group, a pH of 5.3 was best suited as separation was fastest and all analytes were detectable (Figure 3 B). However, arsenobetaine and dimethylarsinic acid of the positive group overlapped at this pH (Figure 3 A). So pH= 6.9 was chosen as a compromise as all analytes could be separated at this pH.

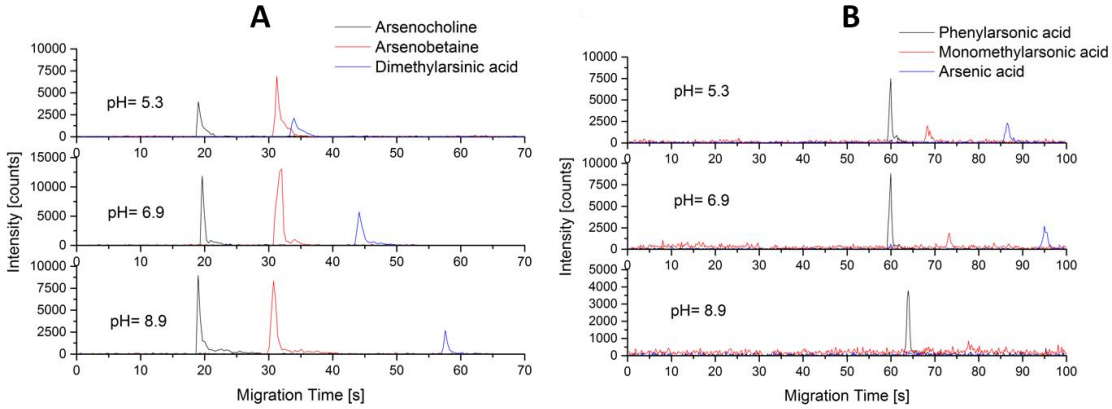


Figure 3: *CE measurements of two standard solutions containing the arsenic species model analytes at different pH values of a 100 mM NH_4Ac BGE. The extracted ion traces are shown. A: Electropherograms of the group detected in positive ESI mode. B: Electropherograms of the group detected in negative ESI mode ($[M-H]^-$). The concentrations were as follows: 25 µM arsenocholine ($[M]^+$, m/z=165.1), 125 µM arsenobetaine ($[M+H]^+$, m/z=179.1), 250 µM dimethylarsinic acid ($[M+H]^+$, m/z=139.0), 1 mM methylarsonic acid ($[M-H]^-$, m/z=138.9), 1 mM arsenic acid ($[M-H_2O-H]^-$, m/z=122.9), and 500 µM phenylarsonic acid ($[M-H]^-$, m/z=201.0).*

As all analytes were separated with CE, no IC separation was necessary for baseline separation in this case. However, the analytes were not detectable with ESI-TOF-MS in one run as some of the analytes were detected in positive ion mode and some were exclusively detectable in negative ion mode. Also the change of the used sheath liquid took about 1 minute for equilibration. Thus, the preseparation with IC enabled the switching procedure of the two ion modes showing the feasibility of the setup.

Modulator characterization

For the comprehensive coupling of two separation techniques, fast separations in the second dimension are required ensuring multiple sampling of the peaks in the first dimension. To decrease the time intervals of the consecutive separations in the second dimension, sequential injection was performed leading to parallel separations of more than one sample plug in one separation capillary. The effect of the decrease of the interval on the separation performance of arsenobetaine and arsenocholine was studied. Therefore, the analytes were pumped into the modulator using the sequential CE-MS setup described in the experimental section (Figure 2) and the waiting time in between the injections was decreased. Exemplarily, parts of the electropherograms for waiting times of 60, 10, and 5 seconds are depicted in Figure 4. Setting a waiting time of 60 seconds, the second sample plug was injected after the first one left the capillary. Using 10 seconds as waiting time, baseline separation was achieved separating four injections in parallel. Decreasing the waiting time to 5 seconds, the limit was reached. The capillary was overloaded and arsenobetaine and arsenocholine could not be separated. Thus, the migration time and the resolution of the analytes was dependent on the injection protocol.

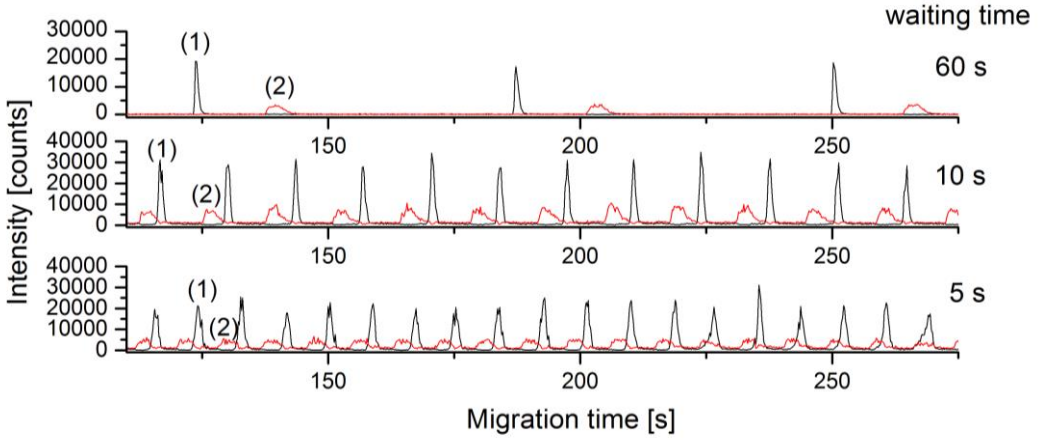


Figure 4: *Electropherograms of sequential injection CE-MS separations using different waiting times between the injections. Extracted ion traces are depicted: (1) arsenocholine 6 µM ($[M]^+$, m/z=165.1), (2) arsenobetaine 31 µM ($[M+H]^+$, m/z=179.1). The BGE was 25 mM NH_4Ac. HV was 22.5 kV.*

In Table 2, the migration times and the resolution of arsenocholine and arsenobetaine are depicted for the different waiting times. For the positively charged arsenocholine, stable migration times were observed for waiting times where the injection interval was longer than the migration times of the two species. Towards lower waiting times, the

migration times were then slightly increasing. The RSDs of the migration times were between 2 and 0.4%. The migration times of the zwitterionic arsenobetaine and the resolution were stable for waiting times greater than 15 seconds and decreased for lower waiting times. Ensuring an appropriate sampling rate, the waiting times should be as low as possible. The minimal suitable waiting time without overloading the separation capillary was 7.5 seconds (see Figure 4). Using this waiting time, a splitting rate of 1:10 of the IC effluent was performed while the transfer capillary was in preinjection position (motor operation times included in the calculations). During injection, another sample split was performed transferring 3% of the sample into the CE separation capillary. This split was necessary as the flow rates of IC and CE are still different even if the use of capillary IC allowed low flow rates of 5 µL/min. It was found that the suitable range for waiting times is dependent on the separated analytes and the separation conditions.

Table 2: *Migration times and resolution of sequential injection CE-MS separations using different waiting times between the injections (n=14). Injection time was 1 s.*

	arsenocholine		**arsenobetaine**		
waiting time [s]	**migration time [s]**	**RSD [%]**	**migration time [s]**	**RSD [%]**	**resolution**
60	28.0	1.9	44.2	1.5	4.8
50	28.6	2.0	45.5	2.1	4.7
40	29.4	1.0	46.5	2.1	5.6
30	30.4	0.7	46.6	1.6	4.9
15	31.2	0.4	43.7	2.1	3.8
10	32.0	0.7	41.1	2.0	3.3
7.5	33.4	1.2	40.1	2.0	2.4
5	34.1	1.2	40.2	1.8	2.8
2.5	31.2	1.4	34.8	3.1	1.9

The characteristics of the modulator were influenced by a complex transport situation and, thus, not easily explainable. The convection in the system introduced by the stirrer and the movement frequency of the transfer capillary determined the amount of sample introduced into the separation capillary. Further, the duration of the injection intervals had an influence on the injected amount. As the solvent of the sample was water, every injection led to an introduction of water into the BGE in the separation capillary causing an inhomogeneity of the electric field. Thus, increasing the injection frequency, the transport situation changed as also the convection and the amount of sample in the capillary increased. Consequently, a compromise between speed and maximum

resilience of the system had to be found. The introduction of water had also an influence on the EOF. Arsenobetaine as a neutral species migrated with the EOF and can be seen as an EOF marker. Thus, an increase in EOF could be observed.

3.2.4.3. ICxCE-MS

A study of a mixture of the arsenic model compounds was performed using the described ICxCE-MS setup. In Figure 5, the raw data of the measurement are shown. The conductivity signal of the internal CD is depicted in the upper graph. At 12.5 min, the baseline change was caused by switching the valve in the IC from the bypass configuration to the suppressor configuration. Arsenocholine, arsenobetaine, and dimethylarsinic acid coeluted in one conductivity peak as expected as they are positively charged or neutral and thus not retarded on the anion exchange column. However, peak broadening was observed for these species. Phenylarsonate and arsenate were separated by the IC and eluted with KOH after switching to the suppressor mode. In the lower graph in Figure 5, the chromato-electropherogram is shown.

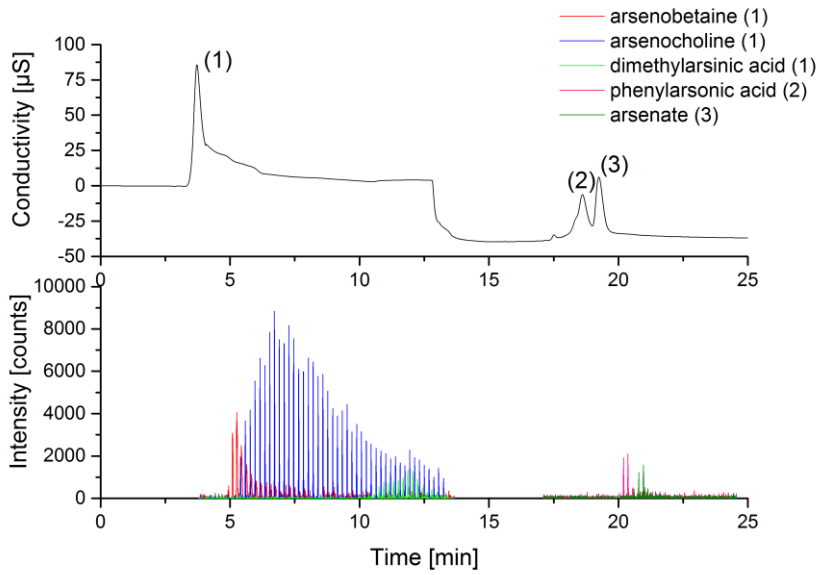


Figure 5: *ICxCE-MS separation of five arsenic species serving as model system. In the upper graph the chromatogram (conductivity signal of the internal CD) after IC separation is shown. In the lower graph the chromato-electropherogram (extracted ion traces) after ICxCE-MS separation is shown. The BGE was 25 mM NH_4Ac. HV was 22.5 kV. The following analyte concentrations were used: 50 µM arsenocholine ($[M]^+$, m/z=165.1), 250 µM arsenobetaine ($[M+H]^+$, m/z=179.1), 500 µM dimethylarsinic acid ($[M+H]^+$, m/z=139.0), 750 µM arsenic acid ($[M-H_2O-H]^-$, m/z=122.9), and 500 µM phenylarsonic acid ($[M-H]^-$, m/z=201.0).*

In Figure 6 the chromato-electropherogram was converted into a three-dimensional contour plot. The y-axes corresponds to the CE migration time, whereas the IC retention time including transfer time is depicted on the x-axes. Thus, summing the times up gives the overall separation time. The dashed line shows the switch from positive to negative ESI mode. The contour plot shows that even if the three positively charged or neutral analytes coeluted in the conductivity detector, the MS detector revealed that arsenobetaine and dimethylarsonic acid were separated by the IC overlapped by a broad arsenocholine peak. Switching to the negative ESI also arsenate and phenylarsonic acid could be detected. As they were eluted with KOH, the suppressor configuration was used for these analytes.

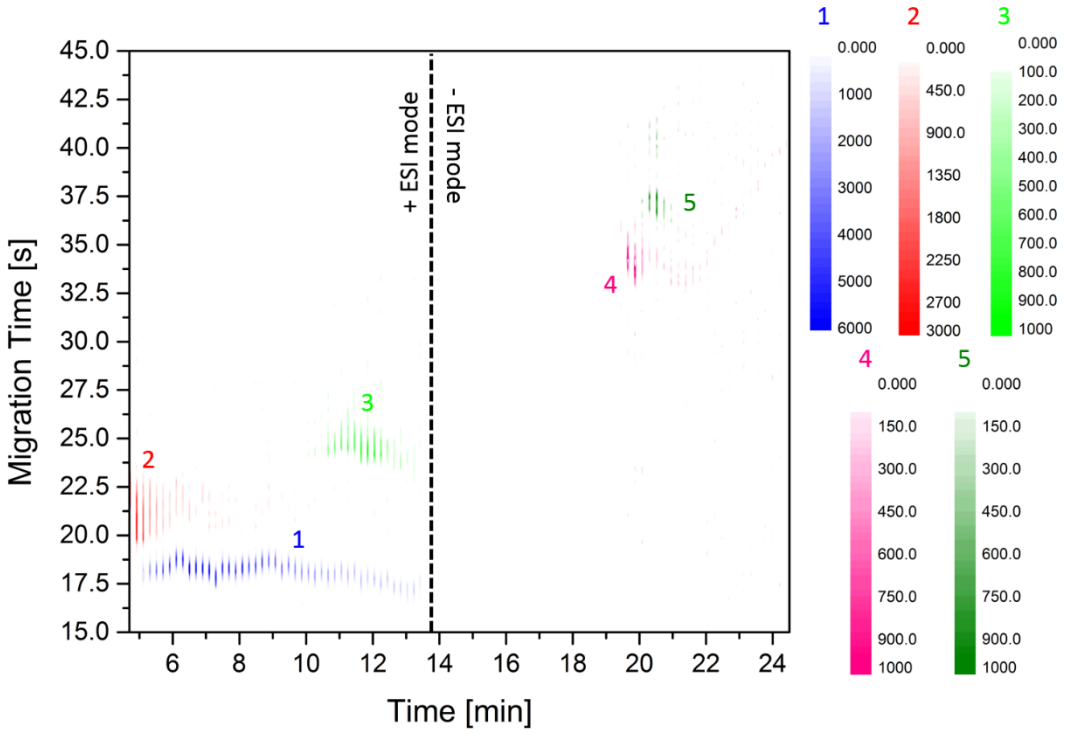


Figure 6: *2D contour plot of the ICxCE-MS separation of five arsenic species serving as model system. The time depicted on the x-axes corresponds to the IC retention time including transfer time. The BGE was 25 mM NH_4Ac. HV was 22.5 kV. Extracted ion traces are shown: (1) 50 µM arsenocholine ($[M]^+$, m/z=165.1), (2) 250 µM arsenobetaine ($[M+H]^+$, m/z=179.1), (3) 500 µM dimethylarsinic acid ($[M+H]^+$, m/z=139.0), (4) 500 µM phenylarsonic acid ($[M-H]^-$, m/z=201.0), and (5) 750 µM arsenic acid ($[M-H_2O-H]^-$, m/z=122.9).*

Notable is further the difference in intensity in the MS between the two groups. This had two reasons. The organic arsenic species were protonated more easily in the ESI as the inorganic species. Moreover, the injection procedure had a discriminating effect depending on the charge. As a high positive voltage (22.5 kV) was applied inlet side

during the measurements, the injection of positively charged species was favored. Consequently, much higher LODs were expected for the negatively charged inorganic species. Methylarsonic acid was not detectable for the measured concentration. A major drawback of the setup was the need of the manual adjustment of the separation and the transfer capillary inside the modulator. Thus, the direct comparison of peak areas of two different measurements was difficult without internal standards (IS). In the contour plot shown in Figure 6, it can be seen that from all peaks coming from the IC at least four segments were injected into the CE. A minimal sampling rate of three injections per peak is recommended [42].

3.2.5. Conclusion

In this work, an approach for the simultaneous analysis of anions and cations with ICxCE-MS was demonstrated. The introduction of a switching valve and a suppressor bypass into the capillary anion chromatography setup enabled the simultaneous determination of anions and cations. With this configuration the cations bypassed the capillary scale suppressor and thus they were not filtered out by the suppressor exchange membrane. The setup was applied to a mixture of different arsenic species. It was shown that it is possible to elute the cations with water from the anion exchange column and introduce them to the CE-MS even if huge peak broadening was observed for arsenobetaine. It was found that the migration times were stable even for high injection frequencies with waiting times that were a factor four shorter than the migration time itself. Intensity was highest for positively charged species caused by the discriminating effect of the electrokinetic injection and the better ionization of the organic arsenic species by the ESI source. Contrary, the intensities for the negatively charged inorganic species were rather poor. However, comparing the intensity of the highest phenylarsonic acid peak in ICxCE-MS to a comparable CE separation, it is just a factor 5 lower not taking into account the multiple ICxCE-MS injections. The study was focused on methodical aspects. The present system had still some limitations in terms of quantitative determinations. The reason here was the need of manual adjustment of the capillary in the modulator before each measurement and the complex transport situation. Thus, for quantitative studies the use of ISs is essential. Therefore, isotope-labeled analytes are recommendable as MS is used as detector.

3.2.6. References

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4. Dual detection combining capacitively coupled contactless conductivity detection and mass spectrometry

4.1. Combining C^4D and MS as a dual detection approach for capillary electrophoresis

4.1.1. Abstract

The hyphenation of two detectors in combination with separation techniques is a powerful tool to enhance the analytical information. In this work, we present for the first time the coupling of two important detectors for capillary electrophoresis (CE), namely capacitively coupled contactless conductivity detection (C^4D) and electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS). The elaborated experimental protocol took into account the requirements of separation aspects and the compatibility with both detectors. ESI-TOF-MS requires background electrolytes (BGE) containing only volatile components such as ammonium acetate or formate. These, however, exhibit a rather high conductivity, which is disadvantageous for C^4D. Thus, the selection of the BGE in an appropriate concentration was undertaken for the determination of various phenolic compounds serving as a model system. The chosen BGE was a 10 mM ammonium acetate/ammonia buffer with a pH of 9. This BGE was a compromise concerning the detection performance of both detectors. The limits of detection (LOD) for m-cresol, m- and p-nitrophenol, and 2,4-dinitrophenol were 3.1 µM (C^4D), 0.8 µM (MS), 0.8 µM (MS), and 1.5 µM (MS), respectively. Moreover, the overall separation efficiency was excellent illustrating that detector-induced band broadening can be neglected in the CE-C^4D/MS system. The analytical characteristics for the determination of phenolic compounds show the suitability of this dual detection approach and demonstrate the complementary use of C^4D and MS detection.

This chapter has been published.

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Author contributions

The experimental work and data evaluation was carried out mainly by *AB*. *RRC* was responsible for the control of the C^4D during the measurements and evaluation of the C^4D data. *AB* wrote the manuscript. The article was revised and discussed by all authors. *FMM* is the corresponding author.

4.1.2. Introduction

CE has become a powerful separation technique due to its high separation efficiency and low sample consumption [1,2]. Capillary electrophoresis with capacitively coupled contactless conductivity detection (CE-C^4D) has gained quite high popularity due to the ease of implementation and low cost [3–5]. Depending on the selected BGE, CE-C^4D provides great potential to perform simultaneous determination of both cationic and anionic compounds [6–11]. C^4D is based on the differences in conductivity of the BGE and analyte zones. It provides a versatile detection approach not depending on structural requirements as in case of UV or fluorescence detection [12–14]. Its linear range covers often high concentrations but the LODs are typically rather high [15]. Moreover, C^4D is not suitable for the identification of the analytes in contrast to MS. The coupling of CE with MS is a well-established hyphenation concept, first introduced by Smith et al. [16]. Since then, it has developed further and can be considered as the ultimate detection technique for CE as it provides higher selectivity than other common detectors such as UV absorbance, laser-induced fluorescence, or electrochemical detection [17,18]. Analytes can be identified providing molecular weight and structural information [18]. Among others time-of-flight mass spectrometry (TOF-MS) is attractive as it offers enhanced resolution, high accuracy of mass determinations, extended accessible mass range, and very high data acquisition rates [17]. The latter aspect is particularly important for fast CE-MS determinations [17, 19]. Despite its high potential there are nevertheless some problems concerning the detection of molecules not amenable to ESI [20]. In addition, for analytes with m/z lower than 50 most TOF-MS devices provide limited sensitivity. The concept of coupling two detectors in conjunction with CE to enhance sensitivity and selectivity is described in various papers. The coupling of fluorescence detection and light scattering was reported by Schrum and coworkers [21]. Moreover, amperometry was coupled to C^4D detection [22, 23], laser-induced fluorescence [24], and electrochemiluminescence [2]. Recently, C^4D was furthermore hyphenated with fluorescence detection [12]. Also a dual electrochemical detection concept [25] and a dual photometric-contactless conductivity detection approach [26] were described. Obviously, hyphenating two different detectors such as C^4D and MS some parameters have to be considered to ensure compatibility [23]. Essential is the right choice of BGE as there are restrictions on both sides. In C^4D mostly BGEs with low

conductivity and relatively high ionic strength are used depending on the conductivity of the analytes to achieve a good signal-to-noise (S/N) ratio [3, 4, 27, 28]. Commonly used buffers are Tris/TAPS, MES/histidine, and boric acid/borate. For CE-ESI-MS, the BGE components should be volatile. Typical examples are ammonium acetate/formate, formic, or acetic acid. They, however, possess high ionic strength and conductivity. Thus, the requests of both detectors have to be compromised. In this work, we present to the best of our knowledge the first approach to couple CE with C^4D and MS in terms of a dual detection concept. This hyphenated separation system is applied to a model system consisting of five phenolic compounds, namely p- and m-nitrophenol, p-chlorophenol, m-cresol, and 2,4-dinitrophenol. It will be shown that C^4D and ESI-MS provide complementary analytical information.

4.1.3. Materials and methods

Ultrapure water obtained from a Milli-Q system was used to prepare all aqueous solutions. Analytical grade ammonium acetate and ammonia (25%) were purchased from Merck (Darmstadt, Germany); ethanol, m-cresol, m-nitrophenol, p-nitrophenol, p-chlorophenol, and 2,4-dinitrophenol from Sigma Aldrich (Missouri, USA).

The overall setup is depicted in Figure 1 consisting mainly of a lab-built capillary electrophoresis device, a C^4D, and a TOF-MS with ESI. The CE capillary **(4)** is guided through the C^4D **(5)** into the ESI source **(6)** of the TOF-MS. Injection into the fused silica capillary was performed by capillary batch injection (CBI). The concept of CBI, thus the injection from one capillary to another, is described elsewhere [19, 29].

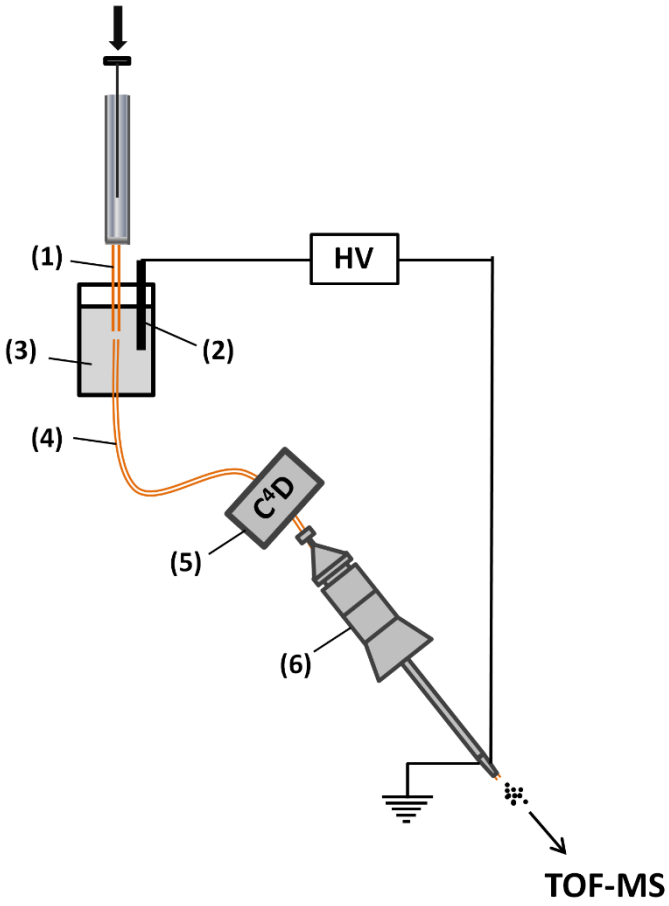

Figure 1: *Scheme of the CE-C^{4}D-MS setup. (1) Injection capillary, (2) high voltage electrode, (3) buffer vial filled with background electrolyte, (4) CE separation capillary, (5) C^{4}D, (6) ESI interface.*

Briefly, the apparatus consists of a vertical and a horizontal positioning unit moving a fixed injection capillary **(1)**. Moreover, it contains an electrophoretic cell **(3)** filled with BGE and equipped with a high voltage electrode **(2)** and a stirrer. The CE separation capillary is located in axial direction on the bottom of the electrophoretic cell. With the help of the positioning units, the injection capillary can be moved toward a sample, which can then be taken up by a microliter syringe (10 µL Nanofil syringe, WPI) using a syringe pump (UMP3, WPI, www.wpiinc.com). Then the injection capillary is moved until aligned with the separation capillary and sample is expelled leading to an injection. Positioning is controlled using a laboratory-modified microscopic video camera (DigiMicro 1.3, dnt, Dietzenbach, Germany). For the electrophoretic separation a fused silica capillary (Polymicro Technologies, Phoenix, AZ, USA) with an ID of 25 µm, an outer diameter of 363 µm, and a length of 28 cm was used. A fused silica capillary (Polymicro Technologies) with ID of 75 µm and outer diameter of 365 µm was used as injection capillary. A high-resolution C^{4}D with a local oscillator, operating at 1.1 MHz using a sine

wave signal with amplitude of 4 Vpp was used as first detector [30]. The C^4D was constructed in the group of Prof. C. L. do Lago, Sao Paulo, Brazil. It was placed along the capillary right before the ESI interface leading to an effective length of 15 cm. A micrOTOF-MS (Bruker Daltonik, Bremen, Germany) with a coaxial sheath liquid electrospray interface (Agilent Technologies, CA, USA) was used as second detector. Nebulizer gas pressure was set to 1 bar. The electrospray voltage was 4 kV. Moreover, the following parameters were set: end plate offset: -500 V; dry gas (N_2) flow: 4.0 L/min; dry gas temperature: 190°C; capillary exit: -105.0 V; skimmer 1: -35.0 V; hexapole 1: -23.0 V; hexapole RF: 75.0 Vpp; skimmer 2: -23.0 V; lens 1 transfer: 40.0 µs; lens 1 prepulse storage: 10 µs; mass-to-charge ratio (m/z) range: 50-550; spectra acquisition rate: 10 Hz. A mixture of 2-propanol, water, and ammonia (49.9:49.9:0.2, v/v/v) was used as sheath liquid at a flow rate of 8 µL/min. Measurements using BGEs with concentrations of 5, 10, and 20 mM of ammonium acetate were performed for optimization of the resolution of the separation and the S/N ratio of the electropherogram of the C^4D. The BGE solutions were prepared by diluting a 100 mM stock solution of ammonium acetate with ultrapure water to the respective concentration adjusting them to a pH of 9.0 using ammonia. A mixture containing 100 µM of m-cresol, p-chlorophenol, m-nitrophenol, 2,4-dinitrophenol, and p-nitrophenol was used to demonstrate the complementary use of C^4D and MS detection. It was freshly prepared just before the experiments diluting stock solutions of 50 mM with ultrapure water. The stock solutions of m-cresol, p-chlorophenol, m-nitrophenol, and p-nitrophenol, respectively, were prepared in ultrapure water. A 50 mM 2,4-dinitrophenol solution was prepared in a mixture of water and ethanol (1:1). All solutions were filtered through a syringe filter (pore size of 0.20 µm, Carl-Roth, Karlsruhe, Germany) before use. The separation capillary was conditioned with 0.1 M NaOH and ultrapure water for 10 min, respectively, and finally BGE for 30 min at every buffer change. With optimized parameters a set of standard solutions containing m-cresol, m-nitrophenol, p-nitrophenol, and 2,4-dinitrophenol was measured and evaluated for calibration. Standard solutions were prepared by dilution of the stock solutions in water to concentrations of 12.5, 25, 50, 75, 100, and 150 µM. To all standard solutions 150 µM p-chlorophenol was added as an internal standard (IS). Moreover, the influence of the stacking effect on the performance of both detectors was investigated.

For this study, a sample containing 100 µM of each model compound and 150 µM of IS was prepared in water and BGE, respectively.

4.1.4. Results and discussion

Three different concentrations (5, 10, and 20 mM) of BGEs were taken in consideration in order to meet the requirements of C^4D and MS detection. For C^4D, a BGE with low conductivity (reduced Joule heating effect) and a relatively high ionic strength (reduction in peak dispersion) is considered as an optimal condition [3, 27]. However, using dual detection there is limited choice as only buffers with volatile components show good compatibility to ESI-MS. These buffers, however, mostly consist of ions exhibiting relatively high conductivity such as ammonium, acetate or formate. The optimum pH for the separation of the model system was found to be 9. Therefore, ammonium acetate buffers of pH=9 with varying electrolyte concentrations were used to examine the influence of buffer composition on the S/N ratio of the C^4D electropherogram.

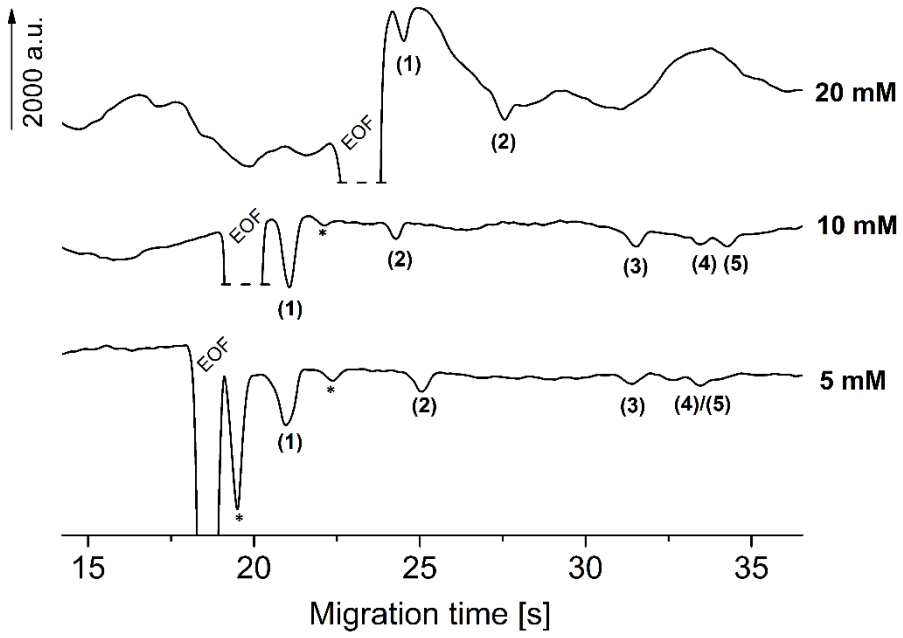

Figure 2: *Influence of the BGE concentration on the analytical performance of C^4D. CE-C^4D recordings of a standard solution containing 100 µM of m-cresol (1), p-chlorophenol (2), m-nitrophenol (3), 2,4-dinitrophenol (4) and p-nitrophenol (5) are shown (* system peak). As BGE 5, 10 and 20 mM solutions of ammonium acetate adjusted with ammonia to pH 9.0 were used. The applied separation voltage was +25 kV (injection site). The overall length of the capillary was 28 cm (equals effective length to MS) with an effective length to the C^4D of 15 cm.*

Figure 2 shows CE-C^{4}D measurements for different concentrations of ammonium acetate (5, 10, and 20 mM). A concentration of 10 mM of ammonium acetate was found to be best suited for the study of the model system. At higher concentrations (20 mM) the S/N ratio decreased drastically due to the high conductivity. At lower concentrations (5 mM) comigration of p-nitrophenol and 2,4-dinitrophenol were found. Moreover, peak shape was best using a BGE concentration of 10 mM. With higher BGE concentrations, the migration time also increased due to a reduced EOF as the zeta potential decreases with ionic strength increase. With selected parameters a sample containing 100 µM of the model compounds and 150 µM of p-chlorophenol (IS) was separated by CE and detected by C^{4}D-MS (Fig. 3).

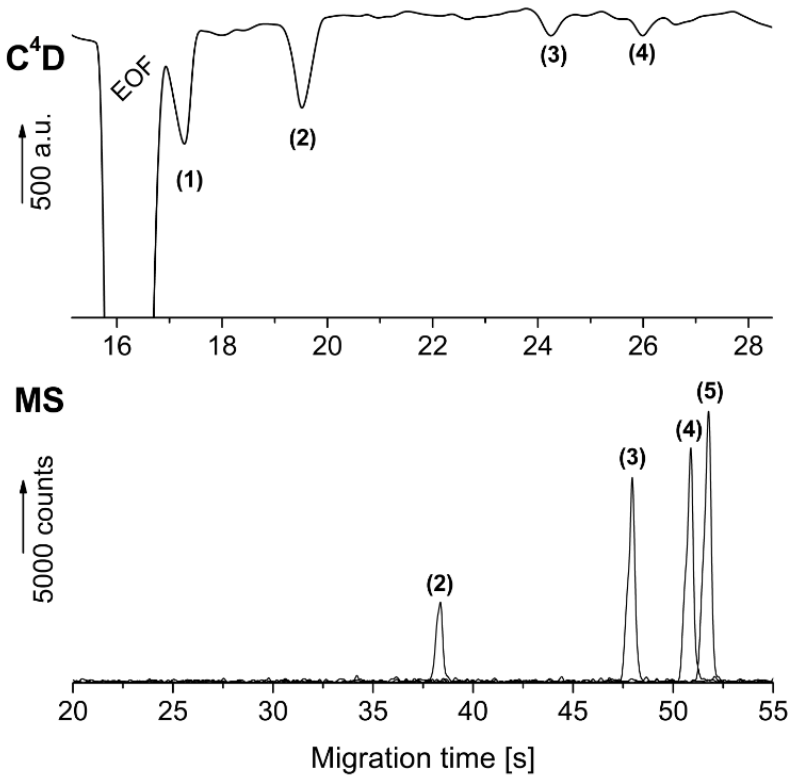

Figure 3: *Dual detection (C^{4}D/MS, selected ion traces [M − H]$^-$ are shown) of the CE separation of a standard solution containing 100 µM of m-cresol (1), m-nitrophenol (m/z=138.01) (3), 2,4-dinitrophenol (m/z=182.99) (4), and p-nitrophenol (m/z=138.01) (5) and 150 µM of p-chlorophenol (m/z=126.99) (2). Separation voltage, +23.5 kV (injection site); capillary length, 28 cm; effective capillary length to C^{4}D, 15 cm. BGE composition, 10 mM of ammonium acetate adjusted with ammonia to pH 9.0.*

An obvious difference between the C^4D and MS response is the enormous signal decrease in C^4D for the water zone (EOF) while MS detection is very insensitive to the water zone, which makes it even difficult to use it as an EOF marker. The analyte m-cresol and p-chlorophenol showed good intensity in C^4D. In case of m-nitrophenol, 2,4-dinitrophenol, and p-nitrophenol poor sensitivity was obtained by C^4D. Contrary, m- and p-nitrophenol and 2,4-dinitrophenol showed very good intensity in the TOF-MS, whereas the sensitivity for m-cresol detection was very poor. As p-chlorophenol was well detectable with both devices, it was chosen as IS for the construction of calibration curves. Comparing the migration times of the species in C^4D and MS, the difference in migration time is in good agreement with the ratio of the effective capillary length for both detectors. As an example the migration time of p-chlorophenol was 19.5 s in the C^4D (effective length of 15 cm) and 38.3 s in case of MS detection (effective length of 28 cm). Standard solutions containing increasing concentrations (12.5; 25; 50; 75; 100, and 150 µM) of m-cresol, m-nitrophenol, 2,4-dinitrophenol and p-nitrophenol, and 150 µM of p-chlorophenol as IS were measured to construct calibration plots.

Table 1: *Analytical characteristics of CE-(C^4D/MS) determination of various phenolic compounds*

	m-cresol		**m-nitrophenol**		**2,4-dinitrophenol**		**p-nitrophenol**	
	C^4D	**MS**	**C^4D**	**MS**	**C^4D**	**MS**	**C^4D**	**MS**
Concentration range [µM]	12.5-150	-	-	12.5-150	-	12.5-150	-	12.5-150
LOD[a] [µM]	3.1	100	50	0.4	75	1.5	50	0.8
R^2	0.995	-	-	0.997	-	0.970	-	0.985
RSD[b] [%] (n=3)	14.5	-	-	6.1	-	7.1	-	5.2

(a) LOD: Peak height equal three times the S/N ratio.
(b) RSD for an analyte concentration of 75 µM.

Table 1 shows the analytical characteristics obtained from these studies for both, C^4D and MS detection. The calibration function was obtained by plotting the normalized areas against concentration. Normalization was performed dividing the respective area through the area of the IS. Moreover, the LODs of the respective compounds using different detectors were compared. The compound m-cresol could not be detected with MS for concentrations lower than 100 µM. However, it showed good sensitivity in the C^4D with a LOD of 3.1 µM. Contrary, the LODs of the nitrophenols were rather high in C^4D. In these cases no calibration could be made without overloading the separation capillary. Thus, for the calibration of nitrophenol determinations the MS was used having LODs equal to or lower than 1.5 µM. Coupling C^4D-MS enabled the quantification of all analytes overcoming the lack of sensitivity of the detectors for the respective analytes. Furthermore, sample stacking and peak broadening were investigated. For this study, two solutions of a mixture of model compounds (one dissolved in water, one in BGE) containing 100 µM of m-cresol, m-nitrophenol, 2,4-dinitrophenol, and p-nitrophenol and 150 µM of p-chlorophenol were used. Comparing the BGE sample and the water sample the peak width at half height of the latter one was 0.3 seconds (for p-chlorophenol) whereas for the BGE sample it was 0.6 seconds. Furthermore, comparing the peak widths of the same compound at both detectors, no significant difference could be observed between C^4D and MS detection. This indicates that both detectors have almost no contribution to band broadening.

4.1.5. Concluding remarks

In this work, we demonstrated for the first time the coupling of CE with dual C^4D/MS detection. The system was optimized and evaluated using a mixture of phenolic compounds as a model system overcoming the limitations of BGE choice caused by the different requirements of the C^4D and MS. As a compromise, a 10 mM ammonium acetate buffer was selected, ensuring an acceptable S/N ratio of the C^4D response and good compatibility with MS detection. C^4D offered good sensitivity towards m-cresol but was rather insensitive for determinations of m-nitrophenol, 2,4-dinitrophenol, and p-nitrophenol. In contrast ESI-TOF-MS detection showed the opposite response behavior. The calibration plots were constructed using the best suited detector for each compound. Fast CE-C^4D/MS measurements of counter-electroosmostic species with

migration times of less than 60 s could be realized with excellent separation efficiency. Both detectors were nearly free from detector-induced band broadening effects. The dual detection arrangement of C^4D/MS provides an attractive complementary approach for CE studies.

4.1.6. References

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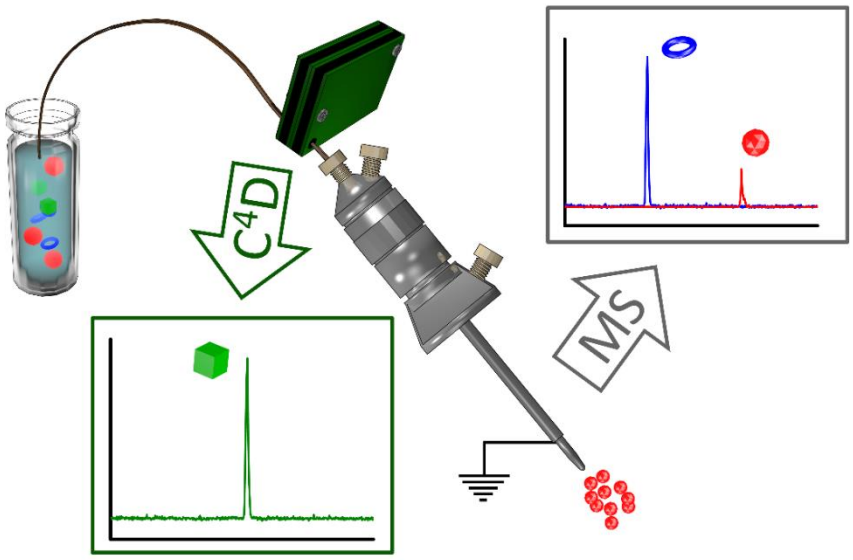
4.2. Dual detection for non-aqueous capillary electrophoresis combining contactless conductivity detection and mass spectrometry

4.2.1. Abstract



Coupling of two detectors is a powerful tool to enhance the overall analytical performance generating complementary information and overcoming the limitations of the single detectors. In this work, capacitively coupled contactless conductivity detection (C^4D) and electrospray ionization mass spectrometry (ESI-MS) were coupled in conjunction with non-aqueous capillary electrophoresis (NACE). Non-aqueous electrolytes are highly compatible with ESI due to their volatility. Moreover, they exhibit low background conductivity, which is essential for the detection with C^4D. A NACE-C^4D-MS method was developed using an acetonitrile buffer containing 2 M HAc and 4 mM NH_4Ac as background electrolyte. The influence of the inner diameter (ID) of the separation capillary on the C^4D was studied and taken into account. A capillary with 50 µm ID was found to be best suited.

The complementarity of the two detectors was shown by determining a sample mixture containing choline, thiamine, nitrate, and chloride as well as bromide and acetylcholine as internal standards (ISs). The C^4D was the detector of choice for the inorganic ions, which were not detectable with the MS whereas the MS had much lower limits of detections for the organic biomolecules. The method was applied on an extract of a food supplement containing the model analytes.

This chapter has been published.

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Author contributions

The experimental work was carried out by *AB*. *BS* was a master student assisting in some experiments under supervision of *AB*. *AB* wrote the manuscript. Results were discussed by *AB* and *FMM*. *FMM* is the corresponding author.

4.2.2. Introduction

Capillary electrophoresis (CE) is a powerful technique combining high separation efficiency, speed, and low sample consumption [1, 2]. A detection strategy to enhance the overall analytical performance is the coupling of two complementary detectors. Thus, the power of both detectors is combined and their weaknesses are compensated. Various detectors were connected to each other such as fluorescence to light scattering [3] or capacitively coupled contactless conductivity detection (C^4D) [4], amperometry to C^4D [5, 6], laser-induced fluorescence [7], or electrochemiluminescence [2]. Moreover, a dual electrochemical detection concept [8] and a dual photometric-contactless conductivity detection approach [9] were introduced. Recently, the two first studies of a dual detection concept of C^4D and mass spectrometry (MS) for CE were presented independently [10, 11] showing the complementary information provided by the two detectors. Beutner et al. [10] applied the developed CE-C^4D-MS system to a model system of five phenolic compounds in aqueous solution. Francisco and do Lago [11] determined sugars in background electrolytes (BGE) with high pH values and biogenic amines in low pH BGEs. C^4D in combination with MS was already used before [12, 13]. However, it was not used for complementary quantitative analysis. In these studies C^4D served for the optimization of the timing of voltage switching.

Using a dual C^4D-MS detection approach, it is important that the CE parameters meet the requirements of both detectors. In terms of BGE, this means on the one hand the need of low background conductivity and high ionic strength for the C^4D [14], but also on the other hand the requirement of high volatility for the electrospray ionization (ESI) process. Non-aqueous separation media satisfy the conditions of both detectors. They possess low electric conductivity and their electric properties can easily be optimized by appropriate conductive additives [15]. Furthermore, due to their low surface tension and high volatility they form stable ESI sprays. Also the ionization process is facilitated for certain applications resulting in higher MS sensitivities [16-18].

In non-aqueous capillary electrophoresis (NACE) a variety of organic solvents is used instead of water in conventional aqueous buffer systems providing a wide range of physicochemical properties such as relative permittivity, viscosity, autoprotolysis

constant, polarity, and volatility [19, 20]. Changing the solvent, selectivity, resolution, and migration time can be adjusted. For very fast separations, acetonitrile is attractive enabling very high electroosmotic velocities [19, 21, 22]. The use of organic solvents leads further to a reduction of electrophoretic current and the ability to separate also water insoluble or structurally very similar compounds [18-20].

NACE was already hyphenated to C^4D and MS separately. C^4D is known as universal detection enabling the determination of small inorganic ions as well as organic ions. Its success is based on its robustness, minimal maintenance demands, and low costs [23]. Moreover, C^4D opens the opportunity to perform the direct analysis in UV absorbing solvents contrary to UV detection [15, 24-26]. Thus, different solvent studies were performed by various groups [24, 25, 27, 28]. Buglione and coworkers [24] analyzed fatty acids in commercial and home-made edible oil using NACE-C^4D. Wang and Pumera [26] performed NACE-C^4D measurements using a microchip to monitor separations in UV-absorbing solvents. Moreover, the use of ionic liquids in NACE was investigated by Vaher and coworkers [15, 29]. A drawback of C^4D detection is the generally relatively high limit of detection (LOD). Buglione and coworkers [30] achieved comparatively low LODs using a deoxycholate salt in a methanol-acetonitrile solvent mixture, however, for unknown reasons. Furthermore, another drawback is the lack of identification of the analytes in more complex mixtures.

Contrary, compared to the C^4D, the MS has higher selectivity and the ability of identification providing molecular mass and also structural information [31, 32]. Time-of-flight mass spectrometry (TOF-MS), inter alia, offers enhanced resolution, accuracy of mass determinations, and very high data acquisition rates enabling the detection of fast CE-MS measurements [32, 33]. The coupling of NACE-MS was first published by Naylor et al. [18, 34] in 1994 for the analysis of metabolites. The high volatility of the organic solvent ensures high compatibility with the ESI and can improve the ionization efficiency [35]. Water insoluble compounds can further be analyzed without MS incompatible additives. NACE-MS was also used for the separation of complex alkaloid mixtures in difficult matrices [36, 37], whereas also a large number of diastereomeric compounds were separated [35]. Further applications are summed up in the review of G. Scriba [18]. However, contrary to the C^4D, most TOF-MS systems have limitations concerning the determination of analytes with low m/z such as inorganic anions.

Moreover, the ionization efficiency of the ESI and, thus, the sensitivity of the ESI-TOF-MS is analyte depending [38]. Therefore, a dual detection approach combining C^4D and MS can overcome the drawbacks of the single detection technique enabling a universal simultaneous analysis and identification of most analytes.

A dual detection approach for NACE was performed by Yuan and coworkers [39, 40]. They used electrochemiluminescence and electrochemical detection simultaneously for the analysis of tertiary amines [39] and for the determination of atropine, anisodamine, and scopolamine in plant extract [40].

In this work, NACE was coupled with a dual C^4D-MS detection system. As BGE an acetonitrile based buffer was used enabling fast separations. NH_4Ac was used as electrolyte due to its volatility ensuring compatibility with the ESI-TOF-MS. The dual detection concept was applied to a model system containing choline, thiamine, chloride, and nitrate determining inorganic ions and biomolecules simultaneously. Moreover, the content of the analytes in a food supplement was determined.

4.2.3. Experimental

4.2.3.1. Materials and Methods

Ammonium acetate, acetonitrile (HPLC grade for liquid chromatography), formic acid (99 %), and sodium hydroxide were purchased from Merck (Darmstadt, Germany). Glacial acetic acid (99,8 %), nicotinamide, acetylcholine chloride, sodium bromide, choline chloride, and thiamine nitrate were provided by Sigma Aldrich (Missouri, USA). Isopropanol (LC-MS grade) was purchased from Carl Roth (Karlsruhe, Germany). Ultrapure water was obtained from a Milli-Q system. If not indicated differently the chemicals were of analytical grade.

4.2.3.2. Instrumentation

For fast non-aqueous electrophoretic separations, a laboratory built miniaturized CE device was used enabling fast high-throughput measurements with low sample consumption using short capillaries and high field strengths. The setup and its detailed characterization were recently described by Schmidberger et al. [41]. The portable miniaturized device (12 cm x 12 cm x 26 cm) basically consists of a sample tray moved

by two motors and an HV electrode (polished syringe needle) connected to a HV source (ESEG, Spezialelektronik GmbH, Dresden, Germany) delivering up to 30 kV. The capillary is guided through a syringe needle serving as HV electrode, which is fixed on a holder. Thus, the only movable part of the system is the turntable of the sample tray, which is moved by two motors. Lifting the sample tray up, the capillary is immersed in the vials performing injection or electrophoretic separation. Rotation to other vials is enabled driving the sample tray down. Injection is performed hydrodynamically by adjusting a height difference of 12 cm between the capillary inlet and outlet and by the suction pressure of the ESI source. The instrument was controlled by a LabVIEW-based software.

As first detector, a high-resolution C^4D was used constructed in the group of Prof. C. L. do Lago, Sao Paolo, Brazil. It was equipped with a local oscillator operating at 1.1 MHz with a sine wave having an amplitude of 4 Vpp [42]. The gap between the two ring electrodes was 0.51 mm. Further details are described elsewhere [42]. It was placed half a centimeter before the fixing screw of the ESI interface.
The second detector was a micrOTOF-MS (Bruker Daltonik, Bremen, Germany) with a coaxial sheath liquid electrospray ionization interface (Agilent Technologies, CA, USA). It was operated in positive ion mode with an electrospray voltage of -4 kV (sprayer tip grounded). A mixture of 2-propanol, water, and formic acid (49.9:49.9:0.2, v/v/v) was used as sheath liquid at a flow rate of 8 µL/min. The nebulizer gas pressure was 1 bar. The MS parameters were set as follows: end plate offset: -500 V; dry gas (N_2) flow: 4.0 L/min; dry gas temperature: 190°C; capillary exit: 75.0 V; skimmer 1: 25.3 V; hexapole 1: 23.0 V; hexapole RF: 65.0 Vpp; skimmer 2: 23.0 V; lens 1 transfer: 49.0 µs; lens 1 prepulse storage: 5 µs; mass-to-charge ratio (m/z) range: 50-550; spectra acquisition rate: 5 Hz.

4.2.3.3. Capillary preparation

Fused silica capillaries purchased from Polymicro Technologies (Phoenix, AZ, USA) with an ID of 15, 25, 50, and 75 µm and an outer diameter of 365 µm were used. The exact lengths and effective lengths are specified in the respective experiments. Before use, both ends of the capillary were polished in a 90° angle to form a smooth surface avoiding any disturbances. Therefore, polishing papers with grid size 32 µm and 12 µm were used. The polyimide coating was removed around 5 mm on both ends using a burner. Before

the measurements, the capillary was conditioned following a defined protocol. First, they were flushed 10 minutes with a 0.1 M sodium hydroxide solution, then rinsed with Millipore water for another 10 minutes, and equilibrated with BGE for half an hour. After use, the capillaries were filled with water for storage.

4.2.3.4. Sample preparation

As BGE, a 4 mM NH_4Ac solution in 2 M HAc/ACN was used. It was prepared diluting a 50 mM NH_4Ac stock solution. The acetonitrile was degassed overnight in a desiccator under reduced pressure (50 mbar) to avoid bubble problems during the measurements. BGEs with varying NH_4Ac concentrations used for optimization measurements were prepared the same way. Moreover, 10 mM standard solutions of nicotinamide, acetylcholine chloride, sodium bromide, and choline chloride were prepared dissolving the respective amount of analyte in 2 M HAc/ACN. A 5 mM thiamine mononitrate solution was prepared using water as a solvent. All solutions were prepared weekly and stored at room temperature.

Solutions for calibration were prepared varying the concentrations of choline, chloride, and thiamine mononitrate. The ISs (acetylcholine chloride and sodium bromide) and the EOF marker, nicotinamide, were added to every calibrant in the same amount. As solvent 2 M HAc/ACN was used. This resulted in the following final sample concentrations: 25 µM of acetylcholine (IS), 125 µM of nicotinamide (EOF), 150 µM of bromide (IS), 50-225 µM of chloride, and 25-200 µM of choline, thiamine, and nitrate, respectively. The calibration solutions were prepared daily.

As real sample, the food supplement “Vitamin B-Loges” (Dr. Loges + Co. GmbH, Winsen, Germany) obtained from a local pharmacy was investigated. First, a tablet was ground homogenously using pestle and mortar. To ensure maximum extraction rate, solubility in the media was tested for the single analytes. Moreover, the expected concentration at 100 % extraction efficiency was calculated to weigh a suitable amount of sample. To avoid high solvent waste rather small aliquots were taken. Thus, 5 aliquots were weighed (2.0-2.4 mg) using a microbalance. All samples were spiked with sodium bromide and acetylcholine used as IS. Then, 2 M HAc/ACN: H_2O (95:5) was added to a total volume of 10 mL. The analytes were extracted in an ultrasonic bath at 40 °C for 2h.

The supernatant was taken and filtered using a syringe filter with pore size 0.45 µm (Carl Roth, Karlsruhe, Germany).

4.2.3.5. Data evaluation

C^4D peaks were integrated using the peak analyzer tool of Origin. MS data was evaluated using the Bruker Compass DataAnalysis 4.0 software. Areas of the MS peaks were normalized by the areas of the IS acetylcholine, areas of the anions detected with the C^4D were normalized with bromide to address the precision of the injections. For that, the areas of the analytes were divided by the respective IS.

4.2.4. Results and discussion

4.2.4.1. NACE-C^4D-MS method development

For method development the influence of the ID of the separation capillary on the C^4D sensitivity was investigated. Therefore, the signal-to-noise (S/N) ratios of the C^4D signals of different IDs of the separation capillary at various NH_4Ac concentrations were determined. Capillaries (*l*= 20 cm) with IDs varying from 15 µm to 75 µm were flushed with an acetonitrile based BGE containing 2 M of acetic acid and ammonium acetate concentrations ranging from 0 to 50 mM. When the baseline was stable, the conductivity was measured for 0.5 min and the mean and the standard deviation were determined. The difference of the mean of the measured conductivity and the conductivity determined for a BGE containing 0 mM NH_4Ac was calculated and divided by the standard deviation to obtain the S/N ratio (Figure 1).

Generally, for the calculation of the S/N ratio the signal of an analyte is divided by the noise of the baseline. However, in this study the difference between the conductivities of BGEs containing no or rather a defined concentration of NH_4Ac was taken as signal simulating conductivity changes of an analyte. In other words a slightly higher or lower BGE concentration can be interpreted as the corresponding conductivity change due to the presence of an analyte zone. Thus, this procedure enabled scanning over a wide conductivity range. Moreover, noise differences using BGEs with varying concentrations and capillaries with different IDs also influence the LODs. Thus, they were also

considered in the calculations. However, it was surprisingly found that the noise did not change significantly in dependence on capillary ID or conductivity of the BGE.

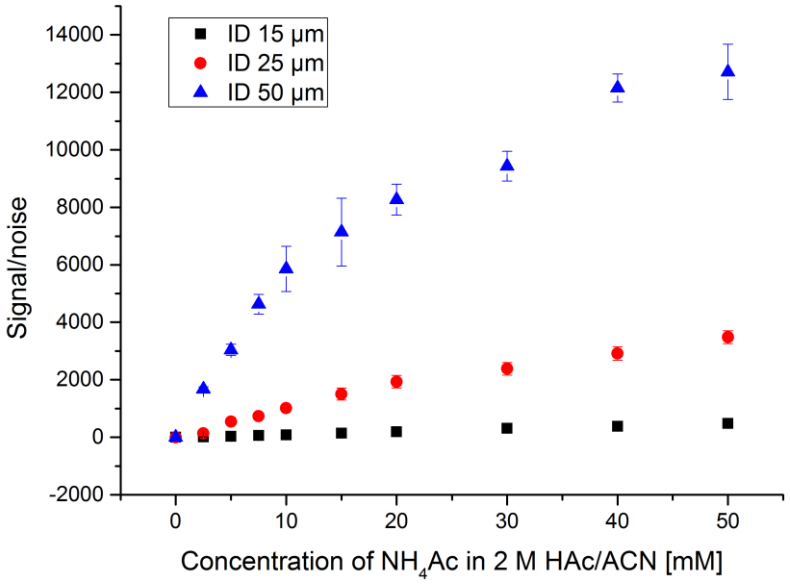

Figure 1: *Dependence of the S/N ratio of C^4D on the background electrolyte concentration for different IDs of the separation capillary. The signal of the background electrolyte with c(NH_4Ac)= 0 mM was taken as baseline.*

The capillary with an ID of 50 µm was found to be best suited concerning sensitivity for the studied conductivity range compared to the capillaries with IDs of 15 and 25 µm. The S/N ratios of the capillary with an ID of 50 µm were about a factor 4-9 higher than with an ID of 25 µm resulting in higher LODs for the capillary with an ID of 25 µm. Using a capillary larger than 50 µm, the S/N ratio increased further for very low concentrations (2.5 mM NH_4Ac). However, for higher electrolyte concentrations the signal stability was poor so that for further experiments the capillary with an ID of 50 µm was chosen. Using the capillary with an ID of 50 µm, the linear range is best in a concentration range between 0 and 10 mM with the highest slope of the S/N with increasing NH_4Ac concentration. For higher concentrations deviations of the linearity were observed as the slope was smaller.

For method development, a model system containing choline chloride, thiamine nitrate, and nicotinamide was used. Acetylcholine chloride and sodium bromide were added as

ISs. Acetylcholine was chosen as it is structurally very similar to choline. BGEs with different NH_4Ac concentrations ranging from 2.5 mM to 10 mM were prepared to investigate the compatibility with the C^4D. It was found that stability of the baseline decreased with increasing NH_4Ac concentration. However, the separation of the model system was improved with increasing electrolyte concentration. Therefore, a buffer containing 4 mM NH_4Ac was chosen for further experiments.

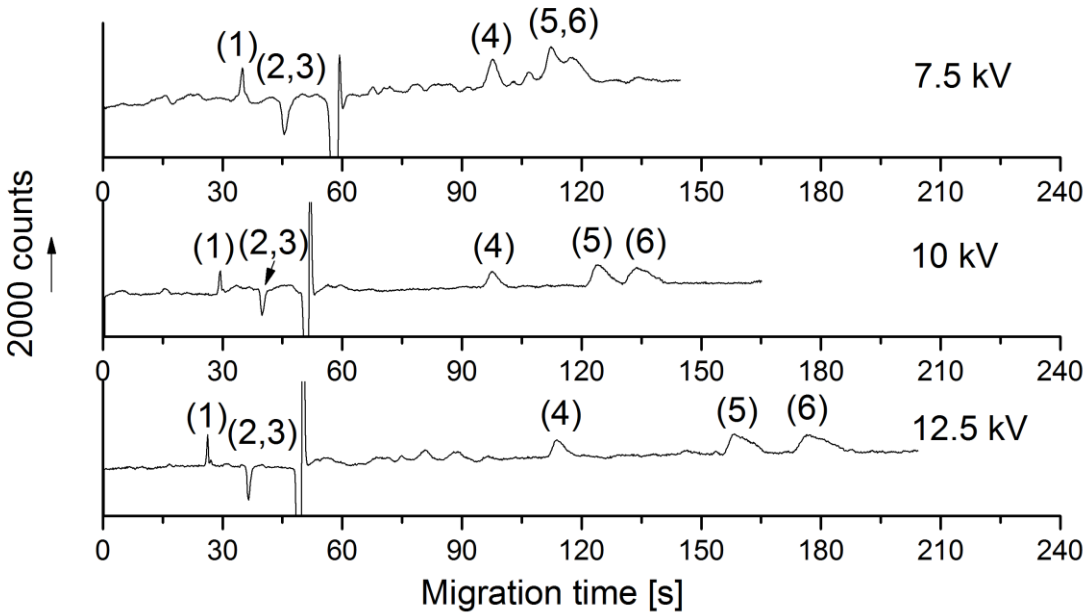

Figure 2: *Electropherograms with C^4D of a model system applying different separation HVs. The model system contained 125 µM choline (1), 125 µM Na^+ (2), 125 µM thiamine (3), 150 µM chloride (4), 125 µM bromide (5), and 125 µM nitrate (6). The length of the separation capillary (ID= 50 µm) was 31 cm. The effective length to the C^4D was 16.2 cm. Injection time was 0.1 s. Separation was performed in a 2 M HAc/ACN background electrolyte containing 4 mM NH_4Ac.*

The HV was varied from 7.5 kV to 15 kV to optimize the separation (see Figure 2). It was found that for HV lower than 10 kV no baseline separation was achieved for bromide and nitrate. However, for lower HV the anions migrate faster and showed less peak broadening as observed applying a HV of 12.5 kV. Thus, a separation voltage of 10 kV was set for all further experiments.

The protocol optimized for C^4D was also applicable to the MS detection. Concerning the ID of the separation capillary an ID of 50 µm is widely used. However, higher voltages can be applied using an ID of 25 µm [33]. The MS performance is less sensitive to buffer concentration changes than the C^4D. The resolution of choline and acetylcholine

increased with increasing ammonium acetate concentration and application of higher HV. The electrolyte containing 4 mM NH_4Ac in 2 M HAc/ACN and a HV of 10 kV were suitable to achieve baseline separation. So it was taken as a compromise meeting the requirements of both detectors.

4.2.4.2. Analytical characterization

The method was applied to a sample containing all model analytes. In Figure 3 an electropherogram is depicted showing the complementary use of both detectors. The C^4D enabled the detection of small inorganic ions such as chloride and nitrate, which are not visible in the MS recordings. Choline (m/z= 104.2) and thiamine (m/z= 265.2) were detectable with both detectors. However, the LODs were better in the MS compared to the C^4D having LODs 15-40 times lower. In case of the IS acetylcholine even a factor 200 was achieved. Moreover, the MS enabled the identification of the compounds. Nicotinamide ($[M+H]^+$, m/z= 123.1) could not be seen in the C^4D as it migrates with the electroosmotic flow (EOF), but serves as EOF marker in the MS. Thus, the combination of both detectors allowed the simultaneous analysis of small inorganic anions and organic biomolecules and their identification by ESI-TOF-MS. For further characterization of the NACE-C^4D-MS method, bromide and acetylcholine were added to the model mixture as ISs for quantification and in order to address the precision of the injection process.

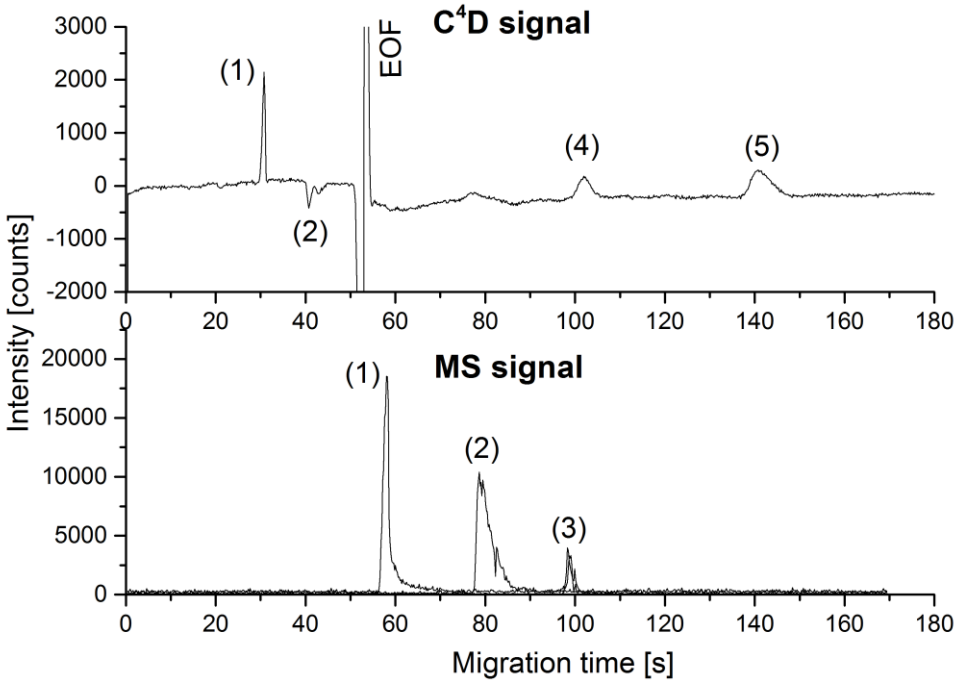

Figure 3: *NACE with dual detection (C^4D-MS) of an extract of a food supplement containing 300 µM choline (1), 60 µM thiamine (2), 75 µM nicotinamide (3, EOF), 165 µM chloride (4), and 130 µM nitrate (5). The concentrations are calculated based on the product specification and the experimental quantification of the inorganic ions. The length of the separation capillary (ID=50 µm) was 31 cm, which was the effective length to the MS. The effective length to the C^4D was 16.2 cm. Injection time was 0.1 s. Separation was performed in a 2 M HAc/ACN background electrolyte containing 4 mM NH_4Ac applying a HV of 10 kV.*

Calibration plots were constructed plotting the normalized areas (by the respective IS) against the analyte concentrations. For the respective analytes, the more suitable detector was chosen for the calibration. The analytical characteristics are shown in Table 1. For the determination of choline and thiamine the MS was used as detector with a linear range from 25 µM to 175 µM. At higher concentrations signs of overload appeared resulting in non-linear behavior. Even if the analytes are also detectable with the C^4D, the LODs were 15-40 times lower in the MS. The LODs for choline were 0.6 µM and 25 µM, the LODs for thiamine 1.3 µM and 18.8 µM, respectively. The anions were not detectable with the MS. Therefore, the C^4D was used achieving a LOD of 30 µM for all studied anions. All LODs were determined without using the IS by measuring the detector signals within a concentration range near the LOD. The linear range measured for the anions was 75-225 µM for chloride and 75-200 µM for nitrate. The precision studies were based on 4 repetitive measurements of a solution containing 175 µM of chloride and 150 µM of nitrate, thiamine, and choline (see Table 1). The relative

standard deviations (RSDs) of the migration times were ≤1% for the cations and 1-2% for the anions under conditions as in Figure 3. The RSDs of the peak areas were 5-6% for the species detected with MS and 16-17% for the analytes detected with C^4D.

Table 1: *Analytical characteristics of the NACE-C^4D-MS method.*

	choline		**thiamine**		**chloride**	**nitrate**
detector	C^4D	**MS**	C^4D	**MS**	**C^4D**	**C^4D**
linear range [µM][a)]	-	25-175	-	25-175	75-225	75-200
R^2	-	0.997	-	0.979	0.983	0.983
slope (norm.)	-	0.004	-	0.027	0.003	0.007
LOD[b)] **[µM]**	25	0.6	18.8	1.3	30	31
RSD of migration time [%][c)]	-	0.3	-	1.0	1.1	1.8
RSD of peak area [%][c)]	-	4.9	-	5.8	15.6	16.7

a) calibration was performed in equidistant concentration steps of 25 µM
b) based on signal/noise=3
c) based on measurements (n=4) of a solution containing 175 µM of chloride and 150 µM of nitrate, thiamine, and choline

RSD values are dependent on concentration of the analyte, the detector itself, and the quantification strategy. Thus, the differences can be explained by the use of different detectors and ISs. Stability of the EOF was also investigated calculating the RSD of the migration time of nicotinamide. The RSD for consecutive measurements (n=4) was 0.4%. The RSD for measurements on three different days (n=3) was 6%. The difference can be explained by the complex transport situation depending on the EOF, the gravity flow, the suction pressure-induced flow of the ESI source, and the conditioning of the capillary.

4.2.4.3. Practical application

The developed method was applied to the food supplement “Vitamin B-Loges komplett” (Dr. Loges + Co. GmbH, Winsen, Germany) to quantify the content of choline, thiamine, chloride, and nitrate. The experimental parameters were the same as for calibration. The linear range of choline and thiamine was limited for MS measurements as at 200 µM signs of overload appeared resulting in a non-linear behavior. Consequently, for quantification sample preparation had to be adjusted appropriately to stay in the concentration ranges.

Choline, thiamine, chloride, and nitrate were quantified using an IS method. A calibration solution containing 125 µM choline, thiamine, and nitrate, respectively, 150 µM chloride, and the ISs acetylcholine and bromide were measured to determine the response factors. Linear behavior of the response factors with changing analyte concentration was proven during calibration. The contents of all analytes were calculated by means of these response factors. All solutions were measured three times. As example one electropherogram with dual detection is shown in Figure 4.

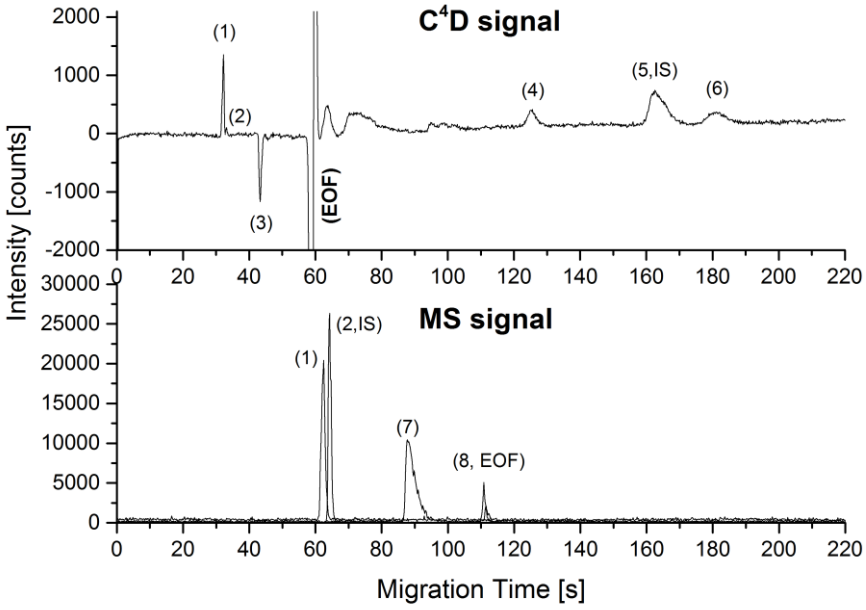

Figure 4: *NACE with C^4D-MS of the real sample containing choline (1), acetylcholine (2, IS), Na^+ (3), chloride (4), bromide (5, IS), nitrate (6), thiamine (7), and nicotinamide (8, EOF). The length of the separation capillary (ID= 50 µm) was 30 cm, which was the effective length to the MS. The effective length to the C^4D was 15.2 cm. Injection time was 0.1 s. Separation was performed in a 2M HAc/ACN background electrolyte containing 4 mM NH_4Ac applying a HV of 10 kV.*

The results are summarized in Table 2. The contents of choline and thiamine were in good agreement with the data given on the patient information leaflet. For the anions no information was available. Furthermore, the content of chloride was determined using a standard addition method. Three samples spiked with same amount of sodium bromide and increasing amounts of acetylcholine chloride were prepared. The normalized areas were plotted against added chloride concentrations and the original content was determined by linear fitting. The found mass was 11.6 mg, which is in good agreement with the results in Table 2 based on the IS method.

Table 2: *Results of the quantification of the organic and inorganic ingredients of the food supplement "vitamin B-Loges komplett".*

	found [mg] (n=3)	standard deviation [mg]	product specification [mg]
Choline	100.1	2.5	100
Thiamine	51.9	2.7	50
Chloride	11.9	1.1	n.a.
Nitrate	16.5	0.9	n.a.

4.2.5. Concluding remarks

In this work, a dual C^4D-MS detection approach for NACE is presented. Complementarity of the two detectors was shown determining sample constituents including inorganic anions as well as organic biomolecules simultaneously. The C^4D detector was the detector of choice for the inorganic anions, which were not detectable by ESI-TOF-MS. However, for the biomolecules MS was the favorable detector with lower LODs. Moreover, MS enabled identification of the compounds.

A NACE-C^4D-MS method was developed and optimized finding a compromise to meet the requirements of both detectors. An acetonitrile based BGE was used leading to fast separations. The organic medium ensures compatibility with both detectors due to its volatility and low background conductivity. For C^4D, the ID of the capillary and concentration changes of the BGE were more critical than for MS detection. Therefore, the dependency of the sensitivity of the C^4D on the ID of the capillary was taken into account. An ID of 50 µm and a BGE containing 4 mM NH_4Ac in 2 M HAc/ACN was found to be best suited as compromise for both detectors. The method was further characterized by performing a calibration determining also the LODs.

Practical applicability of the method was demonstrated determining a food supplement. Choline and thiamine were quantified using the MS, whereas chloride and nitrate were determined by the C^4D.

4.2.6. References

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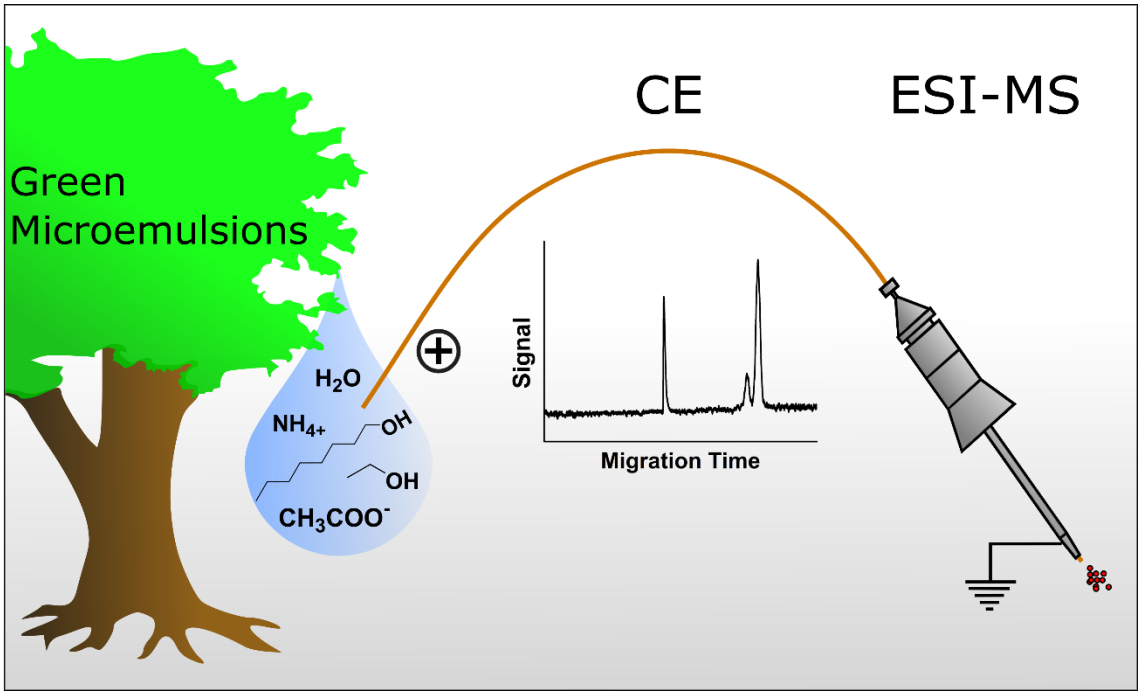
5.Surfactant-free microemulsion electrokinetic chromatography (SF-MEEKC) with UV and MS detection - a novel approach for the separation and ESI-MS detection of neutral compounds

5.1. Abstract



Microemulsion electrokinetic chromatography (MEEKC) is a powerful tool to separate neutral species based on differences in their hydrophobic and hydrophilic properties. However, as a major drawback the conventionally used SDS based microemulsions are not compatible with electrospray ionization mass spectrometry (ESI-MS). In this work, a surfactant-free microemulsion (SFME) consisting of water, ethanol, and 1-octanol is used for surfactant-free microemulsion electrokinetic chromatography (SF-MEEKC). Ammonium acetate was added to the SFME enabling electrophoretic separations. The stability of SFMEs containing ammonium acetate was investigated using small-angle X-ray scattering and dynamic light scattering. A method for the separation of a model system of hydrophobic and hydrophilic neutral vitamins, namely the vitamins B_2 and D_3,

and the cationic vitamin B_1 was developed using UV/VIS detection. The influence of the ammonium acetate concentration on the separation performance was studied in detail. The method was characterized concerning reproducibility of migration times and peak areas and concerning the linearity of the calibration data. Furthermore, SF-MEEKC was coupled to ESI-MS investigating the compatibility between SFMEs and the ESI process. The signal intensities of ESI-MS measurements of the model analytes were comparable for SFMEs and aqueous systems. Finally, the vitamin D_3 content of a drug treating vitamin D_3 deficiency was determined by SF-MEEKC coupled to ESI-MS using 25-hydroxycholecalciferol as an internal standard (IS).

This chapter has been published.

Urška Mohorič, Andrea Beutner*, Sebastian Krickl*, Didier Touraud, Werner Kunz, Frank-Michael Matysik, Surfactant-free microemulsion electrokinetic chromatography (SF-MEEKC) with UV and MS detection - a novel approach for the separation and ESI-MS detection of neutral compounds, Anal. Bioanal. Chem., 408 (2016) 8681-8689 (DOI: 10.1007/s00216-016-0057-0).

**contributed equally*

Author contributions

UM did the experimental work and data evaluation using UV/VIS detection. *AB* and *UM* did the experimental work and data evaluation using MS detection under the supervision of *AB*. *SK* was responsible for the physical-chemical part, DLS, and SAXS measurements. The results were discussed by all authors. *AB* and *UM* wrote the analytical part of the manuscript. *SK* wrote the physical-chemical part of the manuscript. The graphical abstract was designed by *AB* and *SK*. The revision of the manuscript was done by *AB* and *FMM*. *FMM* is the corresponding author.

5.2. Introduction

Capillary electrophoresis (CE) is a well-established analytical technique for the separation of charged analytes. There are special modes of CE that enable also the separation of neutral compounds using micellar solutions as background electrolytes (BGE). The corresponding technique is termed micellar electrokinetic chromatography (MEKC) [1]. In addition, microemulsion electrokinetic chromatography (MEEKC) is another CE separation method that typically uses a BGE consisting of an oil-in-water microemulsion to separate charged and neutral analytes based on electrophoresis and partition chromatography. Charged analytes migrate according to their size and charge whereas neutral analytes can be separated based on their hydrophobic and hydrophilic properties due to different interactions with microemulsion oil droplets [2, 3]. The thermodynamically stable and transparent microemulsions are typically composed of oil, water, surfactant, and co-surfactant forming nanometer-sized stable oil droplets in aqueous buffer [3]. Sodium dodecyl sulfate (SDS) is the most commonly used surfactant in MEEKC leading to negatively charged oil droplets, where the concentration of SDS determines the charge-to-size ratio of the droplet. This ratio affects the electrophoretic mobility and the migration time of the analytes that interact with the charged microemulsion droplets [3]. Apart from SDS, a microemulsion based on sodium bis(2-ethylhexyl) sulfosuccinate was used for MEEKC to determine natural and synthetic estrogens simultaneously by Lucangioli and co-workers [4]. MEEKC has been applied, furthermore, to several model mixtures such as steroids [5], preservatives in pharmaceutical products [6, 7] and foods [8], amino acid derivatives [9], as well as neutral products [10]. Furthermore, the comparison between micellar and microemulsion electrokinetic chromatography for the determination of water- and fat-soluble vitamins has been done by Terabe and Matsubara [11]. It was found that the separation efficiency is higher using MEEKC as the composition of the microemulsion droplets leads to a higher solubility of hydrophobic molecules compared to the micellar structures. MEEKC has also been used as a suitable tool for lipophilicity determination of acidic, neutral, and basic compounds [12]. MEEKC is most commonly coupled to UV/VIS detectors [13, 14]. As many organic compounds show UV absorbance, it is a useful technique for pharmaceutical applications. However, UV/VIS detection has a major drawback due to its relatively low sensitivity and selectivity [15] as a consequence

of the short optical path length of the capillary detection window [13, 14]. Coupling to alternative detection techniques has been described such as laser-induced fluorescence detection [9], electrochemical detection [16], and inductively coupled plasma mass spectrometry [17]. MS detection has major advantages compared to conventional UV detection due to its high selectivity, low detection limits, and versatility [18, 19]. However, MEEKC with typically used SDS-based microemulsions has major limitations when it comes to the hyphenation to electrospray ionization mass spectrometry (ESI-MS), the most powerful detector in combination with separation techniques, due to the nonvolatile properties of SDS [19]. A new generation of surfactant-free microemulsions (SFMEs) has been recently studied by Klossek et al. [20]. Although such SFMEs were already observed and introduced by Barden and coworkers in the 1970s [21], it took a long time to characterize such systems and to understand the origin of their structuring. To get detailed insight into these structures, static and dynamic light scattering (DLS), as well as small-angle X-ray scattering (SAXS) and neutron scattering have been applied [20, 22, 23]. SFMEs basically consist of three components. Component A (usually water), which is completely mixable with a component B (usually a hydrotrope, like ethanol), but poorly miscible with the hydrophobic component C (e.g., 1-octanol), that in turn, is fully miscible with component B. In such systems, fine and remarkably time-stable emulsions can be formed in the two-phasic region, e.g., by adding a sufficient amount of water to a mixture of ethanol and 1-octanol. This phenomenon is known as the ouzo effect [24]. But in particular, scattering experiments and molecular dynamics simulations revealed that in such systems well-defined aggregates on a nanometer scale can occur already in the monophasic region (near the phase separation border, but still far away from critical fluctuations) [22, 25]. This observation was called the “pre-ouzo” effect [20]. Ternary mixtures in this pre-ouzo region are thermodynamically stable, transparent, homogenous solutions, just as classical microemulsions. The aggregates inside are also similar to structures in conventional surfactant-based microemulsions and consist of a highly fluctuating oil-rich core in an outer aqueous pseudo-phase. The hydrotrope is distributed over both pseudo-phases, accumulating at the interface of the oil-rich domain. Furthermore, the thermodynamic stability of these aggregates could recently be explained and even predicted theoretically by an extended Derjaguin-Landau-Verwey- Overbeek (DLVO) theory [26]. Even bicontinuous or reverse aggregates

can be formed depending on the components and compositions. For these reasons, SFMEs provide a promising alternative to surfactant-based microemulsions for several applications [27–29]. In this work, the use of this new generation of SFMEs containing water, ethanol, and 1-octanol for SF-MEEKC is described for the first time. Ammonium acetate was added as electrolyte. The stability of the BGE was investigated using SAXS and DLS. A method for the separation of a model system consisting of hydrophobic and hydrophilic neutral vitamins with SF-MEEKC coupled to UV/VIS detection was developed and optimized. Furthermore, compatibility between the SF-MEEKC and the ESI process was investigated. Finally, the method was applied to a real sample, namely the quantification of vitamin D_3 in a drug treating vitamin D_3 deficiency using an IS.

5.3. Experimental

5.3.1. Chemicals and materials

5.3.1.1. Chemicals

Ethanol was purchased from Sigma-Aldrich (Steinheim, Germany, ≥99.8%). Ammonium acetate (≥96.0%), 1-octanol (≥99.0%), sodium hydroxide solution, formic acid, and propan-2-ol were purchased from Merck (Darmstadt, Germany). All chemicals were used without further purification. Water was purified using a Milli-Q System (Millipore, Bedford, MA, USA). The vitamins B_1, B_2, and D_3 were obtained from Sigma-Aldrich (MO, USA) as well as 25-hydroxycholecalciferol, which was used as an IS in SF-MEEKC-MS experiments. The vitamins were stored at 4 °C and kept in the dark to avoid decomposition.

5.3.1.2. Buffer and sample preparation

SFMEs were prepared by weighing water, ethanol, and 1-octanol in the following proportions: 37.5% water, 43.75% ethanol, and 18.75% 1-octanol (weight fraction). Ammonium acetate was used to prepare the BGE in different concentrations (mmol/kg of SFME). The order of addition was found to be irrelevant for the formation of the SFME, as it could be expected for a thermodynamically stable system. All solutions were filtered through 0.45-µm PTFE Rotilabo syringe filters (Carl Roth, Karlsruhe, Germany).

The amount of ammonium acetate was varied (50, 100, 150, 200, and 250 mmol/kg) to study its effect on the separation performance and is therefore specified further in the experiments. Standard stock solutions of each vitamin were prepared daily by weighing certain amounts of each vitamin and dissolving them in the SFME. The final concentrations of standard stock solution for vitamin B_1 and vitamin D_3 were 10 and 0.085 mg/mL for vitamin B_2. Samples were prepared daily by diluting the stock solutions appropriately with BGE and filtering them through 0.45 µm syringe filters before use.

5.3.1.3. Capillary preparation

Fused silica capillaries were obtained from Polymicro Technologies (Phoenix, AZ, USA). The capillaries (outer diameter (OD)=360 µm, inner diameter (ID)=50 µm) were cut properly (total and effective lengths are specified further in the experiments) and the ends were polished with polishing paper (grid size 12 and 30 µm) to create a smooth surface. For CE-UV/VIS experiments, a detection window (5-10 mm) was made by removing the polyimide coating with a microtorch. For CE-ESI-MS experiments, the polyimide coating was removed from the MS side end of the capillary. Before use, the capillaries were flushed for 15 min with 0.1 mol/L NaOH solution, 15 min with Milli-Q water, and 30 min with BGE. For CE-UV/VIS experiments, the capillary was additionally flushed with BGE for 5 min between the experiments to ensure reproducibility of migration times. After the experiments, the capillaries were flushed with water for storage. All solutions used for flushing were filtered through 0.45 µm filters before use.

5.3.2. Experiments

5.3.2.1. Dynamic light scattering

DLS experiments were performed using a temperature controlled CGS-3 goniometer system from ALV (Langen, Germany) equipped with an ALV-7004/FAST Multiple Tau digital correlator and a vertical-polarized 22-mW HeNe laser (wavelength λ=632.8 nm). All samples were filtered into dust-free cylindrical light scattering cells (10 mm outer diameter) using a 0.2 µm PTFE membrane filter. The sealed measurement cells could be directly placed in the measurement apparatus. Measurements were performed at a scattering angle of 90° and a temperature of 25±0.1 °C. Data points were collected for

300 s. It should be mentioned that for such systems calculations of droplet sizes are not evident. Possible aggregates, which might be formed in such solutions, are usually highly fluctuating systems. So, we used the DLS results only to have a rough, qualitative picture of the occurrence of aggregates.

5.3.2.2. Small-angle X-ray scattering

SAXS measurements were performed on a bench built by XENOCS. A sealed molybdenum tube with a 12:∞ multilayer Xenocs mirror (for Mo radiation) was used, coupled to two sets of scatter-less FORVIS slits, to generate a 0.8 × 0.8-mm^2 X-ray beam (wavelength λ = 0.071 nm) at the sample position. Samples were placed in 2 mm quartz capillaries. Data were recorded using a large online scanner detector (MAR 345, MAR Research, diameter=345 mm). A q-range from 0.24 to 30 nm^{-1} was covered applying off-center detection with an experimental resolution of Δq/q=0.05. Data integration of two-dimensional spectra was performed using FIT2D software. Corrections for background (empty cell and detector noise) as well as normalization with a high-density polyethylene film as a standard were applied. Silver behenate in a sealed capillary was used as the scattering vector calibration standard. All measurements were performed at room temperature and the acquisition time was 3600 s per sample. In order to determine the correlation length and the radii of the aggregates, the Ornstein-Zernike equation was used for data fitting in the low-q-range (0.275-4 nm^{-1}). The Ornstein-Zernike function can be linked to the radius of gyration RG of the aggregates by

$$I(q) = \frac{I_0}{1 + \xi^2 q^2} = \frac{I_0}{1 + \frac{R_G^2 q^2}{3}}$$

where ξ is the correlation length and I0 the intensity for q=0 [30, 31]. The scattering vector q is given by

$$q = \frac{4\pi}{\lambda} \sin\frac{\vartheta}{2}$$

with ϑ being the scattering angle. The average spherical radius R_S of the aggregates can be finally estimated as follows:

$$R_G^2 = \frac{3}{5} R_S^2$$

Hence, the following correlation between correlation length and the spherical radius of the aggregates is valid:

$$R_S = \sqrt{5}\,\xi \approx 2.24 \cdot \xi$$

5.3.2.3. SF-MEEKC-UV/VIS

A laboratory built device consisting of a modified autosampler additionally equipped with a polished syringe needle electrode at the capillary inlet and a 30 kV high-voltage source (ISEG GmbH, Dresden, Germany) was used for all experiments. Control of the CE device was performed using a software developed by the Electronic workshop of the University of Regensburg. For UV/VIS experiments, a second electrode made of a platinum wire was introduced to the system. A Bischoff Lambda 1010 UV/VIS system was used as detector. The wavelength of 272.0 nm was set for all experiments. Data acquisition was performed using a LabVIEW-based software. Injection was performed hydrodynamically by gravity flow. Ensuring equal injection conditions, the difference in height between inlet and outlet vial was kept constant at 10 cm for all experiments. The performance of the SF-MEEKC-UV/VIS setup was investigated concerning selectivity, linearity, limit of detection (LOD), and reproducibility. If not stated differently, a sample containing 500 µg/mL of the vitamins B_1 and D_3 and 77 µg/mL of vitamin B_2 was used. First, the resolution of the neutral species was optimized varying the concentration of ammonium acetate in the BGE (50, 100, 150, 200, and 250 mmol/kg). The reproducibility (n=9) of the migration times and the peak areas was further investigated using a BGE containing 150 mmol/kg ammonium acetate. A set of five standard solutions was measured three times, respectively, examining the linearity of the calibration curve. The concentrations of the samples were between 100 and 1000 µg/mL for vitamin B_1 and between 250 and 1250 µg/mL for vitamin D_3. For vitamin B_2, four standard solutions with different concentrations ranging from 65 to 80 µg/mL were measured.

5.3.2.4. SF-MEEKC-ESI-MS

The ESI-MS system consisted of a Bruker micrOTOF (Bruker Daltonics, Bremen, Germany) time-of-flight mass spectrometer (TOF-MS), equipped with an orthogonal ESI source for coupling with capillary electrophoresis. In detail, a coaxial sheath liquid electrospray ionization interface (Agilent, Waldbronn, Germany) was used. The Bruker software micrOTOF control version 2.3 was used for data acquisition. For optimal ESI conditions, positioning of the tip of the capillary inside the interface was controlled by a microscope camera. Sheath liquid (water/isopropanol/formic acid, 49.9/49.9/0.2, v/v/v) was introduced by a syringe pump (KD Scientific, Holliston, MA, USA) with a flow rate of

0.48 mL/h. Analysis was carried out in positive ion mode. The electrospray voltage was -4 kV (grounded sprayer tip), and other parameters were set as follows: end plate offset: -500 V; dry gas (N_2) flow: 4.0 L/min; dry gas temperature: 190 °C; capillary exit: 75 V; skimmer 1: 25.3 V; hexapole 1: 23 V; hexapole RF: 65 Vpp; Skimmer 2: 23 V; lens 1 transfer: 49 µs; lens 1 prepulse storage: 5 µs; mass-to-charge (m/z) ratio: 220-550; spectra acquisition rate: 3 Hz. The mass traces of the analyte model mixture were selected as follows: vitamin B_1: m/z 265.15; vitamin B_2: m/z 377.19; vitamin D_3: m/z 385.39, and 25-hydroxycholecalciferol: m/z 401.40. The SF-MEEKC system was coupled to ESI-TOF-MS validating the compatibility of SFMEs with ESI by comparing the LODs of the vitamins B_1 and B_2 to a conventional aqueous CE method using 0.1 M acetic acid as BGE. Finally, SF-MEEKC-ESI-TOF-MS was applied for the quantification of vitamin D_3 in a drug treating vitamin D_3 deficiency using 25-hydroxycholecalciferol as an IS. The commercial drug formulation (Vitamin D_3 Hevert 4000 IE, Hevert Arzneimittel GmbH & Co. KG, Nussbaum) for treating vitamin D_3 deficiency was bought in a local pharmacy in Regensburg (Germany). Samples were stored in their original packets at room temperature until the analysis was carried out. Drug tablets were finely powdered and one tablet corresponding to 0.1 mg of vitamin D_3 was dissolved together with the IS in a total volume of 1 mL of the SFME. The solution was vortexed for 5 min, then centrifuged for 10 min at 3500 RPM x g. The supernatant was collected and filtered through a 0.45 µm syringe filter. 520 µL of supernatant was taken and filled up to the total volume of 1 mL for further analysis.

5.4. Results and discussion

5.4.1. Composition and structural investigations of the surfactant-free microemulsion

The SFME chosen for application in SF-MEEKC is composed of water, ethanol, and 1-octanol. Figure 1 shows the ternary phase diagram water/ethanol/1-octanol at room temperature, with α indicating the exact composition of the surfactant-free microemulsion in weight fractions used for SF-MEEKC.

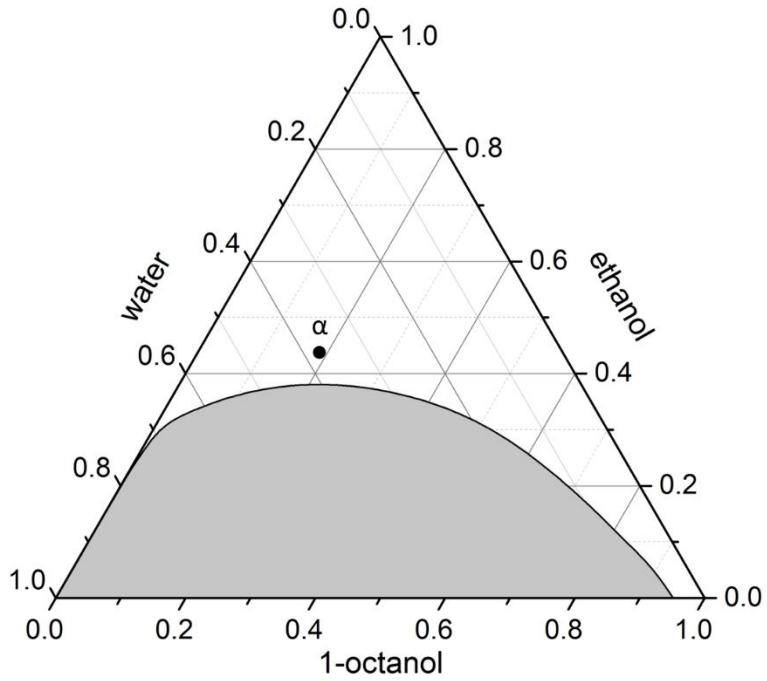


Figure 1: *Ternary phase diagram in weight fractions of the system water/ethanol/1-octanol at 25 °C. The two phasic region (shaded) was redrawn by data of Klossek et al. [20]. The macroscopically homogeneous, one-phase region is given in white. The point α corresponds to the surfactant-free microemulsion used for SF-MEEKC consisting of 37.5% water, 43.75% ethanol, and 18.75% 1-octanol (w/w/w).*

For sakes of clarity, the two-phasic region was redrawn from data given by Klossek et al. [20]. The mixture of 37.5% water, 43.75% ethanol, and 18.75% octanol (w/w/w) (solution α) was chosen, since recent publications revealed the presence of aggregates of well-characterized shape at this composition [20, 22, 23, 25]. To be applicable in electrophoresis, ammonium acetate was added in order to establish a well-defined conductivity and in expectation of charging the interface between the two pseudophases by ion adsorption. In addition, ammonium acetate has the advantage to be compatible with ESI-MS detection. To exclude unwanted effects on the structuring within the SFME, the influence of the added salt on the structuring of the ternary mixture was investigated by DLS and SAXS measurements. The results of the DLS experiments are shown in Figure 2.

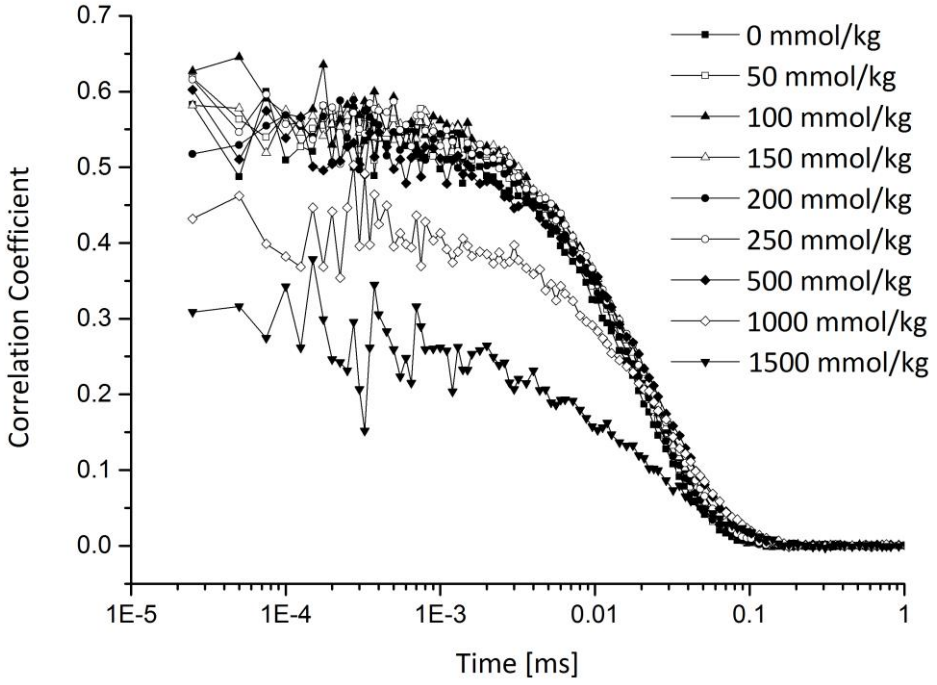


Figure 2: *Time-dependent self-correlation functions obtained by DLS at 25 °C. The symbols correspond to the different molalities of added ammonium acetate in the SFME consisting of 37.5% water, 43.75% ethanol, and 18.75% 1-octanol (w/w/w).*

Correlation functions for 0-250 mmol/kg are not significantly altered. However, correlation functions start to break down at 500 mmol/kg of added salt. For 1000 and 1500 mmol/kg, there are already significant deviations compared to the correlation functions corresponding to samples with lower ammonium acetate concentrations. This means that structures, present in the initial ternary solution α, are maintained over a certain concentration range of ammonium acetate and progressively vanish after adding more than 500 mmol/kg of the salt. To make an independent check of the occurrence of pre-ouzo aggregates and in order to further analyze the influence of the added salt, SAXS measurements were performed. SAXS measurements for 0, 50, 100, 150, 200, and 250 mmol/kg showed a significant low-q scattering accompanied by successively enhanced I_0 values for increasing amounts of ammonium acetate. Radii calculated with the Ornstein-Zernike equation were in the range between 1.9 and 2.3 nm, increasing with increasing amount of ammonium acetate. Addition of the analytes (as described in chapter 5.3.1.2) did not show a notable influence on the radius and on the structuring of the SFME. Further, the scattering results lead to the assumption that the polarizable ions, ammonium, and/or acetate are accumulating at the interface between the two

pseudo-phases. The order of separation of the analytes (see chapter 5.4.2.1) leads to the conclusion that acetate has a higher affinity towards the interface between the water- and octanol-rich domains than the ammonium ions. Consequently, the hydrophobic octanol-rich aggregates can be assumed to be charged negatively overall, which allows successful application in SF-MEEKC. In addition, ammonium acetate could have salted out ethanol towards the interface or into the octanol-rich aggregates [23], which would also explain the increasing radii of these objects.

5.4.2. SF-MEEKC-UV/VIS

5.4.2.1. Chemical properties of the model analytes

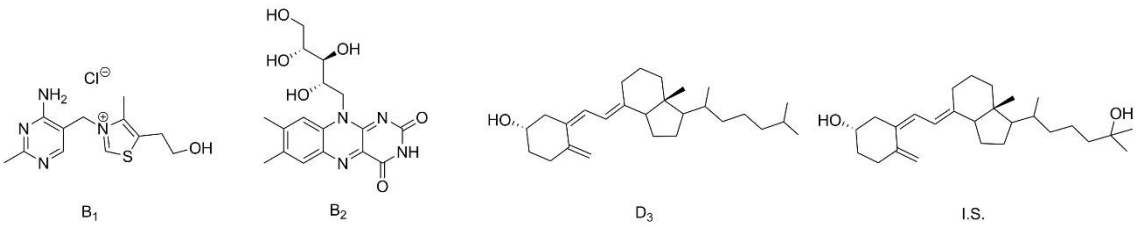


Figure 3: *Molecular structures of the model mixture and the IS: thiamine hydrochloride (vitamin B_1, pK_{a1}=4.8, pK_{a2}=9.2) [32], riboflavin (vitamin B_2, pK_{a1}=1.9, pK_{a2}=10.2) [32], cholecalciferol (vitamin D_3), and 25-hydroxycholecalciferol (IS).*

The structures of the analytes are depicted in Figure 3. The separation in SF-MEEKC is based on the electrophoretic and partitioning behavior of the analytes. The migration order of neutral compounds is therefore determined by their hydrophobicity, indicating that compounds which are more soluble in the oil phase will migrate differently than the ones that partition more into the aqueous phase [33, 34].

5.4.2.2. Optimization of SF-MEEKC-UV regarding electrolyte concentration

Figure 4 shows SF-MEEKC-UV separations of the model analytes using varying concentrations of ammonium acetate. Vitamin B_1 migrates first as it is positively charged. Neutral fat-soluble species are mostly partitioning into the oil droplets, whereas the neutral hydrophilic species reside mainly in the aqueous phase [33]. The neutral and water-soluble vitamin B_2 migrates before the neutral, fat-soluble vitamin D_3. This indicates that the oil droplets are negatively charged and thus the migration time

of the fat-soluble vitamin D_3 is retained. The IS 25-hydroxycholecalciferol is comigrating with vitamin D_3 as it has the same molecular structure apart from an additional hydroxyl group. The concentration of ammonium acetate in SFME was varied between 50 and 250 mmol/kg.

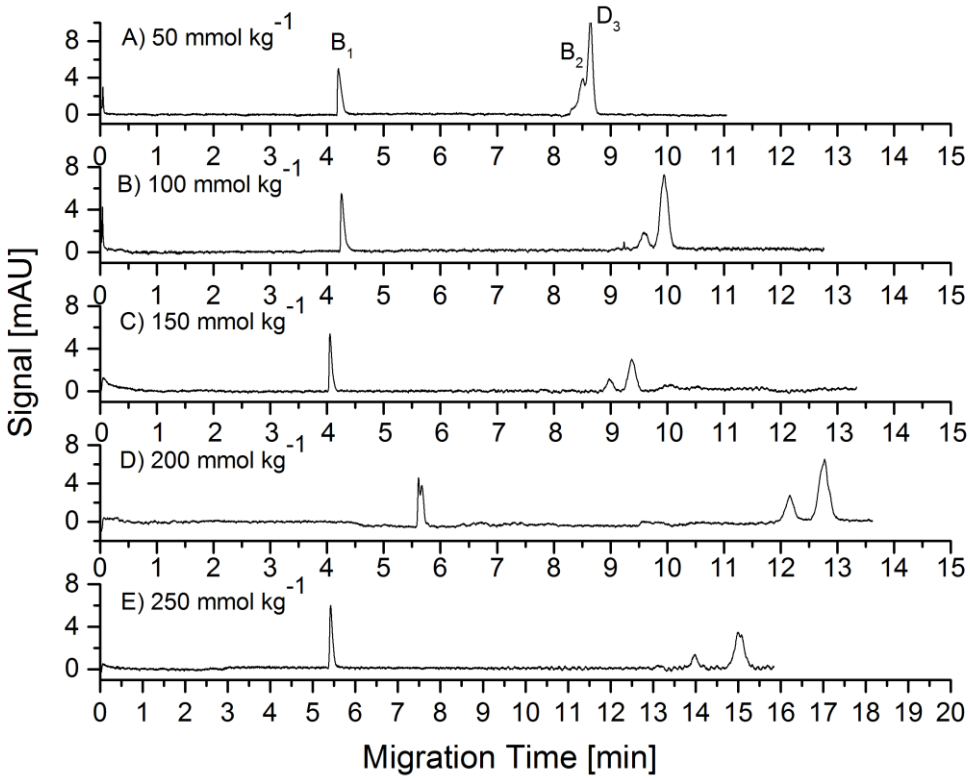


Figure 4: *SF-MEEKC-UV separation of vitamin B_1 (0.50 mg/mL), B_2 (0.077 mg/mL), and D_3 (0.50 mg/mL) using the following concentrations of ammonium acetate in SFME. A) 50 mmol/kg, separation voltage 22.5 kV. B) 100 mmol/kg, separation voltage 22.5 kV. C) 150 mmol/kg, separation voltage 22.5 kV. D) 200 mmol/kg, separation voltage 20.5 kV. E) 250 mmol/kg, separation voltage 20.5 kV. Capillary: total length 31 cm x 50 µm ID (effective length 21 cm). Hydrodynamic injection with a height difference of 10 cm for 30 s. UV detection was set at 272 nm.*

The conditioning of the capillary after changing the BGE was done by flushing 5 min with 0.1 M NaOH, 5 min with water, and 30 min with the new BGE. Some changes in the absolute migration times can occur depending on details of the conditioning protocol. However, the separation performance and the precision of repetitive measurements with the same BGE were quite good as specified later in this section. When the microemulsion containing 50 mmol/kg ammonium acetate was used as BGE in SF-MEEKC, the signals of the vitamins B_2 and D_3 were not completely separated. The resolution of the signals for the two neutral species improved with higher

concentrations of ammonium acetate added to the mixture. Baseline separation of signals of vitamins B_2 and D_3 was first achieved using 150 mmol/kg ammonium acetate. The resolution of signals of vitamins B_2 and D_3 was in fact best when SFMEs with a content of 200 mmol and 250 mmol/kg ammonium acetate were used as BGE. However, due to higher concentrations of ammonium acetate in SFME, rather high electrophoretic currents (≥90 µA) were generated, when applying high voltages (larger than 20.5 kV) leading to a breakdown of electrophoretic current during separation. Therefore, a maximal separation voltage of 19 kV was applicable. However, when applying lower separation voltages, much longer migration times of analytes were observed. Therefore, a compromise between resolution and migration time was made and the SFME containing 150 mmol/kg and applying a separation voltage of 22.5 kV was chosen as the best compromise for separation of the vitamins B_1, B_2, and D_3. Using this protocol, the reproducibility of the migration times and of the peak areas of the different vitamins was characterized by relative standard deviations ranging between 2.3 and 3.5% and between 8.6 and 12%, respectively (conditions as in Figure 4C, n = 9). Further analytical characteristics are specified in Table 1. Analytical characteristics in the concentration range from 100 to 1000 µg/mL for vitamin B_1, from 65 to 80 µg/mL for vitamin B_2, and from 25 to 1250 µg/mL for vitamin D_3, with triplicate injection at each concentration level were determined by SF-MEEKC-UV. The relatively small range of concentrations of vitamin B_2 was due to its low solubility. The linearity was evaluated by plotting peak areas versus concentrations of analytes. The corresponding calibration parameters of the three analytes are summarized in Table 1. Due to its low solubility, quantification of B_2 is limited.

Table 1: *Analytical characteristics for SF-MEEKC determinations of the vitamins B_1, B_2, and D_3 with UV (MS) detection*

Vitamin	Concentration range for calibration [a] (UV)/ [µg/mL]	R[b]	LOD[b] (UV)/ [µg/mL]	LOD[b] (MS)/ [µg/mL]
B_1	100 – 1000	0.9998	7.5	0.5
B_2	65 – 80	0.9755	51.0	7.5
D_3	250 – 1250	0.9881	12.5	2.9

[a] Three data points with three replicate injections at each concentration level (N = 3)
[b] The LOD corresponds to a signal-to-noise ratio of three

5.4.3. SF-MEEKC-ESI-TOF-MS

5.4.3.1. Investigation of the ESI compatibility of the SFMEs

Ensuring compatibility of the SFMEs with ESI, the LODs of the vitamins were compared using SF-MEEKC and a conventional aqueous CE method with 0.1 M acetic acid as BGE. The LODs of the SF-MEEKC-ESI-TOF-MS were determined to be 0.53 µg/mL for vitamin B_1, 7.54 µg/mL for vitamin B_2, and 2.89 µg/mL for vitamin D_3 (Table 1). The LODs of the vitamins B_1 and B_2 for aqueous CE were determined to be 0.63 and 12.5 µg/mL, respectively. Vitamin D_3 is not soluble in water and thus the LOD could not be determined with conventional aqueous CE. Comparing the LODs of CE and SF-MEEKC, it can be stated that the SFMEs are compatible with ESI as the LODs are in the same range. An exact comparison is limited as the injection conditions are different for both methods due to different viscosities of the media.

5.4.3.2. Application of the proposed SF-MEEKC-ESI-TOF-MS method

The potential of the developed method is demonstrated by quantifying vitamin D_3 in a commercial drug formulation. The content of vitamin D_3 in the commercial sample formulation was declared to be 100 µg. The UV/VIS detector was not suitable for the quantification of vitamin D_3 due to problems with matrix effects and limited selectivity compared to MS. In real sample analysis, the noise level of SF-MEEKC-ESI-TOF-MS measurements was also higher than in case of standard solutions. Due to the more complex sample preparation protocol and to improve the precision of results, SF-

MEEKC-ESI-TOF-MS measurements were performed using 25-hydroxycholecalciferol as IS. Five different concentration levels (from 120 to 200 µM for 25-hydroxycholecalciferol and from 90 to 150 µM for vitamin D_3) with triplicate injections were investigated. Different concentrations of standard solutions of vitamin D_3 and IS were chosen to obtain similar MS intensities for both species and to investigate the response factor in this concentration range. Figure 5 shows exemplarily results for real sample analysis by SF-MEEKC-ESI-TOF-MS.

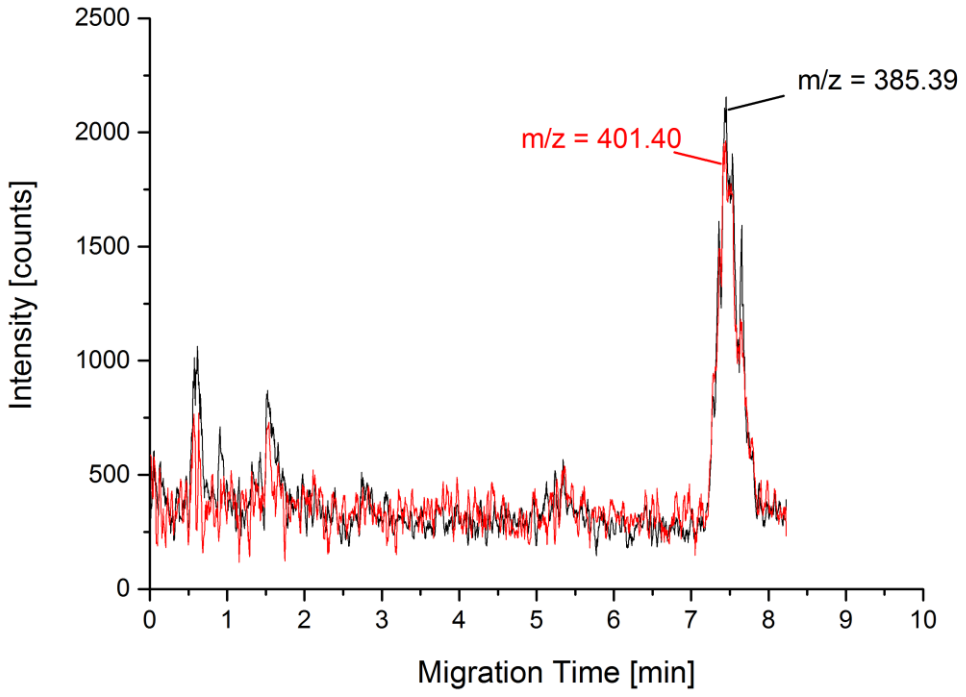


Figure 5: *Extracted ion electropherogram for SF-MEEKC-ESI-TOF-MS determination of the vitamin D3 in a commercial sample (m/z = 385.39, black line) using 25-hydroxycholecalciferol (m/z = 401.40, red line) as an IS. Microemulsion composition: 37.5% of water, 43.75% of ethanol, 18.75% of 1-octanol (w/w/w), and 150 mmol/kg ammonium acetate. Capillary: total length 30 cm x 50 µm ID (effective length 21.5 cm); applied voltage 16 kV, hydrodynamic injection for 10 s.*

The migration times of vitamin D_3 and 25-hydroxycholecalciferol are equal indicating that the latter one is well suited as an IS. The response ratio (D_3/IS) was found to be independent of the concentration of vitamin D_3 and of IS in the studied range. The results of real sample analysis are summarized in Table 2. The contents of vitamin D_3 determined in the individual drug samples ranged between 95 and 113 µg, which is in good agreement to the declared mass of 100 µg.

Table 2: *Determination of vitamin D_3 in a commercial drug formulation (vitamin D_3, 400 IE) by SF-MEEKC-ESI-TOF-MS using 25-hydroxycholecalciferol as IS.*

Sample no.	Declared amount of D_3 / [µg]	Found content of D_3/ [µg]	RSD (n=3)/%
1	100	95	4
2	100	105	5
3	100	113	10.5

5.5. Conclusion

For the first time, we demonstrated the use of SFMEs for electrokinetic chromatography. In the so-called pre-ouzo region, aggregate formation in the water/ethanol/1-octanol microemulsion is occurring spontaneously with no additional surfactant present in the mixture. The DLS and SAXS measurements proved that the addition of ammonium acetate in the chosen concentration range was not altering aggregate formation in the SFME. SF-MEEKC allowed the separation of neutral species in the studied model mixture of vitamins. The compatibility of SF-MEEKC with ESI-MS could be demonstrated. Comparable sensitivity as in aqueous CE-ESI-MS was found. Finally, the practical applicability of SF-MEEKC-ESI-MS was demonstrated determining the content of vitamin D_3 in a commercial drug.

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6.Summary

For the analysis of complex samples such as biological or environmental ones, highly selective analytical strategies are required. The selectivity can be enhanced by means of hyphenation of orthogonal separation techniques or combination of complementary detectors as an alternative concept. In this work, an example for both concepts is presented, respectively. First, methodical and instrumental studies concerning the comprehensive hyphenation of the two most important instrumental techniques in ion analysis namely ion chromatography (IC) and capillary electrophoresis (CE) are described enabling the simultaneous determination of anions and cations. Further, the combination of capacitively coupled conductless conductivity detection (C^4D) and mass spectrometry (MS) as dual detection concept for CE is introduced and applied using aqueous and non-aqueous background electrolyte systems. Another concept to enhance the selectivity is rendering the separation technique compatible to a highly selective detector such as MS. Thus, in a third part, an approach is presented enabling the coupling of surfactant-free microemulsion electrokinetic chromatography (SF-MEEKC) to MS.

For the comprehensive hyphenation of capillary IC and CE, a modulator was developed ensuring well-controlled injections from the first to the second dimension. This was achieved by periodical injection of the IC effluent from a transfer capillary to the CE separation capillary by capillary batch injection. Important for the coupling was the compatibility of the two systems. Thus, the characteristics of the advanced capillary high performance IC with flow rates in the lower µL/min range facilitated the hyphenation being closer to CE conditions compared to IC with conventional columns. Due to the implemented capillary scale suppressor technology, the IC effluent consisted of analyte and pure water avoiding matrix concerned interferences and enabling exploitation of analyte stacking in CE. Further, fast CE measurements in the time range of seconds enabled the comprehensive coupling. Proof-of-concept measurements were performed using a model system containing nucleotides and their cyclic derivates. It was shown that the separation performance could be enhanced in the two-dimensional ICxCE-MS system compared to the single techniques.

The work was further expanded to a methodical study concerning the simultaneous determination of positively and negatively charged analytes. A bypass system for IC was developed using a switching valve. This configuration enabled the cations bypassing the suppressor and thus they were not filtered out by the cation exchange membrane of the suppressor being not selective to eluent cations. Feasibility of the setup was demonstrated separating a model mixture of different arsenic species. The cations were eluted with water from the anion exchange column before driving a KOH gradient elution for the separation of anions. The injection parameters of the modulator introducing the IC effluent into the CE-MS system were studied taking into account the complex transport situation. It was found that the migration times were stable even for highly frequent injections with waiting times between the injections that were a factor four shorter than the migration time itself. Signal intensity of the MS was highest for positively charged species caused by the discriminating effect of the electrokinetic injection and the better ionization of the organic arsenic species by the electrospray ionization (ESI) source.

Further, the coupling of two important detectors for CE is presented, namely capacitively coupled contactless conductivity detection (C^4D) and electrospray ionization time-of-flight MS (ESI-TOF-MS). An experimental protocol was developed taking into account the requirements of the separation aspects and the compatibility with both detectors. ESI-TOF-MS requires background electrolytes consisting of volatile components such as ammonium acetate or formate. These, however, exhibit a rather high conductivity, which is disadvantageous for C^4D. A 10 mM ammonium acetate/ammonia buffer was taken as compromise concerning the detection performance of both detectors. A sample containing various phenolic compounds serving as a model system was determined. The analytical characteristics showed the complementarity and suitability of this dual detection approach as C^4D showed better response behavior towards m-cresol (limit of detection (LOD)=3.1 µM), while MS was more sensitive for determinations of m- and p-nitrophenol (LODs=0.8 µM) and 2,4-dinitrophenol (LOD=1.5 µM). The overall separation efficiency was excellent realizing the separation of counter-electroosmotic species with migration times of less than 60 s and illustrating that detector-induced band broadening could be neglected in the CE-C^4D/MS system.

In a second study, non-aqueous electrolytes were used for CE separations as they are highly compatible with both detectors due to their volatility and low background conductivity. A non-aqueous capillary electrophoresis (NACE)-C^4D-MS method was developed using an acetonitrile based background electrolyte containing 2 M HAc and 4 mM NH_4Ac enabling fast electrophoretic separations. Concentration changes of the background electrolyte and the choice of the inner diameter (ID) of the separation capillary were more critical for C^4D than for MS. To choose a separation capillary with appropriate ID, the dependency of the sensitivity of the C^4D on the ID was studied resulting in the use of a capillary with ID of 50 µm. The complementarity of the two detectors was demonstrated determining inorganic anions such as chloride, bromide, and nitrate (C^4D) as well as organic biomolecules such as choline, thiamine, and acetylcholine (MS) simultaneously. A calibration was performed and the method applied on an extract of a food supplement quantifying the model analytes using the respective detector.

MEEKC is a powerful tool for the separation of neutral species based on differences in their hydrophobic and hydrophilic properties. However, conventionally used SDS-based microemulsions are not compatible with ESI-MS. Enhancing the selectivity of the overall method, a surfactant-free microemulsion (SFME) based on water, ethanol, and 1-octanol was used as background electrolyte ensuring compatibility with ESI-TOF-MS. The small-angle X-ray scattering and dynamic light scattering measurements proved that the addition of ammonium acetate, which was necessary for the electrophoretic separation, was not altering aggregate formation in the SFME in the chosen concentration range. The separation performance of SF-MEEKC was demonstrated separating a model system consisting of hydrophobic and hydrophilic neutral vitamins, namely the vitamins B_2 and D_3, and the cationic vitamin B_1 using UV/VIS detection. The influence of the ammonium acetate concentration on the separation performance was studied in detail. Characterization of the developed method was performed concerning reproducibility of migration times and peak areas and concerning the linearity of the calibration data. Further, the compatibility of SF-MEEKC with ESI-MS was shown determining the content of vitamin D_3 in a commercial drug. Comparable sensitivity to aqueous CE-ESI-MS was achieved.

7.Zusammenfassung

Für die Analyse komplexer Probengemische wie biologische Proben oder Umweltproben sind hochselektive Analysestrategien erforderlich. Die Selektivität kann durch Kopplung zu orthogonalen Trenntechniken oder alternativ durch Kombination komplementärer Detektoren gesteigert werden. In vorliegender Arbeit wird je ein Beispiel für beide Konzepte vorgestellt. Zunächst werden methodische und instrumentelle Untersuchungen zur komprehensiven Kopplung der beiden wichtigsten instrumentellen Trenntechniken der Ionenanalytik, der Kapillarelektrophorese (CE) und der Ionenchromatographie (IC), beschrieben, die die gleichzeitige Bestimmung von Anionen und Kationen ermöglichen. Des Weiteren, wird die Kombination von kapazitiv gekoppelter kontaktloser Leitfähigkeitsdetektion (C^4D) und Massenspektrometrie (MS) als duales Detektionskonzept für die CE eingeführt und unter Verwendung von wässrigen und nicht-wässrigen Hintergrundelektrolytsystemen angewendet. Ferner kann die Selektivität gesteigert werden, indem die Kompatibilität einer Trenntechnik zum MS hergestellt wird. So wird in einem dritten Teil die Kopplung der tensidfreien elektrokinetischen Chromatographie mittels Mikroemulsion (SF-MEEKC) zum MS vorgestellt.

Zur komprehensiven Kopplung von Kapillar-IC und CE wurde ein Modulator entwickelt, der kontrollierte Injektionen von der ersten in die zweite Dimension gewährleistete. Dies wurde durch periodische Injektion des von der IC stammenden Ausflusses mittels einer Transferkapillare in die CE Trennkapillare durch Kapillar-Batch-Injektion erreicht. Wichtig für die Kopplung war hierbei die Kompatibilität der beiden Systeme. Die Eigenschaften der fortschrittlichen kapillaren Hochleistungsionenchromatographie wie zum Beispiel die geringen Flussraten im unteren µL/min-Bereich erleichterten die Kopplung, da somit die Flussbedingungen näher an denen der CE lagen verglichen mit den Flussbedingungen der konventionellen Ionenchromatographie. Aufgrund des implementierten Kapillarsuppressors setzte sich der Ausfluss der IC aus Analyt und reinem Wasser zusammen, wodurch Matrixeffekte verhindert wurden und auch „stacking“ Effekte in der CE ausgenutzt werden konnten. Des Weiteren ermöglichten schnelle CE Trennungen mit Migrationszeiten im Sekundenbereich die komprehensive Kopplung. Anhand von „Proof-of-concept“-Messungen mithilfe eines Modellsystems

bestehend aus Nukleotiden und deren zyklischen Derivaten wurde gezeigt, dass die Trennleistung durch das zweidimensionale ICxCE-MS-System im Vergleich zu Einzelmessungen gesteigert werden konnte.

Die Arbeit wurde um eine methodische Studie zur simultanen Bestimmung von positiv und negativ geladenen Analyten erweitert. Für die IC wurde ein Bypass-System mit Schaltventil entwickelt. Diese Konfiguration ermöglichte, dass die Kationen den Suppressor umgingen und somit nicht von der Kationen-Austauschmembran des Suppressors herausgefiltert wurden. Diese war nämlich nicht selektiv für die Kationen des Eluenten. Die Funktion des Aufbaus wurde demonstriert, indem ein Modellgemisch verschiedener Arsenspezies getrennt wurde. Die Kationen wurden mit Wasser von der Anionenaustauschsäule eluiert, bevor eine KOH-Gradientenelution zur Trennung der Anionen durchgeführt wurde. Die Injektionsparameter des Modulators, der den Ausfluss der IC in das CE-MS-System einführte, wurden unter Berücksichtigung der komplexen Transportsituation untersucht. Es zeigte sich, dass die Migrationszeiten selbst bei hochfrequenten Injektionen mit Wartezeiten zwischen den Injektionen stabil waren, die um den Faktor vier kürzer waren als die Migrationszeit selbst. Die Signalintensität des MS war am höchsten für positiv geladene Spezies, verursacht durch den diskriminierenden Effekt der elektrokinetischen Injektion und die bessere Ionisierung der organischen Arsenspezies durch die Elektronensprayionisations (ESI)-Quelle.

Des Weiteren wird die Kopplung von zwei wichtigen Detektoren für die CE vorgestellt, nämlich der kapazitiv gekoppelten kontaktlosen Leitfähigkeitsdetektion (C^4D) und der Elektrospray-Ionisations-Flugzeit-MS (ESI-TOF-MS). Ein experimentelles Protokoll, das die Anforderungen von Trennaspekten und die Kompatibilität mit beiden Detektoren berücksichtigt, wurde entwickelt. ESI-TOF-MS benötigt Hintergrundelektrolyte, die aus flüchtigen Komponenten wie Ammoniumacetat oder -formiat bestehen. Diese zeigen jedoch eine ziemlich hohe Leitfähigkeit, was für die C^4D nachteilig ist. Als Kompromiss bezüglich der Leistung beider Detektoren wurde ein 10 mM Ammoniumacetat/ Ammoniak-Puffer verwendet. Eine Probe, die verschiedene Phenol-Verbindungen enthält, wurde als Modelsystem verwendet und die darin enthaltenen Analyte analysiert. Die analytischen Charakteristika bewiesen die Komplementarität und Eignung dieses dualen Detektionskonzeptes, da die C^4D ein besseres Ansprechverhalten

für m-Kresol zeigte (Nachweisgrenze (LOD)=3.1 µM), während die MS für Bestimmungen von m- und p-Nitrophenol (LODs=0.8 µM) und 2,4-Dinitrophenol (LOD=1,5 µM) empfindlicher war. Die gesamte Trenneffizienz war hervorragend, da die Trennung von Spezies, die gegen den elektroosmotischen Fluss wanderten, mit Migrationszeiten von weniger als 60 s realisiert wurde und gezeigt wurde, dass detektorinduzierte Bandenverbreiterung im CE-C^4D/MS-System vernachlässigt werden konnte.

In einer zweiten Studie wurden nicht-wässrige Elektrolyte für die CE-Trennungen verwendet, da sie aufgrund ihrer Flüchtigkeit und niedrigen Hintergrundleitfähigkeit mit beiden Detektoren sehr kompatibel sind. Eine Methode für die nicht-wässrige Kapillarelektrophorese (NACE) gekoppelt mit dualer C^4D-MS-Detektion wurde unter Verwendung eines auf Acetonitril basierenden Hintergrundelektrolyten, der 2 M HAc und 4 mM NH_4Ac enthielt, entwickelt, wodurch schnelle elektrophoretische Trennungen ermöglicht wurden. Konzentrationsänderungen des Hintergrundelektrolyten und die Wahl des Innendurchmessers der Trennkapillare waren für die C^4D kritischer als für die MS. Um eine Trennkapillare mit geeignetem Innendurchmesser zu wählen, wurde die Abhängigkeit der Empfindlichkeit der C^4D vom Innendurchmesser untersucht. Dies führte als Ergebnis zur Verwendung einer Kapillare mit einem Innendurchmesser von 50 µm. Die Komplementarität der beiden Detektoren wurde demonstriert, indem gleichzeitig anorganische Anionen wie Chlorid, Bromid und Nitrat (C^4D) sowie organische Biomoleküle wie Cholin, Thiamin und Acetylcholin (MS) bestimmt wurden. Eine Kalibrierung wurde durchgeführt und als Anwendung ein Extrakt eines Nahrungsergänzungsmittels analysiert, wobei die Modellanalyten unter Verwendung des jeweiligen Detektors quantifiziert wurden.

MEEKC ist ein leistungsfähiges Verfahren um neutrale Spezies basierend auf Unterschieden in deren hydrophoben und hydrophilen Eigenschaften zu trennen. Konventionell verwendete SDS-basierte Mikroemulsionen sind jedoch nicht mit ESI-MS kompatibel. Um die Selektivität des Verfahrens zu verbessern, wurde eine tensidfreie Mikroemulsion (SFME) auf Basis von Wasser, Ethanol und 1-Octanol als Hintergrundelektrolyt verwendet, da diese die Kompatibilität mit der ESI-TOF-MS gewährleistete. Die Messungen von Röntgenkleinwinkelstreuung und dynamischer Lichtstreuung zeigten, dass die Zugabe von Ammoniumacetat, das für die elektrophoretische Trennung notwendig war, im gewählten Konzentrationsbereich die

Aggregatbildung in den SFME nicht veränderte. Zur Demonstration der Trennleistung der SF-MEEKC-Methode wurde ein Modellsystem bestehend aus hydrophoben und hydrophilen neutralen Vitaminen, nämlich der Vitamine B_2 und D_3, und des kationischen Vitamins B_1 aufgetrennt, wobei UV/VIS-Detektion verwendet wurde. Der Einfluss der Ammoniumacetatkonzentration auf die Trennleistung wurde im Detail untersucht. Das entwickelte Verfahren wurde hinsichtlich der Reproduzierbarkeit von Migrationszeiten und Peakflächen und hinsichtlich der Linearität der Kalibrationsdaten charakterisiert. Ferner wurde die Kompatibilität von SF-MEEKC mit ESI-MS gezeigt, indem der Gehalt an Vitamin D_3 in einem kommerziellen Arzneimittel bestimmt wurde. Die erreichte Empfindlichkeit war hierbei vergleichbar mit der aus wässrigen CE-ESI-MS-Bestimmungen.

8.Appendix – Developement of a control software for the ICxCE-MS modulator

8.1. General remarks

The described software was written in C# and was developed by Andrea Beutner and Josef Bernhardt (Electronic workshop of the Faculty of Chemistry and Pharmacy of the University of Regensburg). It is partly based on a software developed by M. Grundmann. A. Beutner was responsible for the design and content and J. Bernhardt did the programming.

The task of the software was to control all parts of the modulator for ICxCE-MS measurements (Figure 1). This included the positioning of the motors (2) to move the transfer capillary (3) and the control of the syringe (1), the stirrer (5), and the HV (4). Moreover, also the start of the TOF-MS was triggered by the program to synchronize the time-scales of the MS and the modulator protocol. All data concerning the protocol were saved time-dependent to enable further evaluation of the ICxCE-MS measurements and the calculation of migration times. The software is divided in seven sections, namely “Experimental”, “CBI Stepper Z/X”, “Syringe”, “Stirrer”, “High Voltage”, “TOF Remote”, and “Experimental Data”. In Table 1, an overview of the functions of the different sections is given. In the following, the “Experimental” section and the “CBI Stepper Z/X”-motor section are described closer.

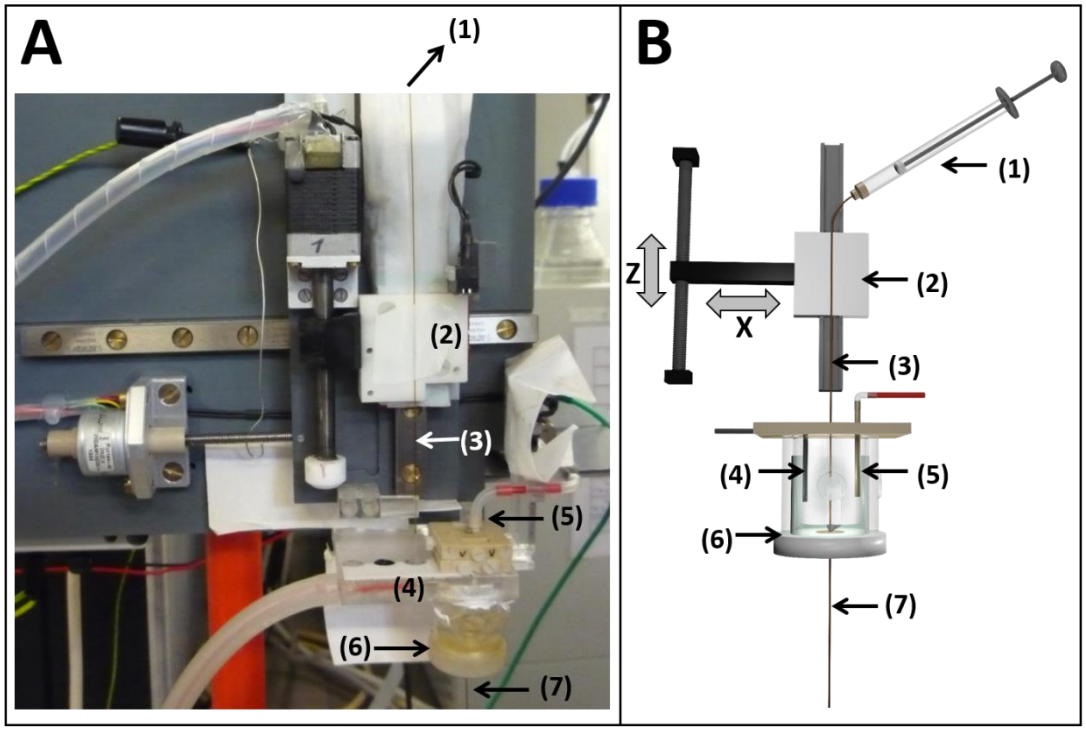

Figure 1: *Photograph* ***(A)*** *and scheme* ***(B)*** *of the modulator. (1) Syringe pump or IC, (2) fixation plate movable by the stepper motors, (3) transfer capillary, (4) HV electrode, (5) stirrer, (6) electrophoretic cell containing BGE, (7) CE separation capillary.*

Table 1: *Overview of the sections of the program.*

Section	Function
Experimental	Automated control of all hardware components
CBI Stepper Z/X	Manual control and initializing of the motors; setting of the motor speed and ramp
Syringe	Manual control of the syringe pump; setting of the type of the used syringe and the rate unit
Stirrer	Manual control of the stirrer
High Voltage	Manual control of the high voltage source; setting of a maximum current
TOF Remote	Sending orders to the TOF-MS software
Experimental Data	Displaying and saving the current, voltage, motor positions, stirrer rate, and syringe rate in dependence of the TOF-MS measurement time. Synchronizing occurs with aid of the stopwatch in the “Experimental” section.

8.2. “Experimental” section of the program

A screenshot of the “Experimental” section is depicted in Figure 2. In this section, experimental methods can be programmed and saved as text-files and measurements are controlled. In Figure 2.1, a table for a experimental procedure is depicted. The table is read row by row from top left to bottom right whereas every field is a separate order. The next order is sent after finishing the previous one. In Table 2, the function of the columns and their allowed entries are described. In Figure 2.2, the feedback of the accessed hardware is depicted. The method defined in Figure 2.1 can be started, stopped or paused using the buttons in Figure 2.3. Moreover, the start of the TOF-MS can be triggered. The functions of Figure 2.3 are explained in Table 3.

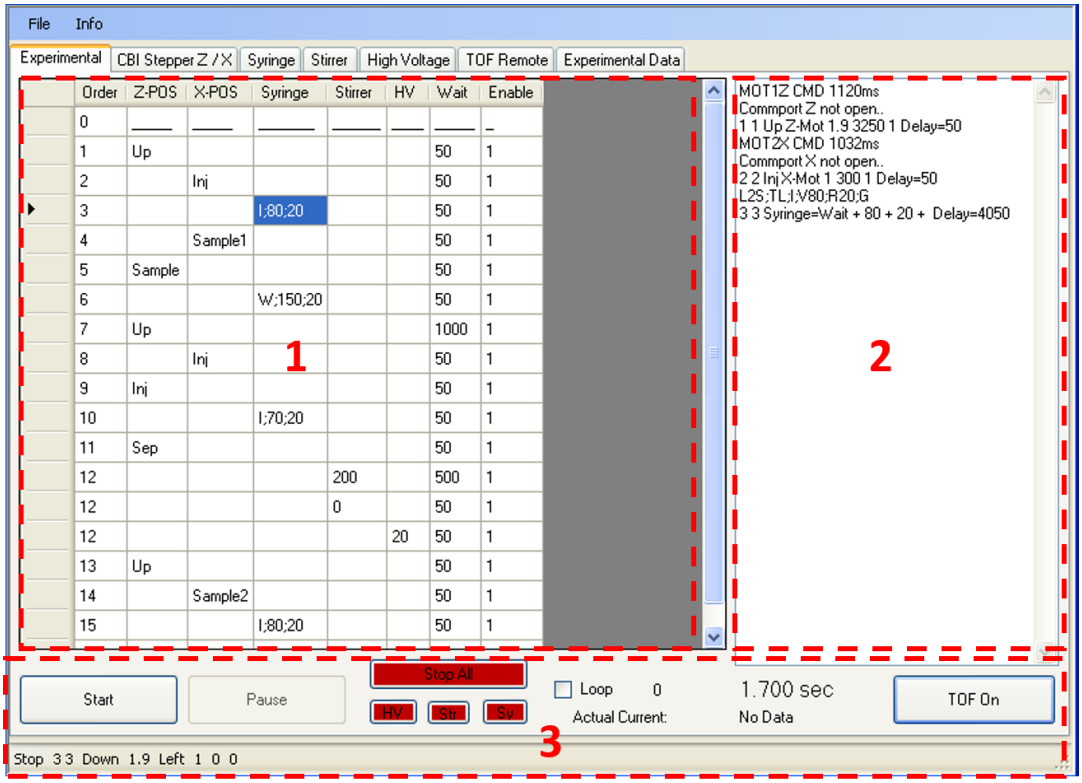

Figure 2: *Experimental section of the software. (1) Experimental protocol, (2) feedback of the accessed hardware, (3) control section.*

Table 2: *Function and allowed entries of the experimental protocol depicted in Figure 2.1.*

Column name	**Function**	**Allowed entries**	**Comments**
Order	None; just for clarity reasons	No limitations	
Z-Pos	Movement of the motor in Z-direction to a defined position	"Name of the position"	Definition of the names in the CBI "Stepper Z/X" section
X-Pos	Movement of the motor in X-direction to a defined position	"Name of the position"	Definition of the names in the CBI "Stepper Z/X" section
Syringe	Withdrawing (W) or injection (I) of a defined volume in a defined rate (completing the order before proceeding with the protocol)	"I or W;integral number; integral number"	mode;volume;rate Definition of units and syringe type in the "Syringe" section
	Start of withdrawing (W) or injection (I) of a defined volume in a defined rate (proceeding with the protocol after starting the order)	"<; I or W;integral number; integral number"	<;mode;volume;rate Definition of units and syringe type in the "Syringe" section
Stirrer	Setting of a defined stirrer speed	"Integral number"	Stirrer speed in turns per minute; to stop the stirrer, it is set to 0
HV	Setting of a defined HV	"Integral number"	HV in kV; to stop the voltage, it is set to 0
Wait	Stopping the program for a defined time interval	"Integral number"	Waiting time in ms
Enable	Activation of the row	"1"	Activation of the row: the orders of the row are carried out
		"0"	Inactivation of a row: the orders of the row are skipped

Table 3: *Explanation of the buttons and functions in Figure 2.3.*

Name	Function	Comment
Start/ Stop	Starts or stops proceeding with the orders of the protocol in Figure 2.1., the button does not stop or start the single hardware components	After pressing “start” the button converts to a “stop” button
Pause/Resume	Pauses or resumes the protocol	After pressing “pause” the button converts to a “resume” button
HV	Stops the HV if switched on	
Str	Stops the stirrer	
Syr	Stops the syringe if in progress	
Stop All	Stops HV, stirrer, and syringe	
Loop	If activated, the protocol is restarted after completion	
Actual Current	Displays the actual current [µA] due to the HV	
TOF On/Off	Triggers the start or stop off the TOF-MS	After pressing “TOF On” the button converts to a “TOF Off” button
Stopwatch	Measures the time interval between start of the TOF-MS and start of the experimental protocol	This interval is needed for synchronization of the data

8.3. “CBI Stepper Z/X”-motor section of the program

In Figure 3, the “CBI Stepper Z/X”-motor section is shown, where parameters of the motors can be set. A number is assigned to every position whereas 0 means the position on the right border (stepper motor X) or the highest position (stepper motor Z). The unit of the numbers is mm, which means that position number “20.033” (stepper motor Z), for example, is located 2 cm and 33 µm under the reference position. Moreover, speed and ramp of the motors are defined. The speed is given in steps per second, whereas 1000 steps (of the stepper Z motor) or 80 steps (of the stepper X motor) are one mm. As ramp, three options can be chosen, namely a trapezoid ramp (“0”), a sinusoidal ramp (“1”), or a jerk-free ramp (“2”). Before using the motors, the comport has to be opened and the motors have to be initialized. Pressing “Initialize Motors” the motors move to the respective reference positions (“RefZ” and “RefX”) and then back to “Setup” for insertion of the capillary. Manual control of the motors is possible choosing a position and pressing “test selected” or clicking on the buttons with the numbers under the “test selected” button.

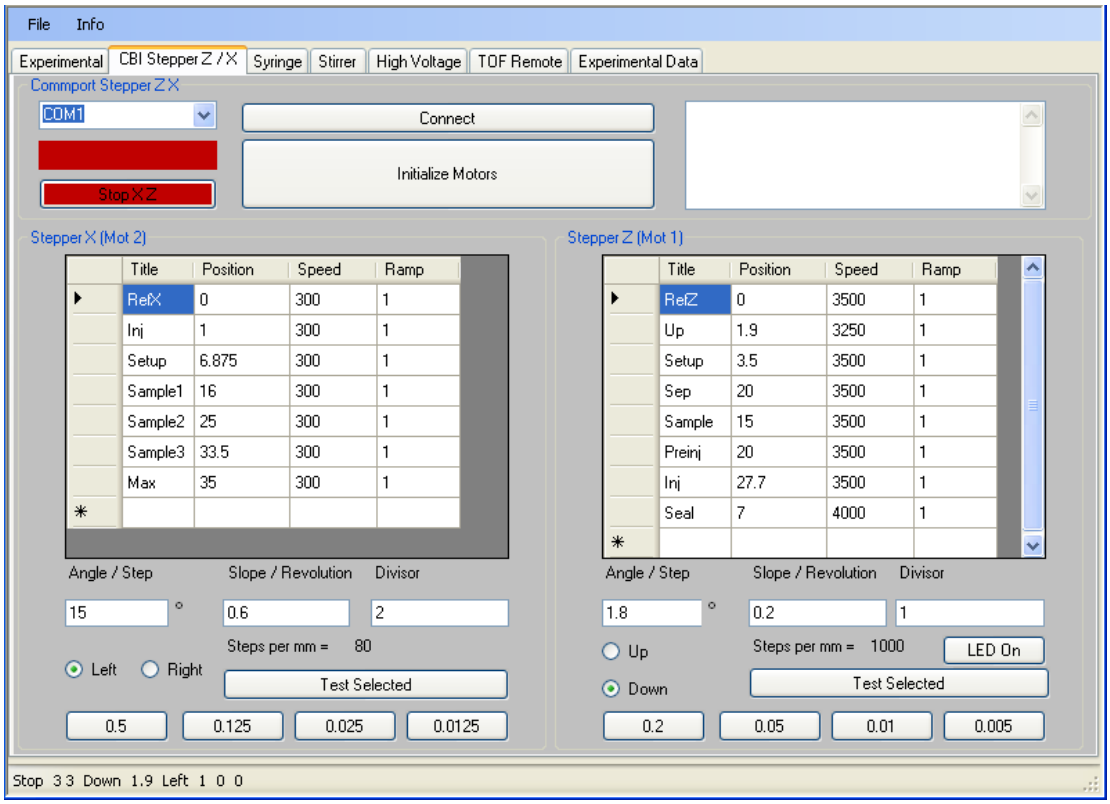

Figure 3: *"CBI Stepper Z/X"-motor section of the software.*

List of abbreviations

ACN	acetonitrile
AD	amperometric detection
AMP	adenosine monophosphate
BGE	background electrolyte
C^4D	capacetively coupled contactless conductivity detector
cAMP	cyclic adenosine monophosphate
CBI	capillary batch injection
cCMP	cyclic cytidine monophosphate
CD	conductivity detection
CE	capillary electrophoresis
CEC	capillary electrochromatography
cGMP	cyclic guanosine monophosphate
cHPIC	capillary high performance ion chromatography
CL	chemiluminescence
CMP	cytidine monophosphate
CSE	capillary sieving electrophoresis
CZE	capillary zone electrophoresis
DAD	diode array detection
DLS	dynamic light scattering
DLVO	Derjaguin-Landau-Verwey- Overbeek
ECL	electrochemiluminescence
EOF	electroosmotic flow
ESI	electrospray ionization
FTICR	Fourier-transform ion cyclotron resonance
GC	gas chromatography
GE	gel electrophoresis
GFC	gel filtration chromatography
GMP	guanosine monophosphate
HV	high voltage

IC	ion chromatography
ID	inner diameter
IEF	isoelectric focussing
IM	ion mobility
IS	internal standard
ITP	isotachophoresis
LC	liquid chromatography
LED	light-emitting diode
LEDIF	light-emitting diode induced fluorescence
LIF	laser-induced fluorescence
LOD	limit of detection
LS	light scattering
µFFE	Micro free-flow electrophoresis
m/z	mass-to-charge
MALDI	matrix-assisted laser desorption/ionization
MEEKC	microemulsion electrokinetic chromatography
MEKC	micellar electrokinetic chromatography
MS	mass spectrometry
NACE	non-aqueous capillary electrophoresis
OD	outer diameter
RPLC	reversed phase liquid chromatography
S/N	signal-to-noise
SAXS	small-angle X-ray scattering
SDS	sodium dodecyl sulfate
SEC	size exclusion chromatography
SFME	surfactant free microemulsion
SF-MEEKC	surfactant free microemulsion electrokinetic chromatography
TOF	time-of-flight
UV/VIS	ultraviolet/visible

Patents and publications

9. A. Beutner, F.-M. Matysik, Selectivity enhancement in capillary electrophoresis by means of dual detection or two-dimensional separation. (submitted to Anal. Chim. Acta)

8. A. Beutner, S.K. Piendl, S. Wert, F.-M. Matysik, Methodical studies of the simultaneous determination of anions and cations by ICxCE-MS using arsenic species as model analytes., Anal. Bioanal. Chem., 2018. (DOI: 10.1007/s00216-018-1241-1)

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Presentations

Oral presentations

2017 **33rd International Symposium on Microscale Separations and Bioanalysis (MSB)**, Noordwijkerhout, Netherlands: Hyphenating IC and CE: The development of a comprehensive system and its application to arsenic speciation analysis.

2017 **Doktorandenseminar des AK Separation Science der GDCh**, Hohenroda, Germany: Teamwork zweier Detektoren – kontaktlose Leitfähigkeit und Massenspektrometrie als dualer Detektionsansatz für die Kapillarelektrophorese.

2016 **CE-Forum**, Regensburg, Germany: Teamwork of two detectors − a dual detection approach for CE combining C^4D and MS.

2016 **Eberhard-Gerstel-Award, Analytica conference**, Munich, Germany: Two-dimensional separation of ionic species by hyphenation of capillary IC×CE-MS

2015 **CE-Forum**, Tübingen, Germany: Two-dimensional ion chromatography x capillary electrophoresis.

2014 **CE-Forum**, Marburg, Germany: Hyphenation of ion chromatography and capillary electrophoresis.

2014 **International Student Conference (ISC)**, Prague, Czech Republic: Hyphenation of ion chromatography and capillary electrophoresis.

Poster presentations

2017 **ANAKON**, Tübingen, Germany: Arsenic speciation analysis by comprehensive hyphenation of ion chromatography and capillary electrophoresis.

2015 **ANAKON**, Graz, Austria: Comprehensive hyphenation of capillary ion chromatography × capillary electrophoresis - mass spectrometry

Eidesstattliche Erklärung

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(4) Die Arbeit wurde bisher weder im In- noch im Ausland in gleicher oder ähnlicher Form einer anderen Prüfungsbehörde vorgelegt.

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