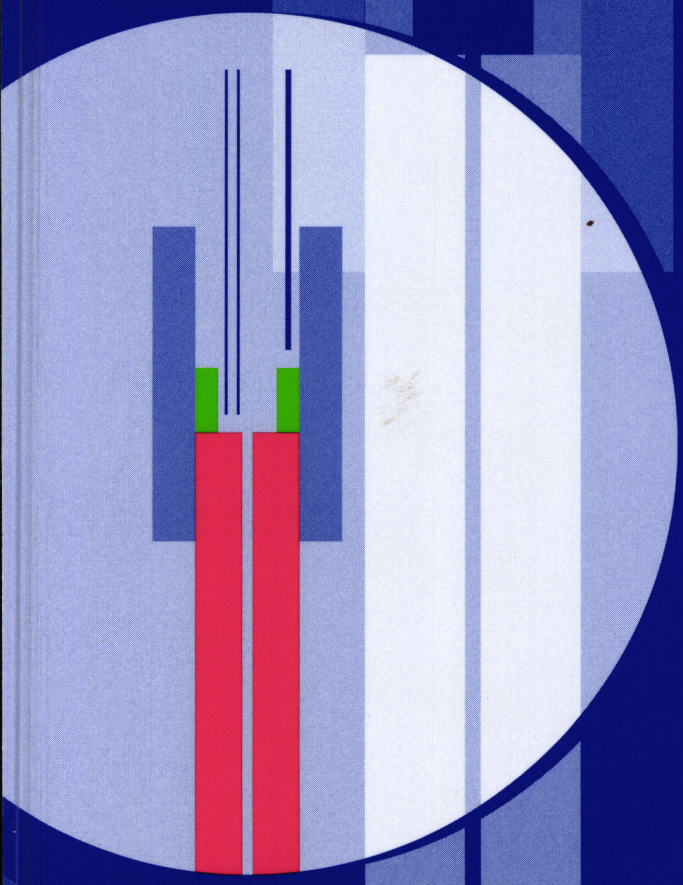
Automated pretreatment of biological samples for capillary electrophoresis



J.R. Veraart

Automated pretreatment of biological samples for capillary electrophoresis

J.R. Veraart

VRIJE UNIVERSITEIT

Automated pretreatment of biological samples for capillary electrophoresis

ACADEMISCH PROEFSCHRIFT

ter verkrijging van de graad van doctor aan
de Vrije Universiteit te Amsterdam,
op gezag van de rector magnificus
prof.dr. T. Sminia,
in het openbaar te verdedigen
ten overstaan van de promotiecommissie
van de faculteit der Exacte Wetenschappen / Scheikunde
op dinsdag 21 november 2000 om 15.45 uur
in het hoofdgebouw van de universiteit,
De Boelelaan 1105

door

Johannes Robertus Veraart

geboren te Bergen op Zoom

Promotoren:	prof.dr. U.A.Th. Brinkman
	prof.dr. N.H. Velthorst
Copromotor:	dr. H. Lingeman

Table of contents

Chapter 1

Introduction

1.1 Capillary electrophoresis

Capillary electrophoresis (CE) arrived on the scene much later than the two main chromatographic separation techniques, gas chromatography (GC) and liquid chromatography (LC). Although the technique was first described in the early seventies, it took another ten years before the ability to obtain high separation efficiency by applying a voltage across a capillary was highlighted. The late eighties saw the advent of commercial CE instrumentation, and the transformation of a research technique into a routine technology in the industrial environment began at about the same time. The early applications of CE focused on the separation of biomolecules such as proteins and peptides where electrophoresis was the traditional method of analysis and use of CE was viewed as an extension of previous methodology. On the other hand the determination of small molecules such as pharmaceuticals and agrochemicals was not routinely performed by electrophoresis and LC was firmly entrenched in these areas as the workhorse technique. Still, CE has gradually become established as an alternative and support technique for LC and it is part of this area, *viz.* the determination of drugs in biological fluids, that will be discussed in the present thesis.

It is often said that CE offers several advantages over LC – the chromatographic technique with which it, for obvious reasons, should primarily be compared. The main and undisputed advantage no doubt is the much higher separation efficiency. An additional, but more modest advantage is the lower, if any, consumption of organic solvents. A third addition to the list, the smaller sample volume required, is by itself correct but its advocates tend to overlook the fact that in many real-life situations, the available sample volume is such that the analyst considers it a drawback that not more, rather than less, sample can be easily introduced. To our opinion, a main benefit of the use of CE over LC on the separation side, is that ionic and ionogenic components can be analysed much better – a statement born out by the list of contents of most treatises on the application of CE.

However, despite the above, it is also true that CE is not as frequently used for the determination of drugs in biological fluids as some authors will have us believed. Especially when trace-level concentrations have to be determined, as is the case with newer rather than older drugs, and with serum or plasma rather than is with urine, the sensitivity or, more correctly the analyte detectability in units of concentration, is often insufficient. As was recently pointed out by Hempel [1], the scope for overcoming this limitation on the detector side is rather limited, a statement with which we agree. Consequently, much of the desired improvement will have to be found on the front end of the system, i.e. by improving sample pretreatment and analyte enrichment prior to introduction. This should simultaneously help to improve

the repeatability of the run-to-run migration times which, with real-life samples, is often poorer than some workers will admit.

In addressing the problem it was our aim to develop a generally valid strategy that can cope with some of the main problems, such as (i) high salt concentrations, which easily adversely affect the determination of the analytes of interest, (ii) the presence of proteins which irreversibly bind to the inner wall of the capillary, which may influence the electro-osmotic flow and, thus, the migration times, and can disturb the analytical results because of drug–protein binding, and (iii) the presence of particulate matter that can clog the CE capillary. And (iv) of course, the sometimes recommended sample dilution cannot be used because the detection limits then will be increased rather than decreased.

Two samples types were selected, serum and urine, and the three classes of drugs, representing amphoteric, basic and acidic compounds. As will be discussed in detail below, and in the next chapters, two approaches were used, an at-line combination of solid-phase extraction (SPE) and CE, and an on-line combination of dialysis, SPE and CE. During the project, setting up fully automated procedures and writing a protocol to be used in future method development, became additional goals of the study.

1.2 Scope of the thesis

The present introductory **Chapter 1** is followed by an extensive review on sample pretreatment of biological fluids for use with CE separation (**Chapter 2**). The various types of interface are categorised, and their several advantages and disadvantages are listed. Next, chromatography-based sample separation techniques such as solid-phase extraction (SPE), solid-phase micro-extraction (SPME) and liquid chromatography (LC) are discussed in detail. The second category of interest comprises electrophoresis-based sample preparation techniques that were combined with CE, such as isotachophoresis (ITP) and special injection techniques (i.e. sample stacking, field-amplified injection and electro-extraction). One main conclusion is that too few systematic studies have been performed in the biological sample preparation–CE field to arrive at definite recommendations and protocols but that – with all due modesty – the dialysis–SPE–CE approach discussed in several chapters of the present study, distinctly merits further attention.

Chapter 3 describes two types of tools that had to be developed prior to starting the sample preparation optimisation. The first topic that is addressed is the distinct need for proper thermostating, and the practical usefulness of several thermostating methods that are used in CE instruments. As the final result, an improved thermostating device is proposed, which was used in all further studies. The second topic deals with the introduction of a stream of liquid (coming from an LC column) into the CE capillary. As an illustrative example, a mixture of chlorinated benzoic

acids was analysed that were dissolved in a series of solutions containing increasing salt concentrations. Without the automated sample pretreatment, separation was only possible for solutions with a low salt content of up to 20 mM, while with the LC column as on-line clean-up device, analysis of samples with salt concentrations of up to 400 mM gave the same result. The interface was used in all subsequent studies reported in Chapters 4 and 5.

The first type of coupling, at-line SPE–CE, is discussed in **Chapter 4**. The general purpose is that an automated robotic SPE device takes care of the sample pretreatment using disposable SPE cartridges. Next, the trapped analytes are desorbed, and the extract is used to fill a 0.1 ml loop. The valve on which the loop is mounted, is now switched and the contents of the loop are flushed to the interface. The injection into the CE capillary was performed by applying a underpressure at the other end of capillary. When the interface is flushed with CE buffer, a voltage is applied over the capillary and CE analysis with UV detection is performed. The practicality of the approach was shown for three groups of analytes in urine and serum. Non-steroid anti-inflammatory drugs (NSAIDs) represent acidic drugs, sulphonamides the amphoteric compounds and tricyclic antidepressants (TCADs) were the model compounds for positively charged molecules. Finally, a real-life application, the determination of phenprocoumon in urine and serum was carried out. With the TCADs, a non-aqueous buffer was used for the CE separation. In that case, the SPE cartridge was also used for the removal of water, because it would interfere with the CE separation. Typical detection limits (LODs) found for various classes of analytes were 10–80 ng/ml with urine and 200-1000 ng/ml with serum. The differences are caused by the fact that up to 8 ml of urine were loaded onto the SPE cartridge, as against 1 ml for serum. The day-to-day and within-day repeatability were 2–10 % for serum samples and 4–11 % for urine samples.

On the basis of the overall successful approach discussed in Chapter 4, we next decided to study a fully on-line technique, dialysis–SPE–CE. The results of that work are presented in **Chapter 5**. The samples are filtered using an automated dialysis module, which has a cut-off membrane of about 15 kDa. Low-molecular-weight material such as the analytes and (in)organic (buffer) salts can pass the membrane to the acceptor phase, but the larger molecules like proteins and particulate matter, will remain on the donor side. Next, the acceptor phase is led through an SPE cartridge to achieve reconcentration of the analytes of interest. Salts and interfering low-molecular-weight material that is still present in the acceptor solution, pass to waste or are removed by flushing of the cartridge with a suitable washing solvent. Subsequently, the analytes are desorbed in a small volume and transported to the interface. When the analytes are passing the capillary, an appropriate volume is injected in a way similar to that described above for the SPE–CE approach. Special attention had to be devoted to the optimization of the start, and the duration, of the injection procedure, to be sure that as much of the passing analytes as possible is

injected in such a way that band broadening effects are minimal. The practicality of the approach was demonstrated by analysing the same classes of compounds as quoted above. For the determination of the TCADs again a non-aqueous buffer had to be used. Methanol had to be used as desorption solvent because its nature does not influence the CE separation. However, because methanol is a non-conducting solvent, an electrokinetic injection technique was used. After clean-up the composition of the extracts is essentially the same for all samples. Therefore, good repeatability and low detection limits were obtained because of the electrokinetic injection. The LODs achieved were 40–100 ng/ml for urine and 50–300 ng/ml for serum. The higher LODs found with serum are caused by the fact that for some compounds it is diffucult to disrupt the analyte–drug binding. The day-to-day and within-day repeatabilities of the analyses were typically 2–10 % for serum and 4–11 % for urine. The optimization was performed using a protocol that can be used for both types of samples. The use of the protocol reduces the number of optimization steps.

In summary, for three groups of drugs - acidic, amphoteric and basic compounds - and for the two most commonly analysed sample types, urine and serum, two combinations of sample treatment combined at-line or on-line with capillary electrophoresis have been systematically studied. Both approaches, at-line SPE–CE and on-line dialysis–SPE-CE can cope with the most serious problems generally encountered, viz. high salt concentrations, presence of proteins and particulate matter, and both can be combined with non-aqueous CE. As a result of the automation of the sample treatment and the robustness of the set-ups, analyses can be conveniently carried out unattendedly and especially with the more sophisticated dialysis-based approach, analyte detectability meets real-life requirements. Finally, designing similar procedures for other applications should be rather rapid if the protocol is used that was developed in the present study.

[1] F. Hempel, Electrophoresis, 21 (2000) 691.

Chapter 2

Coupling of Biological Sample Handling and Capillary Electrophoresis

2

Coupling of biological sample handling and capillary electrophoresis

Summary

The analysis of biological samples (e.g. blood, urine, saliva, tissue homogenates) by means of capillary electrophoresis (CE) requires efficient sample preparation (i.e. concentration and clean-up) procedures to remove interfering solutes (endogenous/exogenous and/or low-molecular/high-molecular-weight), (in)organic salts and particulate matter. The sample preparation modules can be coupled with CE either off-line (manual), at-line (robotic interface), on-line (coupling via a transfer line) or in-line (complete integration between sample preparation and separation system). Sample preparation systems reported in the literature are based on chromatographic, electrophoretic or membrane-based procedures. The combination of automated sample preparation and CE is especially useful if complex samples have to be analysed and helps to improve both selectivity and sensitivity. In this review, the different modes of solid-phase (micro-) extraction will be discussed and an overview of the potential of chromatographic, electrophoretic (e.g., isotachophoresis, sample stacking) and membrane-based procedures will be given.

2.1 Introduction

Capillary electrophoresis (CE) is a highly promising technique for the separation of, especially, ionogenic and ionic compounds. Over the past two decades, much attention has been devoted to its development. The characteristics of CE make it a very useful technique for the determination of charged compounds in biological samples; separation efficiencies are high and separation conditions can easily be adapted to optimize resolution. Furthermore, the separation mechanism of CE is markedly different from that of LC. However, the number of routine applications of biological samples is limited because there still are several problems that have to be solved. No doubt the main problem is that analyte detectability expressed in concentration units, generally is rather poor because of the low volume loadability of the CE capillary. In addition, the high concentrations of inorganic salts present in urine (50–500 mM sodium chloride) and serum (ca. 150 mM sodium chloride) cause the electrical conductivity of the samples to be high, which reduces the efficiency of the CE separation [1–7]. The salt concentration in a matrix can also strongly influence the amount of sample that is loaded when electrokinetic injection is used and the injection profile can be affected in integrated CE devices [164]. Further, the relatively high protein concentrations present in a number of biological samples - e.g. ca. 75 g/l in serum - can cause a nearly irreversible binding of these macromolecules to the free silanols of the fused-silica capillary wall, which may result in significantly changed migration times of the analytes or the presence of interfering peaks in the electropherograms [3, 4, 7, 8]. Another problem is the presence of particulate matter, which can easily clog the CE system. Finally, as in column liquid chromatography (LC), serum and tissue homogenates (e.g., liver, kidney) cannot be injected directly because they are too viscous. Obviously, suitable sample clean-up and analyte-enrichment procedures have to be applied before a sample can be subjected to CE analysis.

Several reviews have been devoted to one or more aspects of sample preparation such as CE combined with microdialysis [9] or in-line solid phase extraction (SPE) [10, 159, 160]. Other reviews deal with typical application fields such as the analysis of serum proteins [11, 12] and clinical [13–15], pharmacokinetic [16], pharmaceutical [15], forensic [15] or metabolic studies [17]. These reviews do not cover the total field of bioanalytical CE procedures. The emphasis of the present paper will be on those sample preparation procedures that can be used for the quantitative determination of organic compounds in aqueous samples, biological fluids and tissue homogenates. The different types of interface that are used to combine sample preparation and CE will be discussed and, next, the various modes of sample preparation will be reviewed. With respect to the latter aspect, the clean-up/concentration procedures will be distinguished on the basis of the separation principle used: chromatography, electrophoresis or membrane transport.

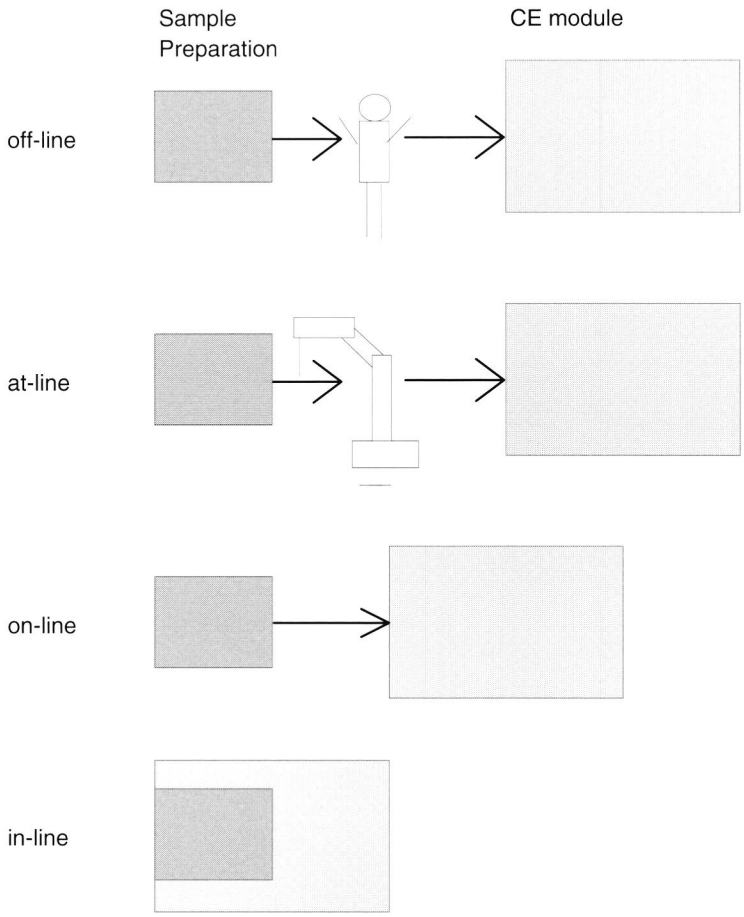


Figure 2.1 Schematic presentation of different types of interfacing between a sample preparation method and CE.

2.2 Interface classification

Combinations of a sample preparation device and a CE separation system can be characterized by the degree of integration between these two units. In principle, four levels of integration, or coupling, can be distinguished (figure 2.1) [18]. The lowest level of coupling is the off-line combination of sample preparation and separation. The two steps are performed separately and the analyte transfer is carried out manually. If a robotic system is used to transfer the samples from one unit to the other, an at-line combination has been achieved. In both the off-line and at-line systems there is no direct stream of liquid between the two units. Combinations in which a direct transport of liquid takes place via connecting capillaries are called on-

line systems. In this case, samples can be processed either parallel - with the second sample being pretreated while the first sample is being analysed - or in series - where samples are pretreated and analysed one after the other (and throughput is lower). The highest level of integration is obtained when the sample preparation and separation units are fully integrated into a new in-line system. The different levels of integration can be characterized and mutually compared as shown in table 2.1. Most of the parameters are self-explanatory, but something should be said with regard to buffer limitation and detector choice.

The selection of the solvents used during sample preparation and separation is rather critical in in-line systems: compatibility problems can occur since both steps are performed in the CE capillary. With on-line systems this is already somewhat less critical, while compatibility problems in off-line and at-line systems can usually be avoided by incorporating a phase-switching step such as evaporation plus redissolution. However, in all off-, at- and on-line systems, the solvent used to transfer the analyte from the sample preparation device to the CE unit should be compatible with the buffer used during the separation step. The present subdivision will be used throughout this review, although different definitions are sometimes used in the literature. For example what we define as in-line SPE–CE, because a single system is used for both sample preparation and separation, is called on-line SPE–CE by several authors [19].

Table 2.1 Relevant aspects of coupled CE systems.

	off-line	at-line	on-line	in-line
Automated coupling between sample pretreatment and analysis system	no	yes	yes	yes
Sample pretreatment performed in series or parallel with analysis	parallel	parallel	series/ parallel	serial
Sample directly injected in analytical system	no	no	no	yes
Complexity of the total system	simple	complex	complex	simple
Robustness when analysing dirty samples	poor/good	poor/good	poor	poor
Multidimensional system can be set up	semi	semi	yes	no
Applicable to in vivo systems	yes	yes	yes	no
Sample loss during transfer between sample pretreatment and analysis system	yes	yes	yes/no	no
CE buffer limitation	no	no	no	yes
Detector choice	free	limited	limited	free

A special problem regarding the combination of a sample preparation procedure and CE via an interface, as in at-line and on-line approaches, is that the inlet of the capillary has to be grounded. Consequently, a high voltage has to be applied at the outlet end of the capillary. When detectors are used which have to be coupled at the outlet of the CE capillary, such as electrochemical (ECD), mass spectrometric (MS) or conductivity detectors, the outlet should be grounded and the high voltage applied at the injection side of the capillary. In other words, these detection techniques cannot easily be applied when at-line and on-line sample preparation procedures are used because in that case both ends of the capillary have to be grounded. No such problems occur with detection techniques are used which can be applied through the capillary silica wall (e.g., absorbance, fluorescence and NMR). Off-line and in-line coupled techniques can be combined with all types of interfaces.

2.3 Chromatography-based sample preparation

Chromatography-based sample preparation for CE has the advantage that the separation can be orthogonal, which implies that a high degree of selectivity can be obtained by combining these two techniques. There are two main approaches in chromatography-based sample preparation: (i) all compounds are desorbed simultaneously, which is the usual case with SPE or (ii) the compounds are eluted sequentially and an additional separation is provided as in LC. With chromatographic techniques the analytes to be separated should preferably be neutral and the pH of the system should be selected accordingly. In CE, however, the analytes have to be charged if a free-solution CE technique is going to be used; as an alternative a micellar system can be applied to separate analytes with a neutral form.

2.3.1 Solid-phase extraction

SPE can be used to simultaneously enrich the trace analytes and remove salts, proteins and a wide variety of other potentially interfering compounds. It can be combined with CE in several ways which vary from off-line to in-line, using a variety of extraction cartridges. A special form of SPE is solid-phase micro-extraction (SPME), which can also be combined with CE and uses small fibres coated with a suitable sorbent.

Off-line solid-phase extraction

Liquid–liquid extraction (LLE) and SPE are nowadays the most frequently applied off-line sample preparation techniques in bioanalysis. In addition to the normal

advantages and limitations of SPE in combination with LC, SPE has a number of distinct advantages over LLE in combination with CE.

- The polarity of the final organic solvent (i.e., acetonitrile, methanol) normally is compatible with the subsequent CE separation and the final volume usually is small. Time-consuming evaporation steps, which may result in analyte losses, can therefore be avoided and improved analyte enrichment obtained.
- The combination of two SPE steps in series will allow the stepwise reduction of the sample volume, while at the same time a higher degree of selectivity (i.e. clean-up) can be obtained.
- Conversion of off-line to at-line or on-line SPE can be achieved by appropriate miniaturization without changing the extraction principle.
- The nature of the final desorption solvent allows the use of subsequent stacking or isotachophoresis (ITP)–CE procedures to improve the final analyte detectability.

Table 2.2 The use of SPE off-line combined with CE.

Matrix	Separation system*	Analyte
Liver	tITP-CE	toxins [72]
Plasma	tITP-CE	enzyme inhibiters [70]
Plasma	CE	acetylsalicylic acid and metabolites [166], phosphorothioate oligonucleotides [30]
Serum	tITP-CE	peptides [69]
Serum	CE	methotrexate/7-hydroxymethotrexate [20], conjugated bile acids [118], pentobarbital [21], D-pen(2,5)enkephalin [22, 119], prilocaine [120], ondansetron [121], cardiovascular drugs [26] pentazonie enantiomers [153]
Tissue homogenates	CE	phosphorothioate oligonucleotides [30]
Urine	spPC–CE	propranolol/doxipin [19]
Urine	tITP-CE	adenosine [68]
Urine	CE	acetylsalicylic acid and metabolites [166], atenolol [122], 7-hydroxycoumarin [123], debrisoquine/4-hydroxydebrisoquine [23], EDTA [29], 3,4-methylenedioxymethaphetamine [124], phosphorothioate oligonucleotides [30], methadone and methabolites [125], organic acids [24], morphine and metabolites [126], cardiovascular drugs [26], hypoglycemic drugs [48], benzophetamine [148]

* spPC, solid-phase preconcentration , tITP, transient isotachophoresis

An early application of off-line SPE–CE [20] dealt with the determination of methotrexate and its major metabolite in serum. By using 0.5 ml of serum and reversed-phase (RP)-SPE, followed by an evaporation/redissolution step and, finally, oxidation of both the analyte and its metabolite, LODs of 3–5 nM were obtained using laser-induced fluorescence (LIF) detection. Analyte recoveries were 80–90%. An overview of the various combinations of off-line SPE and various modes of CE is given in table 2.2. The determination of xenobiotics like drugs and their metabolites in serum, plasma and urine obviously is the main application area, and modified silicas (e.g. C18) [21–28] or ion exchangers [29, 30] are the preferred sorbents. The purpose of the off-line SPE method is to remove particulate matter, interferences, salts and proteins and, also, to enrich the analytes. The limited enrichment factor of the off-line method can be improved by evaporation/redissolution or by combining it with another additional sample preparation method (like transient ITP/in-line SPE). Because off-line SPE is rather robust if dirty samples have to be analysed, it is one of the most frequently used sample preparation techniques for CE.

At-line solid-phase extraction

To overcome the main disadvantage (laboriousness) of the off-line approach and to maintain the advantages of disposable cartridges, an SPE system can be used that can be coupled at-line to the CE device (figure 2.2) [31–33]. A robotic system is used to perform the SPE procedure. Next, a loop is filled with the effluent of the SPE cartridge

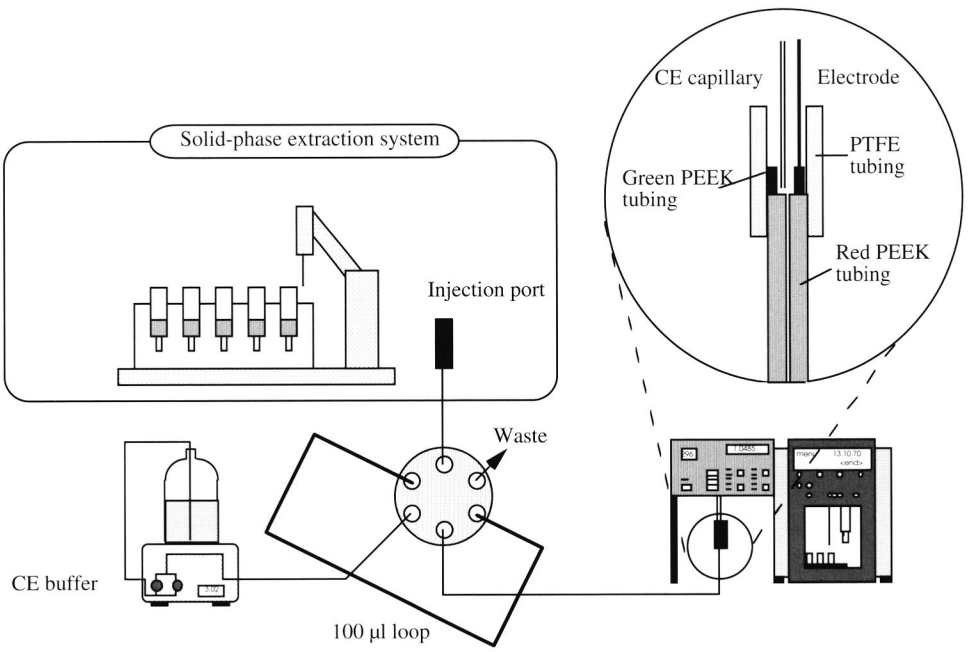


Figure 2.2 At-line SPE–CE set-up.

and by switching a valve, the contents of the loop are flushed to the interface. In the interface, the separation capillary is positioned in a piece of PEEK tubing, which serves as the injection vial. When the compounds of interest pass the tip of the separation capillary, the analytes are injected hydrodynamically by applying an underpressure at the other end of the capillary. After injection the interface is flushed with the CE buffer and the analysis is started. The determination of non-steroidal anti-inflammatory drugs [32], sulphonamides [33], the anti-coagulant phenprocoumon [34] and tricyclic antidepressants (TCADs) [163] in both serum and urine has been reported. CE of the TCADs was performed using a non-aqueous CE buffer. Most of the water had to be removed in the SPE step [163]. All methods were linear over at least two decades and the samples could be simultaneously concentrated (up to 20 times) and desalted while the drug–protein bindings were disrupted. Detection limits, when using UV detection, were ca. 0.1 μg/ml. Because this procedure is performed parallel to the CE analysis, the sample throughput is the same as with direct CE analysis [32].

A set-up similar to the above was presented [167] which uses a robotic arm with two needles of different lengths both connected to the SPE system. The longer one was used to fill the vial of the CE autosampler and the shorter one to drain it. While the manifold is working, the needles are positioned in the vial; when at rest the needles are up, above the vial. The desorbant containing the analytes, was flushed into the vial; next the vial was transported to the CE capillary by using the carrousel of the CE system and CE was performed. The detection limit was about 0.01 μg/ml for chlorophenols in urine, which is quite good compared with the earlier system. However, the electropherograms show the presence of many interferences, which will be problematic for samples of unknown origin. Furthermore, so far the only reported application was for urine.

The most recent approach is based on a small vial as interface [168]. The desorption solvent from an SPE cartridge is flushed to this vial. Next, the sample is injected, the vial is filled with CE buffer and the analysis is performed. Serum samples must be deproteinized and their pH adjusted off-line, which makes the system less than fully automated. By combining the SPE step with field-amplified electrostacking, a detection limit of about 0.01 μg/ml was found for pseudoephedrine in human plasma.

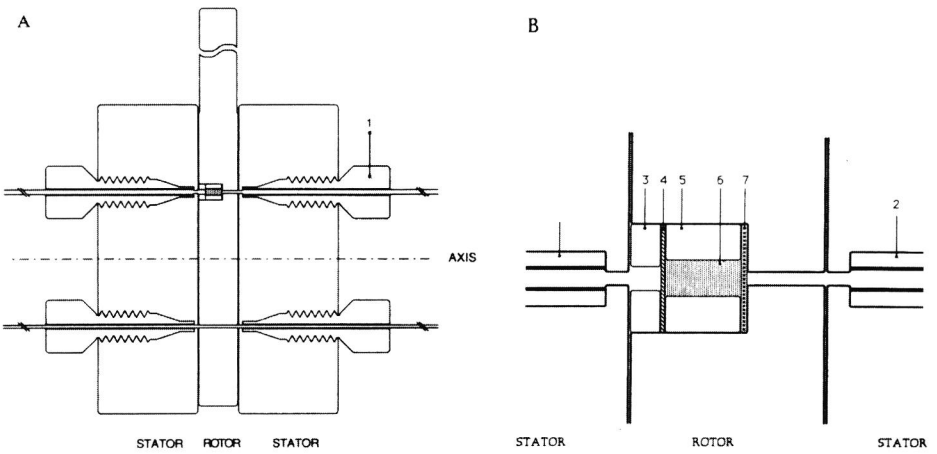


Figure 2.3 Rotary valve injector with A) cross-sectional view of the switching valve with B) the precolumn. 1 = finger-tight connections; 2 = capillary inlet/outlet; 3 = 1.5 x 0.3 mm I.D. PTFE tubing; 4 metal screen; 5 = 1.5 mm x 0.5 mm I.D. PEEK tubing; 6 = PLRP–S packing material; 7 PTFE screen [36].

On-line solid-phase extraction

Two on-line SPE–CE systems have been described in literature. One approach uses an SPE column built into the rotor of an injection valve. In this case the SPE column is loaded in the parallel mode. Another approach is to use a SPE–CE system in the serial mode.

The rotary SPE injector, developed in the early nineties (figure 2.3) [35–38], consists of an injection valve in which a small column containing the sorbent, is positioned. The column can be conditioned and loaded in the loading position. Up to 100 ml can be loaded onto this column at a flow of up to 5 ml/min [36]. Higher flows will result in poorer precision [36]. Next, the column is positioned between two CE capillaries by switching a valve. The analytes are eluted during the first seconds after applying the voltage and, next, the CE analysis is started. The loadabilty of the capillary is increased by 2–3 orders of magnitude compared with direct injections [36]. The analysis of aqueous solutions [36–38], and the direct injection of spiked biological matrices such as serum [35] and urine [35] have been reported. LODs were 50 nM for aqueous solutions of papaverine [36] and 50 ng/ml for quinidine and hydroquinidine in urine and serum [35] using UV absorbance detection in all cases. The determination of desipramine in serum was also reported, but here the system was mainly used to remove the proteins [35]. A main drawback of the system is the excessive band broadening, caused by the large amounts of desorption solvents needed. This probably explains why this approach is not used anymore.

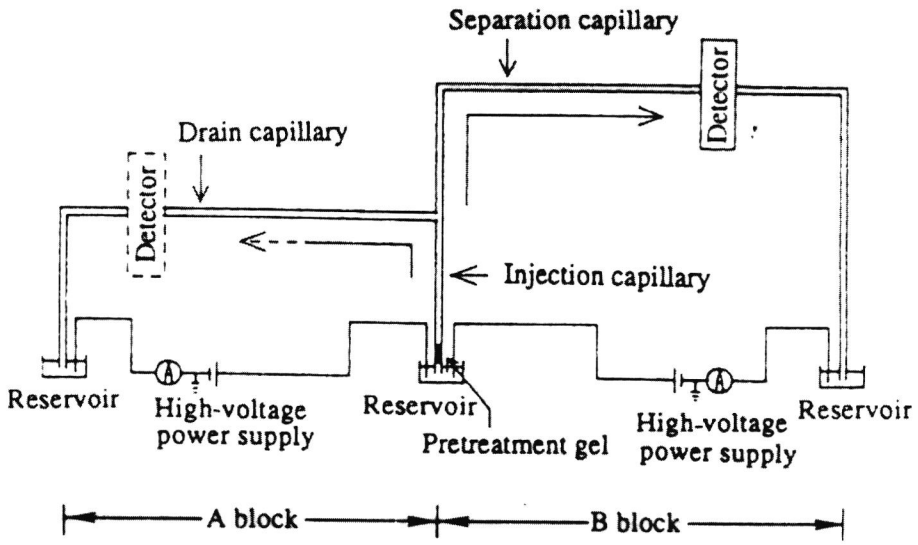


Figure 2.4 On-line SPE–CE set-up using connected capillaries [39].

In the second approach, three capillaries are connected via a T-piece: the injection capillary, in which a sorbent bed is positioned, the drain capillary and the separation capillary (figure 2.4). After conditioning of the capillaries, a voltage is applied over the injection and drain capillaries and the analytes are enriched. Next, the injection capillary is placed in a desorption solvent and for a short period of time a voltage is applied between the injection and the separation capillary. After exchanging the elution solvent with CE buffer, the CE analysis is started [39]. The system was tested by determining propranolol in serum. Up to 1 ml of sample could be loaded and a LOD of 0.15 mg/ml (UV absorbance detection), was obtained. This was a 100-fold improvement compared with direct injection [39]. The response was linear between 100 fg and 2 ng injected. The analyte recovery in serum was about 95% and the clean-up and separation each took about 11 min. However, the technique is not in use anymore, probably because of the complexity/fragility of the set-up.

In-line solid-phase extraction

A wide variety of SPE methods has been reported which use the in-line approach (table 2.3). Two set-ups can be distinguished: (i) an open-tubular capillary coated with a sorbent and coupled with a CE capillary and (ii) a small SPE unit positioned between two CE capillaries.

With the former SPE technique, capillaries coated with C18-bonded silica [40] or a metal chelate [41] are used. Between two analyses, stabilization of the open-tubular capillary column and a time-consuming flushing procedure, which can take up to 10–20 min, are necessary [41]. Even so, the open-tubular capillary is easily contaminated by (non-soluble) sample components. Another disadvantage is that the capacity of the

open-tubular cartridge is limited; the gain in sensitivity is therefore less than in off-line SPE.

The alternative approach, which uses SPE devices, can be (iia) a solid-phase preconcentration CE (spPC–CE) system using a short bed (length, 1–2 mm) of sorbent or (iib) a membrane preconcentration CE (mPC–CE) system using a particle-loaded membrane positioned between two CE capillaries, as is shown in figure 2.5 [42].

For spPC–CE, C18-bonded silica, SDB (styrene–divinylbenzene copolymer) and IgE-coated packing materials have been reported as suitable phases. However, only SDB can be used for mPC–CE because of its higher capacity, which is necessary because of the small dimensions of the membrane. After washing of both the capillary and the SPE module, the SPE cartridge is loaded by flushing the sample through the capillary using the pressure device of the CE system. Next, the capillary and the SPE module are flushed with the CE buffer. Finally, a small plug of the desorption buffer is introduced into the capillary and the CE analysis is started [10, 43, 44].

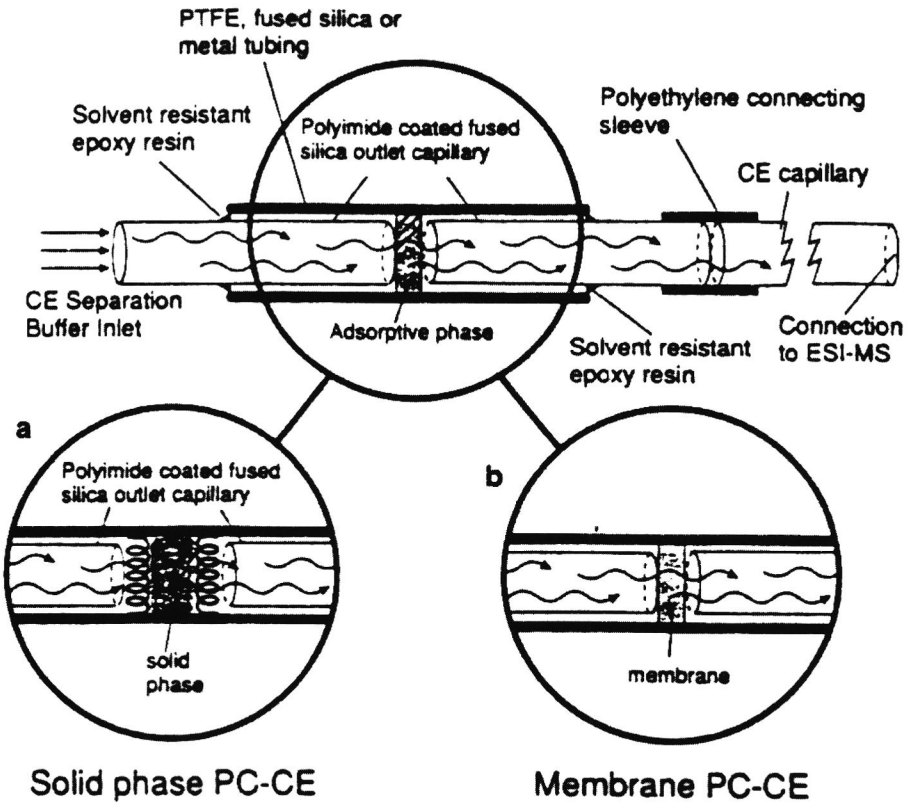


Figure 2.5 Different types of in-line SPE–CE set-up: (a) spPC–CE and (b) mPC–CE [43].

Table 2.3 In-line SPE–CE

Sample	Matrix	Pretreatment before injection	Sorbent	Detection	LOD	Volume loaded/eluted	Reference
spPC							
Doxepin/ propanolol	urine	off-line SPE + reconstitution	C18	UV	0.5 µg/ml -	n.g./n.g. 200 nl/n.g.	[19] [43]
Haloperidol	liver	$ZnSO_4$ precip.	C18	UV	-	80 nl/300 nl	[45]
	water	none	C18	MS	0.1–1 µM 0.1–1 µM	100 nl/100 nl 350 nl/200 nl	[128] [127]
Hypoglycemic drugs	water	none	C18	UV	5 ng/ml	200 µl/71 nl	[129]
Metallothionein	liver	LLE	C18	UV	0.3 µg/ml	27 µl/23 nl	[130]
Peptide mixture	water	NDA deriv.	C18	UV/LIF	<1 nM	100:1	[131]
	water	none	C18	UV	- - 5 ng/ml	9 µl/67 nl n.g./n.g. 20 µl/ 75 nl	[47] [28] [46]
Tryptic digest	water	none	C18	MS	0.1 nM	5 µl/ 50 nl	[132]
	water	HPLC fract.	C18	UV	1–10 ng/ml	50 µl/75 nl	[46, 43]
	water	NDA deriv.	C18	UV/LIF	-	n.g./n.g.	[131]
	water	none	C18	UV	-	40 µl or 100 µl / 65 – 170 nl	[170]
mPC							
Bench Jones proteins	urine	none	SDB	MS	-	1 µl/80 nl	[10, 42, 43]
Haloperidol	urine	$ZnSO_4$ precip.	C18	MS	-	10 µl/250 nl	[10, 42, 43]
Haloperidol	water	none	SDB	UV	-	n.g./50 nl	[10, 133]
LEP digest of apomyoglobin	water	none	SDB	MS	0.5 µM	1 µl/200 nl	[127]
Peptide mixture	cell surfaces	IA + HPLC fract. + dilution	SDB	MS	-	50 µl/ 80 nl	[134]
	water	none	C18	MS	20 nM	5 µl /100 nl	[128]
	water	none	SDB	MS	-	300 nl/70 nl	[10, 135]
	water	none	SDB	UV	-	n.g./55 nl	[10, 133]
3-Phenylamino-1,2-propanediol	liver cells	centrif. + filtr.	SDB	MS	2 µg/ml	200 nl/100 nl	[137]
Proteins	humor	dilution	C8	MS	-	7 µl/80 nl	[43, 150]
	humor	dilution	C2	MS	-	1 µl/ 60 nl	[151]
	water	none	C2, C8, C18, SDB	MS	-	1 µl/ 60 nl	[151]
Terbutaline	water	none	C18	UV	0.6 nM	n.g./n.g.	[179]
Coated/ connected capillary							
Triazine	water	none	C18	UV	0.1 µg/ml	10 nl/120 nl	[40]
Proteins	water	none	metal chelate	UV	1 µg/ml	390 nl/16 nl	[41]

IA = immuno affinity, mPC = membrane preconcentration, n.g. = not given, spPC = solid-phase preconcentration

The main advantage of using this system is that the SPE step provides up to 1,000-fold enrichment of the analytes. Nevertheless, there also are some drawbacks:

- Analysis of biological samples can result in contamination of the CE capillary or the SPE module, due to irreversible binding of the proteins onto the sorbent or to clogging of the capillary due to particulate matter. Flushing with sodium hydroxide, as is frequently used in CE, to remove bound material from the capillary wall is difficult or impossible because of dissolution of the sorbent or destruction of the capillary coating.
- The choice of the CE buffer is limited because it should be compatible with the packing material (cf. above). Furthermore, the analytes should not desorb from the cartridge during the washing procedure. Therefore, high concentrations of organic solvents or micelles cannot be used in the CE buffer.
- Desorption should take place in a volume that is as small as possible. Typical volumes are 100–300 nl for cartridge-based and 50–100 nl for membrane-based set-ups [45]. Although the use of a large desorption volume can be partly compensated by using an additional in-line ITP step [45] to concentrate the analytes after desorption, in most cases a decreased resolution is found when the sample volume is increased [46].
- Analyte breakthrough can occur if sample volumes are loaded that are too large. This effect is shown in figure 2.6 for the separation of nine peptides using an mPC–CE system. At a concentration of 1 μg/ml, all peptides were detected, while with the same amount of analyte, but at a lower concentration, a partial loss of two and a complete loss of five further compounds was observed [47].

The majority of the applications described deals with test compounds dissolved in water or buffer and only a few real-life applications have been reported. Usually, the real-life samples are treated by means of an off-line sample preparation step (e.g. off-line SPE or LLE or protein precipitation) prior to injection into the in-line SPE–CE system. Or, in other words, the method is not sufficiently powerful to prevent clogging and other interferences, but is purely a concentration method. The applicability is often somewhat questionable because of a lack of validation data (e.g., linearity, accuracy, precision). The same conclusion was drawn by Hempel [175]. Furthermore, generally no data are provided about the number of samples that can be analysed with a single cartridge. LOD values vary between 1–2,000 ng/ml or 0.1–1000 nM depending on the compound, detection method and concentration factor (table 2.3). Because of the in-line character of the sample pretreatment, both the through-capillary (UV and LIF) and at-the-end-of-the-capillary (MS) detection systems can be used. MS is primarily used here to identify peptides and proteins. Because of the analyte enrichment due to the in-line SPE–CE approach, full-scan mode MS could be used to reconstruct unresolved CE peaks.

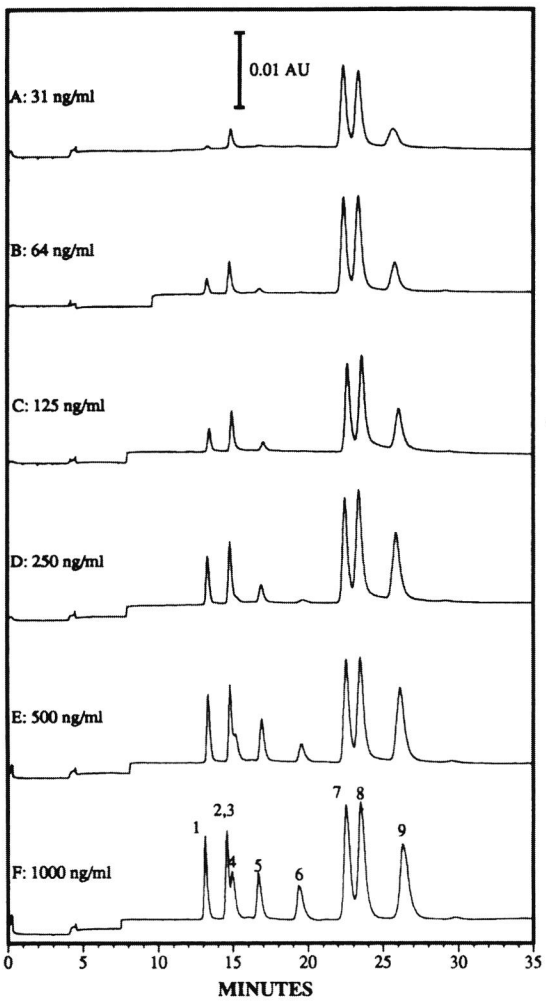


Figure 2.6 Effect of volume of sample injected onto a mPC–CE system. The absolute amount of each compound loaded onto the membrane is the same for A–F (injected amount 228 ml for A; 9 ml for F). (1) Bradykini, (2) angiotensin, (3) a-melanin-stimulating hormone, (4) thryptopin, (5) luteinizing-hormone-releasing hormone (6) leucine enkephalin, (7) bombesin, (8) methionine enkaphalin, and (9) oxytocin [47].

An interesting effect of in-line SPE–CE is that the decrease of the EOF is proportional with the length of the SPE cartridge in the CE capillary, and can cause an increased [28] or a decreased [45] resolution. The explanation of this effect which is only observed when the cartridge is washed with an ammonium-containing buffer or when the cartridge is loaded with a peptide mixture, is rather difficult. This suggests that the surface of the packing material is changed by amine-containing compounds in such a way that the EOF is changed [28]. The SPE cartridge can also improve separation: glipizide and glyburide, for example, are unresolved when using CE

without an in-line SPE pretreatment, but they are baseline separated when using spPC–CE [48].

The overall conclusion is that mPC–CE and spPC–CE can be used to concentrate analytes from aqueous or pretreated samples. Robustness still has to be improved to enable direct injections of real-life samples. Furthermore, more attention will have to be devoted to quantitative analysis.

Solid-phase micro-extraction

Only a few papers discuss the use of solid-phase micro-extraction (SPME) for CE. Because of the small dimensions typical of CE, a properly automated interfacing which does not cause a loss of sensitivity, is rather difficult to design. Because of this, until now only an off-line set-up has been used [49, 149].

Ten barbiturates were enriched ca. 10-fold after 5 min extraction and ca. 50-fold after 30 min extraction. The recoveries from serum were 60–90% compared with those from standard solutions, the differences being due to protein interaction. With urine samples the use of SPME resulted in a better separation than with direct analysis, because salts and many other interferences are not extracted. The LOD for UV detection were about 1 μg/ml for serum samples and ca. 0.1 μg/ml for urine samples [49]. The use of different types of SPME devices was also discussed [149, 161, 162]. This system was able to concentrate tryptic digest of bovine serum albumine. Further research should be devoted to the analysis of larger sample volumes of biological origin and the use of an automated procedure.

2.3.2 Liquid chromatography

LC and CE can be coupled in two different ways. One approach is to use the LC system to create a preseparation. One or a few heart-cuts which contain the compounds of interest are then subjected to CE analysis. Alternatively, the LC effluent is divided into series of small fractions which are all analysed by CE, the result being a comprehensive two-dimensional separation.

Heart-cut LC–CE

A heart-cut LC–CE system contains three valves, an LC column and an interface (figure 2.7) [1]. The interface is the same as was used for at-line SPE–CE (cf. figure 2.2) [32, 34]. First, the sample is loaded onto the column and salts are flushed to waste. Next, valve V2 is switched, the compounds are eluted to the interface and the analytes are injected into the CE capillary. Finally, the interface is flushed with CE buffer and the CE analysis is started. By selecting the proper injection time, after the switching of valve V2, the compounds of interest are injected selectively into the CE capillary by applying an underpressure at the other end of the CE capillary. The

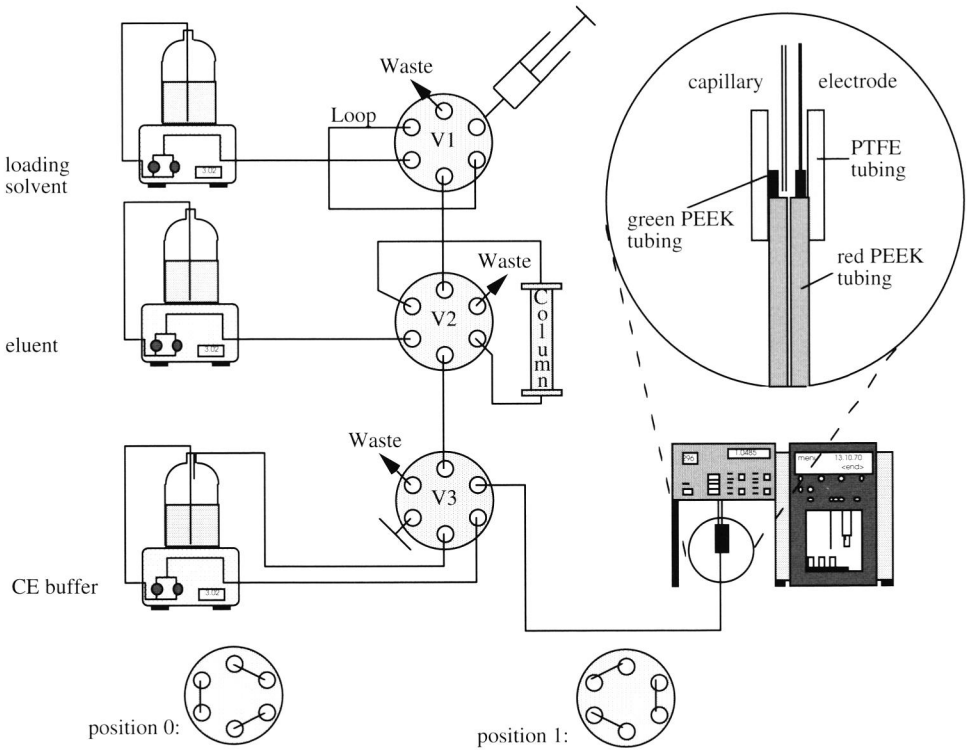


Figure 2.7 On-line LC–CE set-up [32– 34].

approach was successfully used for benzoic acids in samples containing up to 400 mM of sodium chloride. Removal of the inorganic salts was found to be nearly quantitative. The LODs were about 50 ng/ml using UV detection, and linearity was satisfactory over about two orders of magnitude. Unfortunately, the system was not really robust when particulate matter and/or proteins were present; and therefore, an additional dialysis step had to be introduced (cf. section 2.5.1).

In another study, a protein G immunoaffinity (IA) column was used to determine insulin in serum [50]. The LC system was used both to preconcentrate (up to 1,000-fold) and to clean the sample. The eluted plug containing the insulin (1 µl) was flushed to the CE column, and a part was injected into the CE capillary. The insulin concentration was much lower than expected which probably indicates that other proteins from the serum sample competed for binding to the antibody under the conditions applied. The LOD using UV absorbance detection, was about 25 nM of insulin. In each electropherogram a peak of the antibody was found indicating a poor binding constant for the carrier sorbent–antibody complex.

Comprehensive LC–CE

When fractions of the LC effluent are continuously injected into a CE system and analysed, a two-dimensional separation is obtained which can be presented as a 2D chromatoelectropherogram.

Various interfaces can be used to transfer liquid from the LC to the CE part of the system. Two main problems arise when coupling these parts. One is that the volume of liquid eluting from the LC system is larger than the volume that can be injected into the CE capillary. The second problem is that in order to obtain a good separation, the LC effluent has to be divided into very small fractions. While one fraction is being analysed by the CE system, the next fraction should not enter the CE capillary, otherwise the CE analysis will be destroyed.

An early LC–CE interface was based on an electrically actuated six-port valve (figure 2.8A) [51–53]. The LC effluent is trapped in a loop during the CE analysis of the previous fraction. When the valve is switched, the contents of the loop are flushed to the inlet of the CE capillary and part of the liquid is injected into the CE capillary. Next, the valve is switched again and the CE analysis is started. The remaining effluent is flushed to waste via a T-piece positioned between the valve and the CE capillary. This set-up did not result in a really sophisticated 2D analysis because the effluent that is trapped during the CE run (which typically takes a few minutes) is injected as a single fraction in the CE capillary. The total analysis time was on the order of 2–4 h.

Another approach is to use a microtiter plate to collect the LC fractions which can then afterwards be analysed at-line or off-line [171]. The simplicity of the set-up is a distinct advantage and the system can be implemented rather quickly in a laboratory. Up to 96 fractions can be collected on one well plate. Unfortunuately, because the CE analysis time is some 15 min for a single fraction, the total analysis time can be up to 24 h, which is unduly long.

A faster analysis can be obtained by using an optical-gated CE system (figure 2.8B). The LC effluent is flushed continuously to the CE capillary. Most of it is flushed to waste via a splitter, but a small part is always injected into the capillary. Just after the inlet of the CE capillary, a powerful laser gating beam photolyses the analytes [54, 55]. When a CE injection is performed, the gating beam is switched off for a very short time and a small plug is injected. Because of these very narrow injection plugs, a short capillary can be used, which results in short CE analysis times (ca. 10 s). As a result of the faster CE analysis, the LC effluent can be divided into more fractions. This results in either a 2D analysis featuring better resolution, or in a faster 2D analysis, i.e. when the LC flushing is accelerated [56, 57].

Another interface is the flow-gating interface shown in figure 2.8C [50, 58]. It is constructed from two stainless-steel plates, held at a distance of 127 μm. The inlet of the CE capillary is just opposite to the exit of the LC column, both of which are

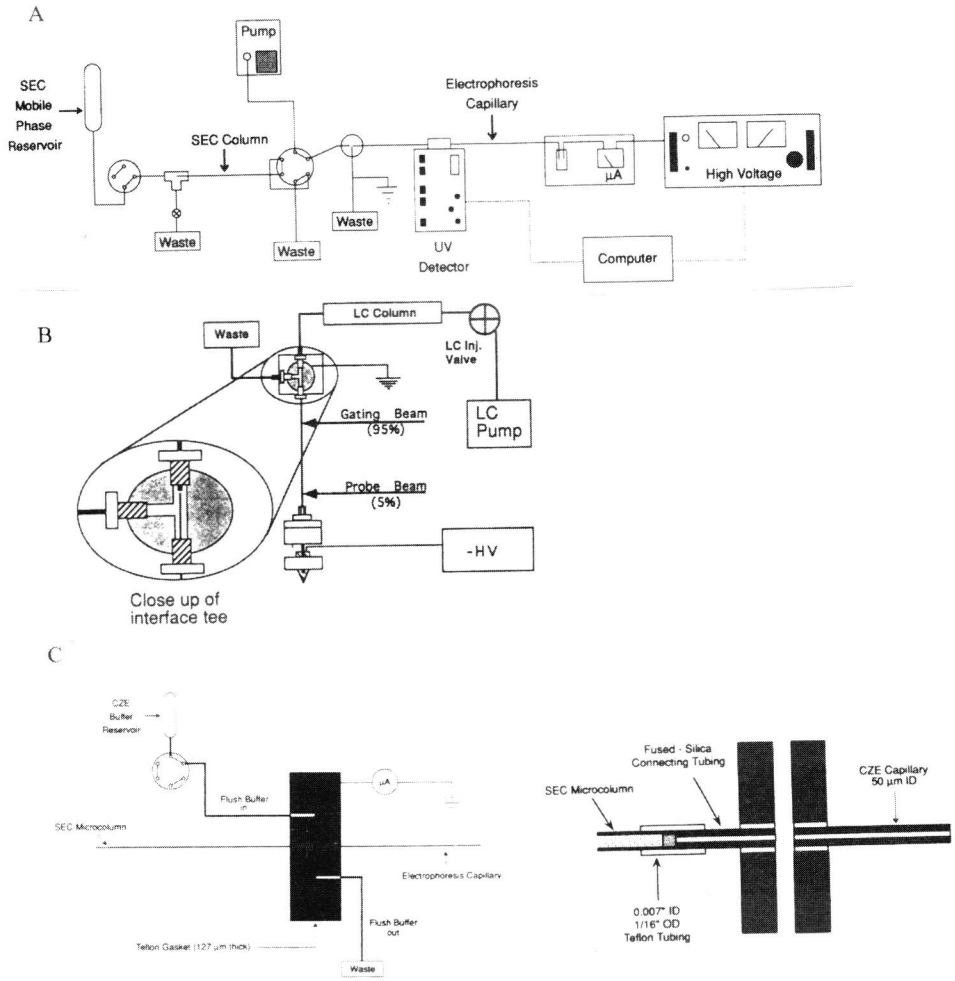


Figure 2.8 Different interfaces used for the on-line LC–CE set-up (A) loop/valve interface [52] (B) optical floating interface [56] and (C) flow gating interface [58].

mounted on one of the two plates. When there is a flow of CE buffer between the plates, no LC effluent will enter the CE capillary. Only when this flow is stopped, can LC effluent be injected into the CE capillary. The main advantage of this interface is that it reduces extra-column dilution. The more efficient transfer from the first to the second separation step results in an analyte detectability which is about one order of magnitude better than with the other two interfaces [50, 58]. Because the interfacing has to be performed at the grounded side of the capillary, only through-capillary detection methods can be used.

The complementarity of the LC and CE separations was demonstrated by comparing the determination of proteins with ITP and LC [59]. In the multidimensional LC–CE systems, various sorbents were used (see table 2.4) such as C8- or C18-bonded silica and size-exclusion (SEC) materials. The advantages of

these systems are the dramatic increase of peak capacity and separation power. Even a 3D system has been designed, viz. by combining SEC, RPLC and CE [60]. The 3D analysis was performed using both types of interface: an injection-port-based interface between the SEC and RPLC column and an optical-gated interface between the RPLC column and the CE capillary. Because of the fractionation, plus interfacing problems of the 2D set-ups, only concentrated samples (10 g/l) can be analysed. Therefore, the approach is used only for the analysis of proteins, tryptic digests and the profiling of human, horse and bovine serum. As an example, figure 2.9 shows that whereas the analysis of a peptide sample by either RPLC or CE did not result in a complete separation, the combination of both techniques provides a characteristic 2D fingerprint.

Complex mixtures like tryptic digests, which cannot be separated with a single technique, can be successfully characterized by combining LC and CE. In the near future, more attention should be devoted to the interpretation of the complex 2D data sets and the repeatability and linearity of the procedures should be demonstrated. If satisfactory analytical data can be generated, LC–CE will have a bright future.

Table 2.4 On-line comprehensive LC–CE.

Sample	Technique	Interface	Label	Detection technique	Analysis time (h)	Reference
Peptides	RPLC–CE (C18)	Loop type	fluorescamine	FLU	4	[51]
Protein mixture	RPLC–CE (C8)	Loop type	TRITC	FLU	1	[76]
	RPLC–CE (C18)	Gating beam	FITC	FLU	0.25–0.5	[76]
	SEC–CE	Loop type	-	UV	16	[136]
	SEC–CE	Loop type	-	UV	2–4	[76]
	SEC–CE	Gating flow	-	UV	2-6	[58]
Serum proteins	SEC–CE	Loop type	-	UV	2	[52]
Tryptic digest of cytochrome C	RPLC–CE (C8)	Loop type	TRITC	FLU	1	[76]
	RPLC–CE (C18)	Loop type	fluorescamine	FLU	4	[53]
	RPLC–CE (C18)	Gating beam	FITC	FLU	0.14	[57]
	RPLC–CE (C18)	Gating beam	FITC	FLU	0.25–0.5	[76]
	RPLC-CE (C18)	Micro titer plate	-	UV	Up to 24	[171]
Tryptic digest of ovalbumin	RPLC–CE (C18)	Loop type	fluorescamine	FLU	4	[51]
	SEC–RPLC–CE (C8)	Gating beam	FITC	FLU	8	[137]
Trypic digest of myoglobin	RPLC–CE (C18)	Microtiter plate	-	UV	Up to 24	[171]

FITC = fluorescein isothiocyanate, TRITC = tetramethylrhodamine isothiocyanate isomer-5

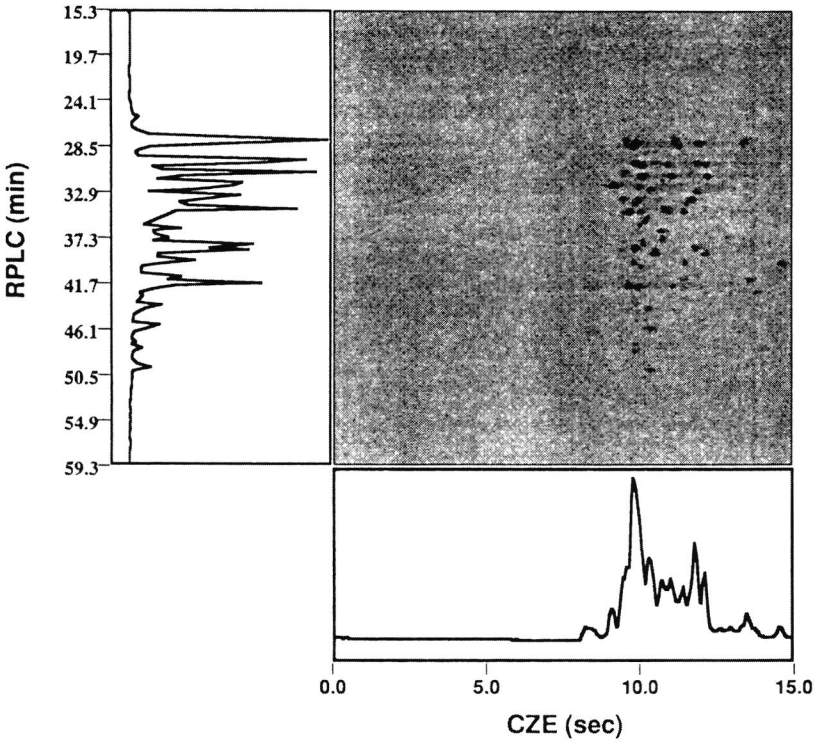


Figure 2.9 Separation of TRITC-labelled tryptic digest of horse heart cytochrome C using 2-dimensional RPLC–CE. 2 mg were loaded onto the RPLC column [56].

2.4 Electrophoresis-based sample preparation

Electrophoresis-based techniques such as ITP or sophisticated injection techniques for CE, can also be used for sample preparation prior to CE analysis.

2.4.1 Isotachophoresis

In ITP the analyte-containing plug is positioned between two different buffers, the leading buffer (with ions with a higher mobility than those of the analytes) at the detector side, and the terminating buffer (with ions with a lower mobility than the analytes) at the injection side. Two approaches of coupling ITP with CE can be distinguished, an on-line multiple capillary system called coupled-capillary transient ITP (cITP), and the in-line combination of ITP and CE in a single capillary, called transient ITP (tITP).

Multiple capillary isotachophoresis–CE

Two set-ups are used for multiple capillary cITP–CE. In one of these a T-piece based interface is used to connect the capillaries and, in the other, an injection-split based interface.

A cITP–CE system using a T-piece consists of three capillaries [60–64]. The sample is introduced between the leading and terminating buffer in a capillary with a large I.D. (800 μm) and the ITP process is started by applying a voltage over the ITP and waste capillaries (figure 2.10A). When the compounds of interest pass the T-piece (as monitored by UV detection), the ITP process is terminated. The voltage is now applied over the ITP and CE capillaries (I.D. 300 μm), which results in injection of the analytes into the CE capillary. Next the voltage is applied again over the ITP and

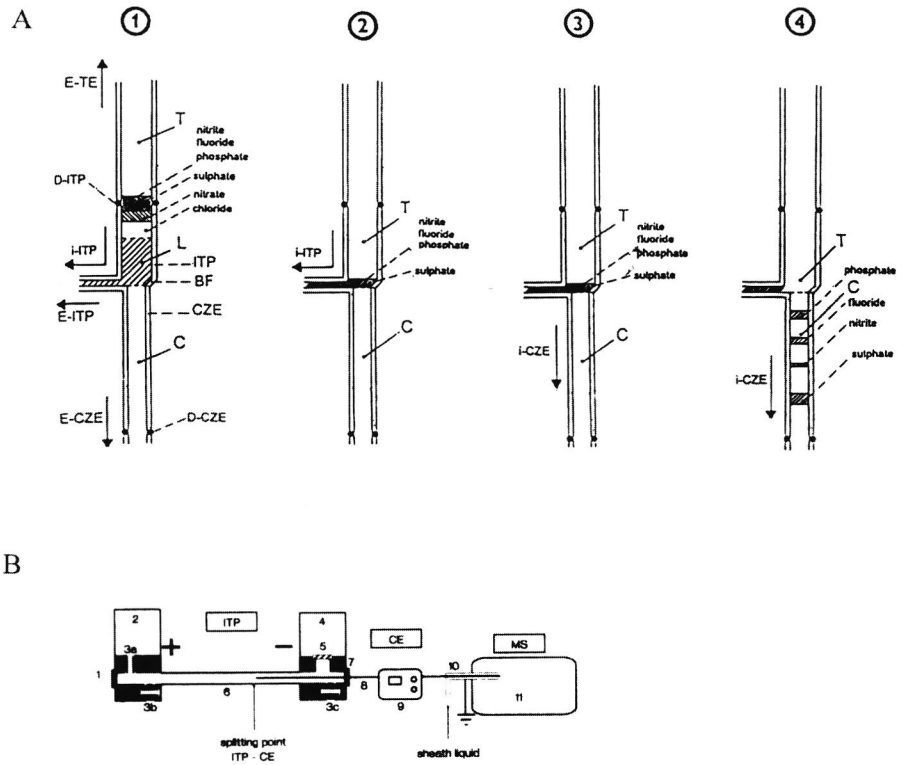


Figure 2.10 On-line ITP–CE set-up of (A) a T-piece-based interface with (1) ITP stage, (2) removal of the macroconstituents, (3) transfer of the sample microconstituents into the CE capillary and (4) CE separation of the microconstituents [61] (B) injection split interface with 1 = injection septum; 2 = terminating buffer; 3a-c = valves; 4= leading buffer; 5 = membrane (to prevent EOF); 6 = ITP capillary; 7 = septum; 8 CE capillary; 9 = UV detector; 10 = sheath needle; 11 = electrospray MS [65].

waste capillaries to remove the remaining matrix compounds. When the terminating buffer has passed the T-piece, the CE run is started by applying the voltage over the ITP and CE capillaries and detection is performed with a second detector.

The injection-split method is based on the two-capillary system shown in figure 2.10B. The sample is injected in the ITP capillary (typically I.D. 500 μm [7]) and the ITP process is started. When the analytes have migrated to the tip of the CE capillary (typically 50 μm I.D. [7]), the ITP process is stopped and about 10 % of the analytes is injected into the CE capillary. The remaining compounds are removed from the ITP capillary and the CE run is started. The potential of this method has been shown in a few papers dealing with aqueous samples [7, 65, 66] and one paper describing the analysis of a biological sample [67]. With aqueous samples, the analytes can be concentrated 100–1,000-fold. Biological samples have to be pretreated first by off-line precipitation or dilution. In the example discussed, a derivatization step was incorporated to increase the detection selectivity and/or sensitivity (UV absorbance, fluorescence) prior to injection into the ITP–CE system [7, 67]. MS detection was also used. When using UV absorbance detection, the LODs of the derivatized amino acids were as low as 10^{-7} M even in the presence of a 105-fold excess of macroconstituents in the sample. The area RSD values were 1–5% [64].

Applications reported using both methods are shown in table 2.5. The main advantage of the T-split injection approach is that all the analytes injected into the ITP capillary, are introduced into the CE capillary and, consequently, less sample is necessary to obtain the same sensitivity. In practice, cITP–CE is not often used because it can be applied only to aqueous samples. Therefore, additional (off-line) sample pretreatment is necessary for biological fluids.

Single-capillary isotachophoresis–CE

The set-up for tITP–CE is simpler than that for cITP–CE because a single capillary is used for both the ITP sample pretreatment and CE separation. Three buffer combinations can be envisaged (figure 2.11) [66]. A buffer system as depicted in figure 2.11A can be used when the co-ion of the background electrolyte, C, has a mobility which is higher than that of the sample components, S, and can therefore serve as the leading ion. In the configuration of figure 2.11B, the mobility of C is lower than that of the sample ions and, as a result, a suitable leading ion, L, must be added to the sample. A combination of both systems is shown in figure 2.11C. The analysis can be divided in two parts: the focusing step in which the ITP effect concentrates the analytes and the CE step in which the compounds are separated. A focusing step which is too short, i.e. incomplete, will result in loss of resolution [68].

Published applications of tITP–CE are summarized in table 2.6. Sugars, peptides, proteins and tryptic digests were analysed in standard solutions. Up to 1.4 ml can be injected in the CE capillary. Injecting plasma or urine extracts (after off-line SPE

pretreatment) resulted in an overall decrease of the efficiency [68–70]. The determination of cardiovascular drugs in serum and urine also required an additional SPE procedure and LODs were about 50 mg/l using UV detection [71]. Toxins in paralytic shellfish liver tissue homogenates were determined after off-line SPE on C18-bonded silica [72]. tITP–CE can also be used in a capillary filled with gel to analyse polymer chain reaction (PCR) products [73, 74].

Various detection systems, such as UV absorbance, LIF and MS were used. Table 2.6 shows that, with UV detection, analytes in water can be determined down to about 1 nM, while analysis of biological samples results in 100-fold higher LOD values. In the latter instance, the ITP step is mainly used to reconcentrate the sample after dilu-

Table 2.5 Applications of cITP–CE.

Analyte	Matrix (Pretreatment)	Concentration factor	Buffer system	Detector	LOD ng/ml	Ref.
-split interface						
Organic acids	water	not mentioned	LE: 10 mM alanine (pH 3.9), TE: HAc BGE: 10 mM alanine (pH 3.9)	UV	-	[62]
Small anions	water	10^5–10^6	LE: 8 mM alanine-Cl (pH 3.4), TE: aspartic acid (pH 4), BGE: aspartic acid (pH 3.4)	Conductivity	0.6	[61]
Sulphanilate	urine (diluted 1:25-250)	not mentioned	various	UV	150	[63]
Amino acids	water (derivatization with DNP)	100-1000	LE: 20 mM CL-HIS (pH 5.5), TE: 10 mM MES-HIS (pH 5.5), BGE 50 mM MES-HIS (pH 5.5)	UV	70	[64]
njection split interfase						
Amino acids	water (derivatization with OPA/FITC)	100	LE: 5 mM borate (pH 9.5), TE: 5 mM ACES (pH 10), BGE: 5 mM borate (pH 9.5)	UV/ LIF	7	[7]
Angiotensin	serum (precipitation + derivatization with FITC)	1000	LE: 10 mM TRIS (pH 9.2), TE: 10 mM alanine (pH 10.4), BGE: LE 10 mM TRIS (pH 9.2)	LIF	0.1 nM	[67]
Proteins	water	1000	LE: 10 mM NH_4AC (pH 4.8), TE: 20 mM EACA (pH 4.4) BGE: 20 6-aminocaproic acid (pH 4.4)	UV	100	[66]
Anthracyclines	water	200	LE: : 60% MeOH + 10 mM phosphate (pH 7.5),TE: 60% MeOH + histidine (pH 7.2), BGE: 60% MeOH + 10 mM phosphate (pH 7.5)	MS	300	[65]

DNP = 2,4-dinitrophenyl.

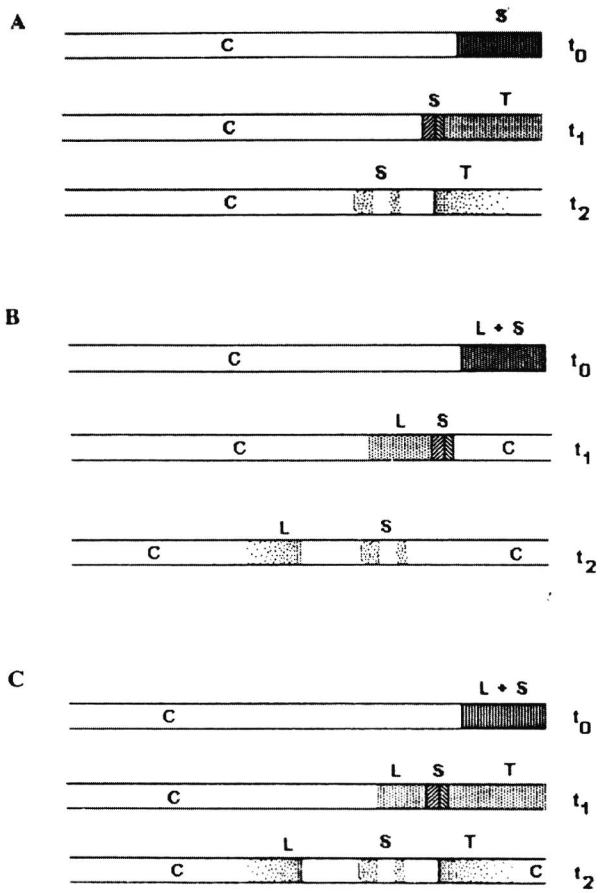


Figure 2.11 Three different buffer systems for in-line ITP–CE, where C = background electrolyte, L = leading buffer, S = sample and T = terminating buffer. t_0, t_1 and t_2 represent the situation before analysis, during the ITP step and during the CE analysis, respectively[99]. For further details, see text.

tion and an off-line sample preparation (SPE or LLE) procedure. When using LIF, analytes, admittedly, frequently have to be derivatized, but the LODs typically are 100–1,000-fold lower than with UV detection. In addition, ribonucleosides could be detected much more selectively in the presence of adenosines [75]. With MS detection, LOD values of 10–30 nM in the SIM mode [72] and 100 nM in the full-scan mode [76, 77] can be obtained for peptide-type compounds. With all detectors, quantitative analyses showed satisfactory precision (RSDs, 1–6%) [63, 68, 72] and linearity over some three orders of magnitude [63, 68, 70, 72, 75, 78–80].

Table 2.6 Applications of tITP-CE.

Analyte	Matrix pretreatment	Concentration factor	Buffer system	Detector	LOD (nM)	Ref.
β-Agonists	water	35	LE: 80% MeOH + 50mM NH_4Ac (pH 4.8), TE: 80% MeOH + 50mM alanine (pH 4.8)	MS	60	[77]
Adenosine	urine (off-line SPE + CCA deriv)	70	LE: TRIS (pH 7.0), TE: 80mM borate (pH 10)	UV	4	[68]
				LIF	60	[68]
				UV	28	[172]
Amino acids	water (OPA deriv)	100	various	LIF	5	[75, 78]
Antimuscarinic drugs	water	100–500	LE: 10mM TEA (pH 5) in 50% MeOH, TE:10mM alanine (pH 5) in 50% MeOH, BGE: not given	UV	1	[138]
	plasma/urine (LLE)	50–100	LE: 50% MeOH + 10mM TEA (pH 5), TE: 50% MeOH +10 mM alanine (pH 5)	UV	100	[71]
Catechols	water	70	LE: TRIS (pH 7.0), TE: 80mM borate (pH 10)	LIF	-	[68]
DNA fragments	water	20	LE: 80mM TRIS (pH 8.3), TE: 80mM TRIS/butyrate (pH 8.3)	UV	-	[73]
Enzyme inhibitor	plasma (off-line SPE)	100	LE: 20 mM EACA (pH 3.4),TE: 10mM HAc, BGE: 20mM EACA (pH 3.4)	UV	50	[70]
Oligonucleotides	water	20	LE: 12% PEG + 100mM ammonium formate (pH 4.5), TE: 100mM MES (pH 4.5)	UV	1000	[74]
Peptides and proteins	water	up to1000	various	UV	-	[79]
Peptides	water	30	various	UV	-	[139]
	serum (off-line SPE)	100–1000	LE:10 mM NH_4Ac (pH 3.6), TE: 50 mM HAc (pH 3.1), BGE: 20 mM EACA (pH 3.6)	UV	30	[69]
Proteins	water	100	LE: 0.01% Brij35 + 10mM 2-10mM TEA (pH 5), TE: 0.01% Brij35 + 10mM alanine (pH 5), BGE: not given	UV	10	[80]
	water	50	LE: 20mM TEA (pH 4.4) , TE: 10mM HAc, BGE: 20mM TEA (pH 4.4)	UV	30	[66]
	water	100-1000	various	UV	1	[140]
	water	100	LE: 10mM NH_4Ac (pH 5), TE: 1M HAc, BGE: 20mM EACA-HAc (pH 4.4)	MS	100	[76]
Toxins	liver (off-line SPE)	100	LE: 35 mM morpholine (pH 5), TE: 10 mM formic acid	UV/MS	20	[72]
Sugars	water (aminopyridine deriv)	70	LE: TRIS (pH 7.0), TE: 80mM borate (pH 10)	LIF	-	[68]

BGE = background electrolyte, CCA = chloroacetaldehyde, EACA = ε-aminocaproic acid, LE = leading electrolyte, OPA = *o*-phthalaldehyde, TE = terminating electrolyte, TEA = triethylamine.

2.4.2 Special injection techniques

In CE, special injection techniques are frequently used to increase the sensitivity. Strictly speaking, these techniques are no real sample preparation procedures, but they are often used, and combined, with other techniques with the same 'enrichment purpose' in mind. Therefore, they are included in this review. Enrichment is based on the difference in analyte velocity in the sample and the buffer when a voltage is applied during or after injection, which results in concentration of the compounds of interest. Several methods can be distinguished: sample stacking, field-amplified injection and electro-extraction.

Sample stacking

One of the most frequently used concentration techniques in CE is sample stacking. It is based on the principle that the velocity of an ion is the product of its mobility and the electric field strength. If the conductivity of the sample plug is lower than that of the CE buffer, the electric field strength and, consequently, the velocity of the ions in the sample plug are higher than in the CE buffer, and the ions are focused at the sample/buffer boundary [81, 82]. The main advantage of stacking is that it can be applied both in free-solution and micellar CE systems [83] and that it is a rather simple technique. Positive and negative compounds can be stacked within one run. However, to avoid adverse effects on the resolution and linearity, optimization is important when sample stacking is used [84–86]. The main problem of sample stacking is that the sample/CE buffer conductivity ratio is a rather critical parameter which has to be as low as possible, because ratios higher than 0.3 cause peak deterioration [83, 86] and large fluctuations of the sample conductivity will cause a matrix-dependent efficiency [84, 85]. As for biological samples, which often contain a high concentration of salts, dilution is frequently used to lower the conductivity. Unfortunately, this also results in a decrease of the analyte concentrations which counteracts the stacking process. Proteins can bind to CE capillary walls and disturb the analysis; they have to be removed before injection [87–89]. One way to remove the proteins is to add acetonitrile to the sample, which will cause their precipitation [176]. Acetonitrile will also reduce the conductivity of the sample which will result in an improved stacking performance of the analytes.

Sample stacking enables rather large volumes to be concentrated in the CE capillary, viz. up to 60% of the total capillary volume [90]. Improved analyte detectability has been obtained for β-blockers (120-fold [174], derivatized phenolic compounds (100-fold) [91], clenbuterol (80-fold) [92], cobalt (10-fold) [92] and small peptides (10-fold) [90]. The use of a capillary coated with an ethylene vinyl acetate (EVA) copolymer instead of a bare silica capillary resulted in an increased stacking effect for phorphyrins in water [93]. However, with urine samples stacking was found

to occur using bare silica capillaries but not when applying EVA-coated capillaries [93]. No explanation has been given for this phenomenon.

Although stacking can result in a 10–100-fold concentration, it cannot be used for the direct injection of biological samples, because of their high salt concentrations and the presence of proteins and other macromolecules. Stacking proceeded with a prior salt- and/or protein-removal procedure, such as LLE, SPE or protein precipitation, is then required to obtain a stable, low-conductive matrix.

Field-amplified injection

Field-amplified injection (FAI) is similar to stacking and is based on the same principle: the velocity difference of the analyte in the sample and the CE buffer. The main difference is that FAI is performed when the injection part of the capillary still is in the sample vial, while with stacking the sample vial is replaced by a buffer vial before the focusing process starts.

FAI procedures can be used for the determination of positively and negatively charged compounds and their mixtures [94]. The main advantage of FAI is a large preconcentration factor of ca. 100 because the whole sample is processed. The most important disadvantages are the dependence of the concentration factors on the charge of the analytes and the strong influence of the conductivity of the matrix. Because of this, FAI is not often used for the analysis of biological samples, but mainly for the efficient concentration of analytes from aqueous solutions. Published applications include the determination of derivatized amino acids [94–97], small inorganic [98–100] and organic [100] ions in water, opiates in plasma, serum and urine [180], metformin in plasma [152] (after an LLE step) and antiarrhythmic drugs [101] (after extraction) in serum. The LODs using UV detection were in the 1–40 ng/ml range. The use of a water plug can improve the detection limits and the repeatability [173]. The determination of neutral compounds by means of micellar electrokinetic chromatography was also reported [154–157].

Electro-extraction

LLE is an often used sample-handling procedure which is, however, laborious and time consuming. As an alternative, electro-extraction (EE) can be used to speed up the process. A non-miscible solvent is added to the sample and a voltage is applied over the two phases. The extractable analytes move into the added solvent and are sampled into the capillary. For EE to be efficient, the extraction solvent should have a very low conductivity. In other words, it should be relatively non-polar, like e.g. an ethyl acetate extract [94]. Because the analytes have, at the same time, to be present in their ionic form, this is a quite complicated situation. However, somewhat surprisingly the quoted paper does not discuss this aspect. The injection procedure consists of three steps (figure 2.12). In the first step (A), a capillary filled with the leading buffer and a

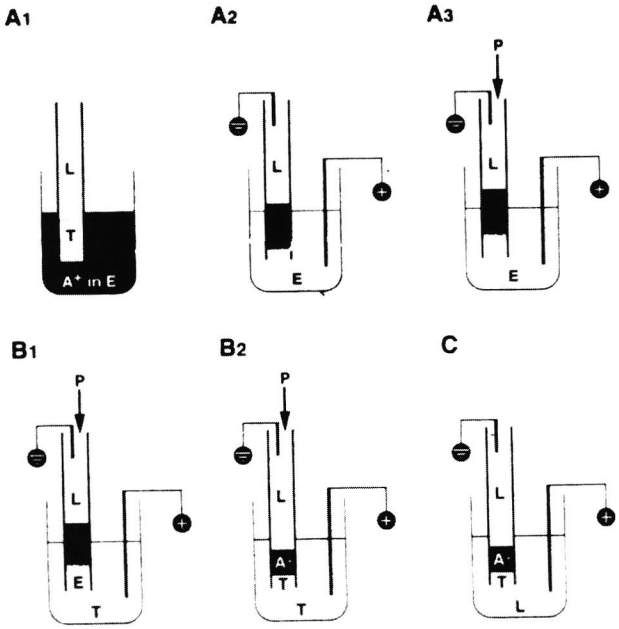


Figure 2.12 Electroextraction isotachophoresis procedure. L = leading buffer, T = terminating buffer, E = ethyl acetate, A+ = analyte and P = pressure [94]. For more details, see text.

small plug of the terminating buffer is placed in the injection vial and a voltage is applied. At the same time, pressure is applied to obtain a pressure-induced counter flow to avoid injection of a large volume of ethyl acetate in the CE capillary. Next, the capillary is placed in a vial filled with the terminating buffer and the focusing step (B) is started. During this ITP focusing step the remaining ethyl acetate plug is pushed out of the capillary. After a steady state has been reached, the CE capillary is placed in the leading buffer vial and the CE analysis is started (C). High concentration factors of over 1,000-fold can be established. The only known application [94] is the determination of β-antagonists in water which resulted in an LOD of 2–5 nM using electrospray-MS in the SIM mode for detection.

2.5 Membrane-based techniques

Dialysis is normally used to remove particulate matter or high-molecular-weight compounds and can be coupled with a wide variety of separation techniques [102, 103]. The principle is to use a semi-permeable cut-off membrane, which can be used to separate small and large molecules. Four approaches are found in the literature, dialysis, microdialysis, supported-liquid membrane systems and electrodialysis.

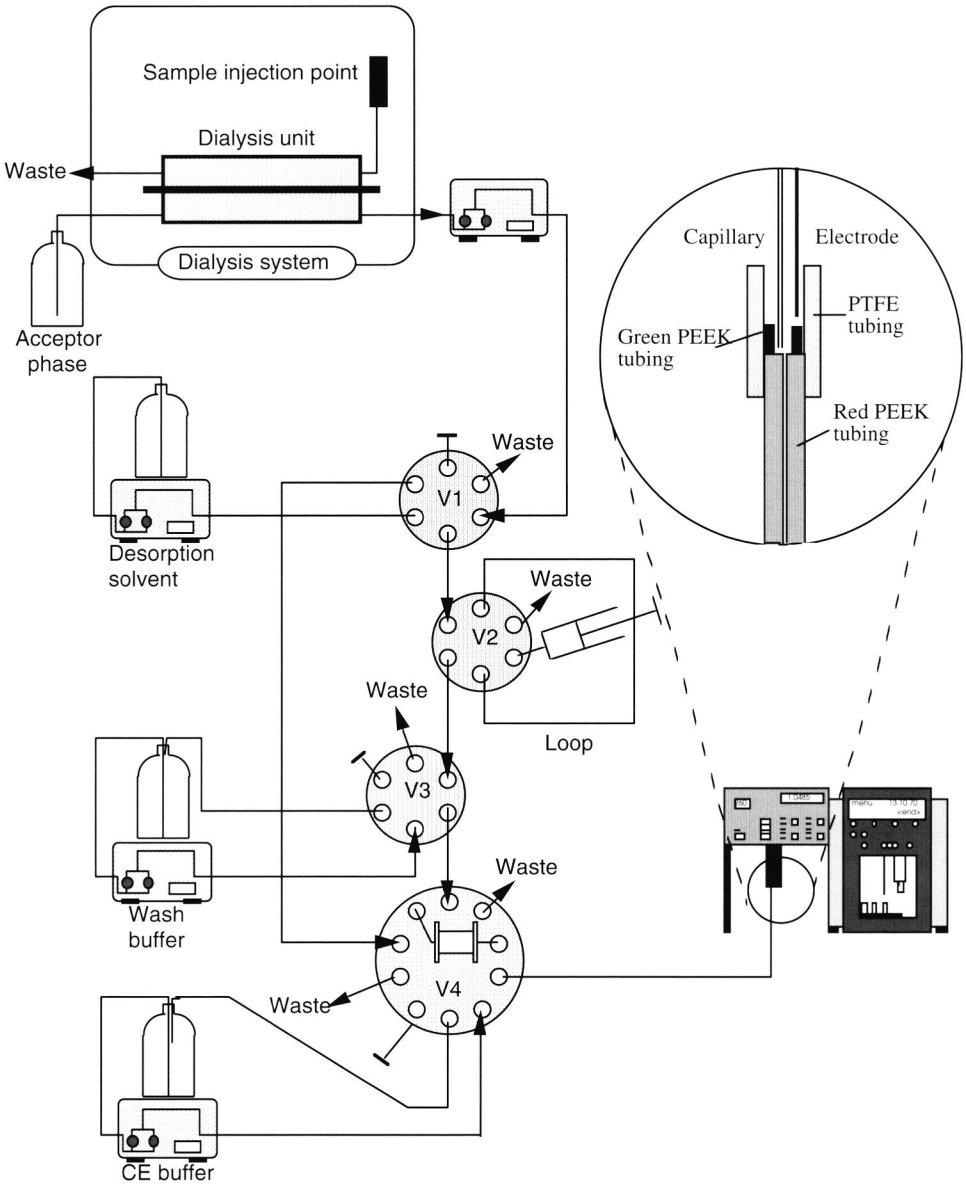


Figure 2.13 Dialysis–SPE–CE set-up [104].

2.5.1 Dialysis

A dialysis-based sample preparation procedure for CE which features an automated dialysis unit is shown in figure 2.13 [104]. The sample is injected in the donor phase of a dialysis module. A membrane with a molecular-weight cut-off

(MWCO) of 15 kDa is positioned between the donor and acceptor phase. Small molecules can pass the membrane, while larger molecules (including proteins) and particulate matter will remain in the donor phase. To speed up the dialysis process the acceptor and/or donor phase can be displaced continuously. Renewal of the acceptor phase causes the total volume in which the analytes are dissolved to increase up to 10–100-fold. Therefore, the acceptor solvent is flushed over a small (typically 50 x 2 mm I.D.) trace-enrichment column to trap the analytes of interest. Next, the analytes are eluted in a small volume (typically 50–100 µl) and injected via the interface as discussed for the at-line SPE–CE set-up (cf. section 4 [1, 32, 34]). In the automated system all pumps, switching valves and the synchronisation of the dialysis and CE units are controlled by the dialysis system. As an example, figure 2.14 shows the determination of sulphonamides in urine [104]. Within-day and day-to-day precision were about 7%. The LODs (UV detection) were 0.05–0.3 µg/ml in urine and serum. The method was linear over two decades and was used for unattended analysis. The on-line dialysis–SPE–CE method was able to separate also acidic [177] and basic drugs [178] in both serum and urine.

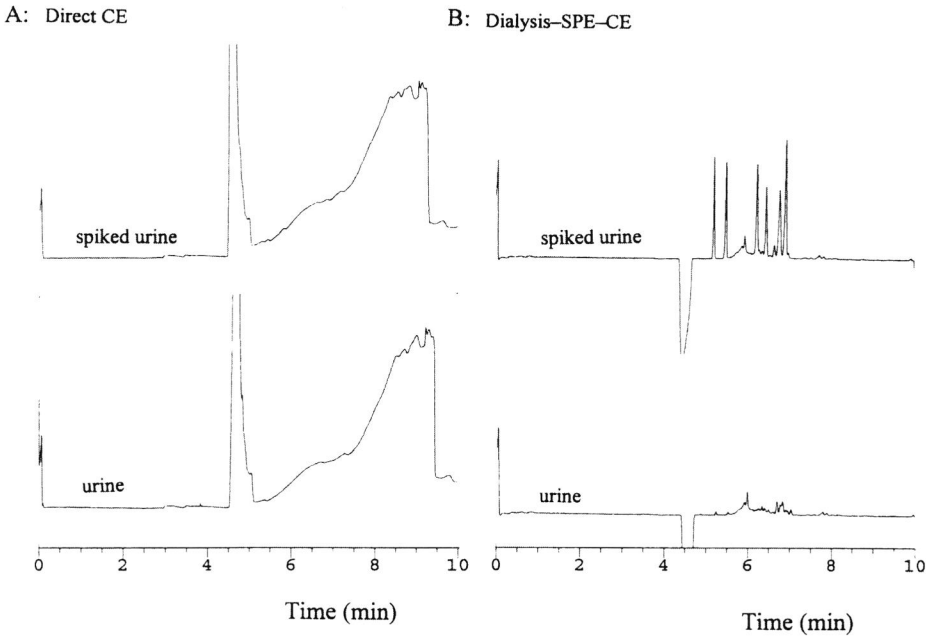


Figure 2.14 Separation of six sulphonamides in urine (blank and spiked, 10 µg/ml each) using direct injection into the CE system and using the dialysis–SPE–CE set-up [104].

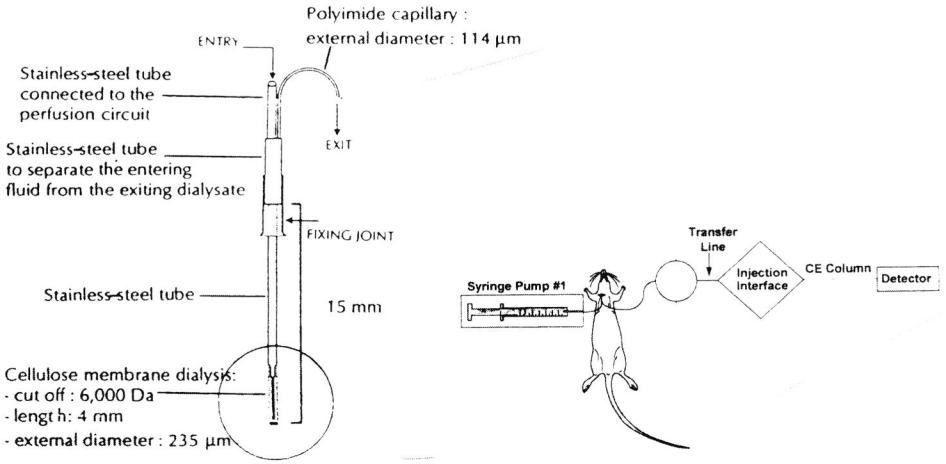


Figure 2.15 Schematic diagram of microdialysis probe (left) [117] and on-line microdialysis–CE system (right) [108].

2.5.2 Microdialysis

In microdialysis a probe constructed from a small piece of dialysis fibre, with a length of 2–5 mm, an I.D. of 75 µm and an MWCO of about 20 kDa is used. The fibre is positioned between two capillaries, consisting of PTFE, PEEK, polyethylene or fused silica (figure 2.15). During the dialysis process, the acceptor liquid is flushed through the capillaries and the permeated analytes are removed from the dialysis fibre. The probe can be used for both *in vitro* (placed in a vial) and *in vivo* (placed in the tissue, mostly the brain, of an animal) analysis. Because the probe is placed in the animal and transport of the sample only takes a few minutes, the method can be used for real-time sampling and analysis.

The use of an implanted dialysis probe (figure 2.15) has several advantages: the number of animals that have to be used for a study can be reduced, the sampling frequency can be increased, the animals can be sampled while they are awake and they can move freely in their cage [99]. Beside the analytes, salts and other small molecules can also pass the membrane which will result in a decrease of their tissue concentrations. Since this can affect the concentration of the analytes, an isotonic salt solution is normally used as the acceptor phase [105] to prevent exhaustion of the animal.

Table 2.7 Applications of off-line and on-line microdialysis–CE.

Analyte	Sample	Microdialysis–CE interface	Label	Detection technique	LOD	Reference
ff-line microdialysis						
α-Difluoromethyl-ornithine	blood		NDA	UV	5 μM	[105]
Amino acids	brain		-	MS	10 μM	[9, 141]
	brain		NDA	ECD	0.1 μM	[96, 142]
	brain		NDA	LIF	5 nM	[111, 143]
	brain		CBQCA	LIF	0.3 μM	[95]
	brain		-	ECD	10 nM	[9, 144]
	urine		-	ECD	-	[142]
Amphetamine	brain		FITC	LIF	3 nM	[145]
Glucose	blood		-	ECD	0.9 μM	[142]
Glutathion	brain		-	ECD	21 nM	[142]
Isoproterenol (catecholamine)	blood		-	ECD/UV	0.6 ng/ml	[146]
L-Dopa	brain		-	ECD	4 ng/ml	[9, 142-144, 147]
Peptide	water		-	ECD	0.7 μM	[142]
Phenobarbital	brain/blood		-	UV	2 μg/ml	[117]
Tryptophan, kynurenine	brain		-	ECD	4 nM	[110, 143]
n-line microdialysis–CE						
Amino acids	brain	micro injection valve	NDA	LIF	0.5 μM	[9, 143]
	water	flow-gated	OPA	LIF	1 μM	[106]
Antineoplastic + metabolites	brain	micro injection valve	-	LIF	1 μM	[9, 108, 143]
Ascorbic acid	brain	flow-gated	-	UV	30 μM	[109]
					10 μM	[9, 107]
Lactate	brain	flow-gated	-	UV	3 mM	[109]

CBQCA = 3-(4-carboxybenzoyl)-2-quinoline carboxaldehyde, FITC = fluorescein isothiocyanate, NDA = naphthalenedialdehyde, OPA = *o*-phthaldehyde

As shown in table 2.7, both dialysis coupled off-line and on-line with CE are used. The on-line dialysis system can be connected with the CE system by the flow-gating approach [106, 107] or via a micro-injection valve-based interface [100, 108]. The flow-gating interface is the same as that presented by Jorgenson et al. for connecting LC and CE [50, 58]. The micro-injection valve interface collects the acceptor solvent after valve switching, which is then flushed to the CE system [9, 108]. Both interfaces are grounded to prevent a high-voltage leak to the animal. With this on-line set-up, measurements can be performed every 45 s [109].

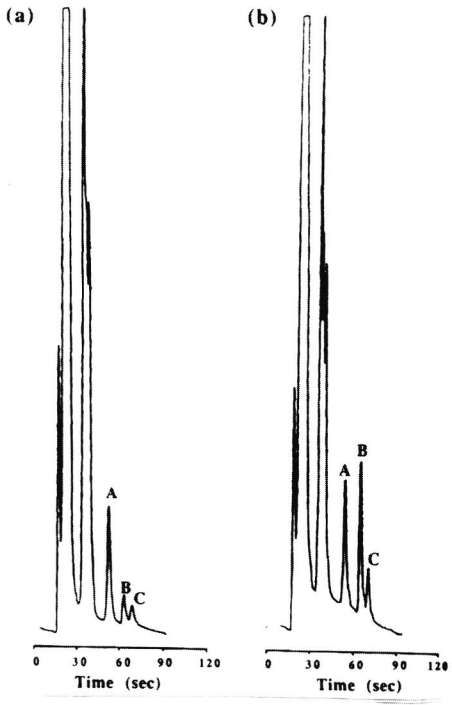


Figure 2.16 Electropherograms obtained (a) prior to and (b) after stimulation with high concentration ACSF using on-line microdialysis–CE. A = fluorescein (internal standard), B = glutamate and C = aspartate [107].

Typical analytes that are determined by this approach are drugs and their metabolites and endogenous compounds such as amino acids, detected with UV or LIF, are frequently used. The combination of CE with MS or ECD detection is used only in off-line set-ups because both the dialysis–CE interfacing and the MS or ECD detectors require a grounded electrode and can therefore not be coupled easily.

Figure 2.16 shows the on-line determination of glutamate/aspartate released from the brain, prior to and after stimulation with a high concentration of artificial cerebrospinal fluid (ACSF). The amino acids were on-line derivatized with naphthalenedialdehyde (NDA) and detection was performed using LIF. Forty analyses/hour could be performed.

Reported LODs for the off-line mode are in the 1–100 nM range, while with the on-line mode typical values are 0.5–30 μM. With both modes, linearity was observed over 2–3 orders of magnitude with RSD values of 4–5% [96, 105, 106, 110, 111]. The main advantage of the on-line system is the temporal resolution (the time in which an analysis is performed of a collected sample) which can be down to every 0.7–1.0 min [107, 109], while the temporal resolution for the off-line method is about 5–20 min [96, 105, 110, 111]. However, the detection limits of the off-line method are better and the set-up of the method is simpler.

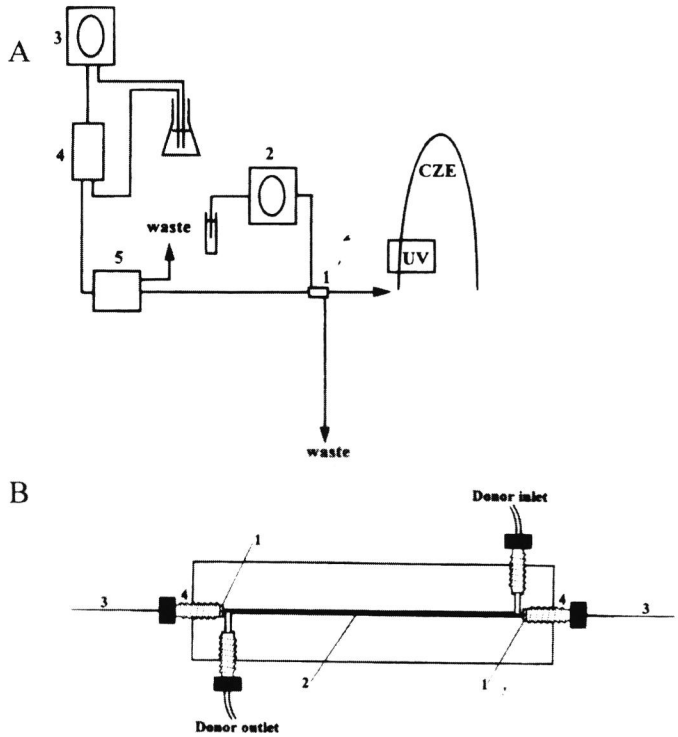


Figure 2.17 (A) Membrane extraction set-up with (1) hollow fiber membrane unit, (2) sample processor, (3) pump, (4) flow processor, and (5) miniaturized switching device. (B) Hollow fiber unit with (1) compartment for inner O-ring, (2) polypropylene hollow fiber, (3) fused silica capillaries, and (4) positioning nuts [101].

2.5.3 Supported-liquid membranes

Dialysis can also be performed by using a hollow-fibre miniaturized-supported-liquid membrane (SLM) device. This consists of a polypropylene fibre which is impregnated with a hydrophobic organic solvent such as 6-undecanone and is placed in a polymer block (figure 2.17) [101, 112, 169, 165]. If basic analytes have to be determined, the donor compartment is filled with the sample adjusted to a rather high pH (e.g. pH 10–12) while the acceptor phase has a rather low pH (e.g. pH 2–4). As a result the basic analytes can easily pass the membrane in their neutral form. However, they will immediatelly become charged in the acceptor phase and will, therefore, not diffuse back into the membrane. A similar procedure can be used for acidic compounds, but now a low pH should be used in the donor phase, and a high pH in the acceptor phase. In both cases the analytes will be concentrated in the acceptor phase.

So far, reported applications are the determination of the weak base bambuterol in plasma [101, 112–114], of alkylphenones in aqueous solutions [169] and of NSAIDs in urine [165]. To determine bambuterol, the sample pH was adjusted to 10 (which caused a 1:2 dilution of the sample) before injection of the sample in the donor phase. During enrichment the sample was pumped through the donor phase for 20 min at 25 µl/min while the acceptor phase, a phosphoric acid solution, was held stagnant. After enrichment the analytes were desorbed in an acceptor phase were volume of 10–15 µl. From this acceptor phase 1–4 µl was pumped to the CE capillary. After (manual) disconnection of the capillary, the large plug of acceptor phase was concentrated using a stacking procedure. The LOD of bambuterol in plasma (UV detection) was 4 nM, with a linear response over almost two orders of magnitude [101, 112–114]. The LODs of the NSAIDs are in the 2 ng/ml range, but many interferences are present in the electropherogram when using UV as detection method. The impregnated polypropylene fibre can be used for 6 h without becoming exhausted. SLM–CE probably has some potential, but the manual handling which is required, detracts from its usefulness for routine analysis.

2.5.4 Electrodialysis

Electrodialysis is a well-known process in biotechnology and is used to neutralize and to enrich and/or purify bioanalytical samples. The electric field which is applied over the membrane, not only increases the mass transfer of charged compounds compared to a diffusion-controlled process, but also effects a charged-dependent dialysis speed. Three devices are reported in the literature: (i) an electrodialysis device (EDD) which is used to remove large particles from a sample [98], (ii) a concentration electrodialysis device (CEDD) for the removal of large molecules and salts [115], and (iii) a post-capillary dialysis unit, to remove salts after an isoelectric focusing (IEF) step [116].

The off-line coupling of EDD with CE has been used for the CE determination of inositol phosphates in a fermentation broth [98]. The EDD consisted of a donor and an acceptor chamber, each with a volume of 0.5 ml, separated by a dialysis membrane with an MWCO of 30 kDa (figure 2.18). The CE capillary was positioned in the acceptor phase, water, and the electrode in the donor chamber, which was filled with the sample. Upon application of an electric field, the analytes will pass the membrane and enter the CE capillary. After completion of the electrodialytic process, the CE capillary was placed in a vial containing the CE buffer and the analysis was started. The applicability of the method was tested for the determination of ATP in test solutions in the presence of human serum albumin (HSA). The HSA concentration could be increased to 50 g/l without any adverse influence on the separation of ATP, although the sample had to be diluted 10-fold in advance. Unfortunately, the method

was not sensitive enough to determine ATP at levels low enough to analyse serum samples. For the rest the system has the distinct disadvantage that the CE capillary has to be removed from the EDD and inserted into the CE vial by hand between sample pretreatment/injection and the CE analysis.

A CEDD device consists of three compartments [115]. The sample (about 50 µl) is injected in the first compartment which also contains the anode. The CE capillary is

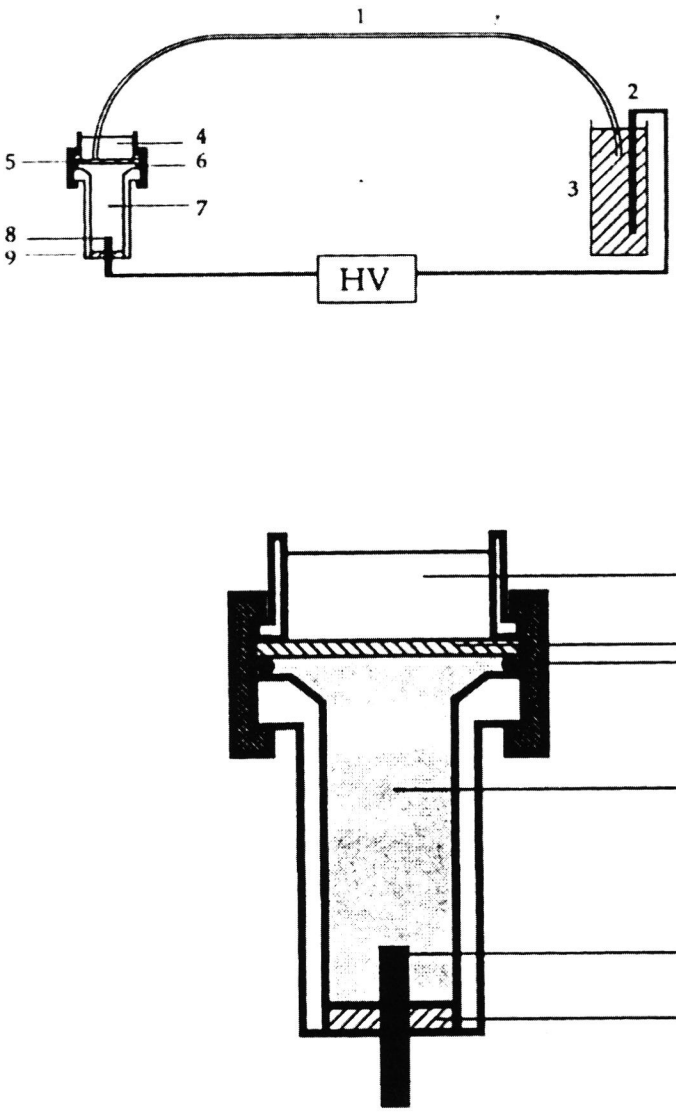


Figure 2.18 EDD coupled with a CE system. 1 = fused silica capillary; 2 = anode; 3 = buffer vial; 4 = acceptor compartment; 5 = membrane; 6 = O-ring; 7 = donor compartment; 8 = cathode; 9 = septum [98].

placed in the second compartment and the cathode is positioned in the third compartment. A 30-kDa cut-off membrane is placed between the first and the second compartment, and a 500-Da membrane between the second and third compartment. After sample injection a voltage is applied between the anode and cathode. During equilibration (about 10 min), small, medium and large molecules will migrate to the third, second and first compartments, respectively. Next, the sample is injected electrokinetically into the capillary and the CE analysis is started. The method has been used to determine inositol phosphates in serum. LODs of about 3–7 μM were obtained when MS in the SIM mode was used for detection. Unfortunately, this is too high for the analysis of real-life samples.

IEF is an electrophoretic technique which separates compounds on the basis of their isoelectric points. Unfortunately, when MS is used for detection the electrolytes used for the IEF process contaminate the ionisation chamber of the detector. A direct IEF–MS set-up is, therefore, not recommended. To prevent problems, an alternative set-up is presented in the literature [116]. The IEF capillary is connected to a transfer capillary via a dialysis fibre (MWCO, 5,000). The fibre is placed in a dialysis device, which contains an electrode and is filled with the acceptor solvent, which can be displaced by a syringe pump. The IEF capillary is filled with a mixture of carrier ampholytes and the sample. During IEF, the electrode in the dialysis device is grounded. When the carrier ampholytes reach the dialysis unit, the electrode is disconnected and the voltage is applied over both the IEF and the transfer capillary. The carrier ampholytes are dialysed through the membrane and replaced by the acceptor phase (2% acetic acid). The focused proteins are transported through the transport capillary to the MS by applying a small pressure (25 mbar) and voltage. In the single paper devoted to this approach, the separation of aqueous solutions of proteins was much better than with direct IEF–MS. No real-sample analyses were reported.

2.6 Conclusions and trends

The most important conclusion to be drawn from the present review is that biological sample handling for CE still is in its infancy. Despite the fact that a wide variety of procedures, which have been published in over 170 papers, have been combined with CE, the vast majority is either laborious or time-consuming, or deals with simple aqueous solutions. Because of the specific problems related to CE: (i) small injection and detection volumes, (ii) limited applicability of organic solvents, (iii) necessity of pH adaptation, and (iv) application of high voltages, most of the automated sample procedures applied so far are not robust, selective or sensitive enough to obtain reliable quantitative results for the determination of organic compounds in biological samples. This is demonstrated by the overview of the

characteristics of the different chromatographic, electrophoretic and membrane-based sample preparation procedures presented in table 2.8.

When the papers quoted in this review are studied in some more detail, one finds that less than 50% deals with the analysis of biological samples and that of those 50%, over 40% does not need any additional sample preparation such as precipitation, LLE or SPE. The two techniques that are most frequently used are off-line SPE–CE (29 papers) and microdialysis–CE (25 papers). The latter approach is mainly applied for *in vivo* analysis, while the former is primarily used for *in vitro* studies. Other relatively new techniques which are potentially useful but not (yet) widely applied, are at-line SPE–CE and dialysis–SPE–CE.

Techniques like FAI, sample stacking and in-line SPE–CE can be used to improve the sensitivity of the CE analysis, but if particulate matter or proteins are present additional sample preparation steps are necessary. Furthermore, the presence of a large concentration of salts can disturb the sample stacking and FAI processes.

In most cases detection limits are on the order of 100–1,000 ng/ml for real-life samples when using UV absorbance detection. The sensitivity can be improved by using more sensitive/selective detection principles like fluorescence or MS However, the application of fluorescence or LIF has the disadvantage that most analytes of interest do not possess native fluorescence. The use of MS detection still is not without problems because not all MS modes can be coupled to the electrophoretic system since the injection point of the capillary has to be grounded.

The overall conclusion is that although a large number of papers have been presented which discuss the combination of sample preparation and CE, the number of applications related to the direct injection of biological samples is limited. Or, in other words, much effort will have to be devoted to the developement of more robust and userfriendly sample preparation techniques which should take into account the specific features of CE. The two techniques which have already proven their applicability are off-line SPE–CE and microdialysis–CE. Having read the studies presented in Chapters 4 and 5 of the present thesis, one may add that at-line SPE–CE and on-line dialysis–SPE–CE can be regarded as worthwhile additions to this list.

Table 2.8 Sample preparation coupled to CE: general conclusions

Technique	Trace enrichment	Desalting	Robustness	Typical LOD (ng/ml)	Sample type	Special features	Additional sample preparation	Number of applications	
								total	applied to biological sample
biological samples									
Off-line SPE–CE	10–100	++	++	1–100	urine, serum, liver plasma	-	-	29	29
At-line SPE–CE	10–20	++	++	100	urine, serum	-	-	6	6
On-line SPE–CE	10–100	++	+/-	10–200	urine, serum	-	-	5	2
In-line SPE–CE	1–1000	++	--	10–10,000	urine, liver	-	off-line SPE, LLE	29	10
SPME	50–100	++	++	100–1000	serum, urine	-	-	4	2
Heart-cut LC–CE	1–1000	++	--	50	aqueous	-	-	2	0
Comprehensive LC–CE	-	++	--	-	aqueous	2D data	-	9	0
cITP–CE	100–1000	+/-	--	10–100	serum, urine	-	precipitation	8	2
tITP–CE	100–1000	+/-	--	1–10	liver, plasma, urine, serum	-	off-line SPE	18	6
Stacking	10–100	--	--	10–100	urine, serum	-	off-line SPE, LLE	18	4
FAI	20–100	--	--	1–40	serum	-	off-line SPE, LLE	17	5
Electro-extraction	100–1000	++	-/+		ethyl acetate	-	-	1	0
Dialysis–SPE–CE	1–10	++	++	40–300	urine, serum	-	-	3	3
Microdialysis–CE	-	+/-	++	100–3000	brains	*in vivo* sampling	-	25	25
SLM–CE	1–10	+/-	-/+	5	plasma	-	-	4	3
Electrodialysis–CE	1	+	++	1000–10,000	plasma	-	-	4	4

List of abbreviations

ACSF	artificial cerebrospinal fluid
ATP	adenosine triphosphate
BGE	background electrolyte
BSA	bovine serum albumine
CCA	chloroacetaldehyde
CCD	charged coupled device
CE	capillary electrophoresis
CEDD	concentration electrodialysis device
cITP	coupled capillary transient isotachophoresis
2D	two-dimensional
3D	three-dimensional
DNP	2,4-dinitrophenyl
EACA	e-aminocaproic acid
ECD	electrochemical detection
EDD	electrodialysis device
EE	electroextraction
EOF	electroosmotic flow
EVA	ethylvinylacetate copolymer
FAI	field-amplified injection
FITC	fluorescein isothiocyanate
FLU	fluorescence
GSH	reduced glutathione
HIS	histidine
IEF	isoelectric focusing
ITP	isotachophoresis
LE	leading electrolyte
LEP	lysine-C endopeptidase
LIF	laser-induced fluorescence
LLE	liquid–liquid extraction
LOD	limit of detection
MES	2-(N-morpholino)propanesulphonic acid
mPC–CE	membrane preconcentration–CE
MS	mass spectrometry
MWCO	molecular weight cut-off
NDA	naphthalenedialdehyde
NMR	nuclear magnetic resonance
ODS	octadecyl-bonded silica
OPA	o-phthalaldehyde
PEG	polyethylene glycol

RPLC	reversed-phase liquid chromatography
SDB	styrene-divinylbenzene copolymer
SEC	size-exclusion chromatography
SLM	supported liquid membrane
SPE	solid-phase extraction
SPME	solid-phase micro-extraction
spPC–CE	solid-phase preconcentration–CE
TAPS	N-tris[hydroxymethyl]methyl-3-aminopropanesulphonic acid
TEA	triethylamine
TE	terminating electrolyte
tITP	transient isotachophoresis
TRIS	tris(hydroxymethyl)aminomethane
TRITC	tetramethylrhodamine isothiocyanate isomer-5
UV	ultraviolet

References

[1] J.R. Veraart, C. Gooijer, H. Lingeman, N.H. Velthorst and U.A.Th. Brinkman, Chromatographia, 44 (1997) 581.

[2] J. Boden and K. Bächmann, J. Chromatogr. A, 734 (1996) 319.

[3] Z.K. Shihabi and M.E. Hinsdale, Electrophoresis, 16 (1995) 2159.

[4] L.L. Garcia and Z.K. Shihabi, J. Chromatogr., 652 (1993) 465.

[5] Z.K. Shihabi and M.E. Hinsdale, J. Chromatogr. B, 669 (1995) 75.

[6] M.J. van der Schans, J.K. Allen, B.J. Wanders and A. Guzman, J. Chromatogr., 680 (1994) 511.

[7] D.G. Stegenhuis, H. Irth, U.R. Tjaden and J. van der Greef, J. Chromatogr., 538 (1991) 393.

[8] Z.K. Shihabi, J. Chromatogr., 652 (1993) 471.

[9] S.M. Lunte and C.E. Lunte, Adv. Chromatogr., 36 (1996) 383.

[10] A.J. Tomlinson, N.A. Guzman and S. Naylor, J. Cap. Elec., 2 (1995) 247.

[11] M.A. Jenkins and M.D. Guerin, J. Chromatogr. B. 699 (1997) 257.

[12] R.P. Oda, R. Clark, J.A. Katzmann and J.P. Landers, Electrophoresis, 18 (1997) 1715.

[13] M.A. Jenkins and M.D. Guerin, J. Chromatogr. B. 682 (1996) 23.

[14] Z.K. Shihabi and M.A. Friedberg, Electrophoresis, 18 (1997) 1724.

[15] L.A. Holland, N.P. Chetwyn, M.D. Perkins and S.M. Lunte, Pharm. Res., 14 (1997) 372.

[16] D. Leveque, C. Gallionrenault, H. Monteil and F. Jehl, J. Chromatogr. B, 697 (1997) 67.

[17] S. Naylor, L.M. Benson and A.J. Tomlinson, J. Chromatogr. A, 735 (1996) 415.

[18] G.W. Somsen, C. Gooijer, N.H. Velthorst and U.A.Th. Brinkman, Trends Anal. Chem., 17 (1998) 129.

[19] M.E. Swartz and M. Merion, J. Chromatogr., 632 (1993) 209.

[20] M.C. Roach, P.Gozel and R.N. Zare, J. Chromatogr., 426 (1988) 129.

[21] K. Srinivasan and M.G. Bartlett, J. Chromatogr. B, 703 (1997) 289.

[22] C.P. Chen, D. Jeffery, W. Jorgenson, M.A. Moseley and G.M. Pollack, J. Chromatogr. B, 697 (1997) 149.

[23] A. Cifuentes, J. Valencia, E. Sanz, M.J. Sanchez and M.A. Rodriguez-Delgrado, J. Chromatogr. A, 778 (1997) 389.
[24] M. Willetts, P. Clarkson and M. Cooke, Chromatographia, 43 (1996) 671.
[25] F. Vonheeren, R. Tanner, R. Theurillat and W. Thormann, J. Chromatogr. A, 745 (1996) 165.
[26] J. Sadecka and J. Polonsky, J. Chromatogr. A, 735 (1996) 403.
[27] W.C. Brumley, C.M. Brownrigg and A.H. Grange, J. Chromatogr., 680 (1994) 635.
[28] A.J. Tomlinson, L.M. Benson, R.P Oda, W.D Braddock, M.A. Strausbauch, P.A. Strausbauch, P.J. Wettstein and S. Naylor, J. High Resol. Chromatogr., 17 (1994) 669.
[29] R.L. Sheppard and J. Henion, Anal. Chem., 69 (1997) 2901.
[30] S.H. Chen, M.X. Qian, J.M. Brennan and J.M. Gallo, J. Chromatogr. B, 692 (1997) 43.
[31] D.P. Bogan, B. Deasy, R. O'Kennedy, M.R. Smyth and U. Fuhr, J. Chromatogr. B, 663 (1995) 371.
[32] J.R. Veraart, C. Gooijer, H. Lingeman, N.H. Velthorst en U.A.Th. Brinkman, J. Chromatogr. A. 719 (1998) 199.
[33] J.R. Veraart, C. Gooijer, H. Lingeman, N.H. Velthorst, U.A.Th. Brinkman, J. High Resol Chromatogr., 22 (1999) 183.
[34] J.R. Veraart, C. Gooijer, H. Lingeman, N.H. Velthorst en U.A.Th. Brinkman, J. Pharm. Biomed. Anal., 17 (1998) 1161.
[35] A.J.J. Debets, K.–P. Hupe, U.A.Th. Brinkman and W.Th. Kok, Chromatographia, 29 (1990) 217.
[36] A.J.J. Debets, M. Mazereeuw, W.H. Voogt, D.J. van Iperen, H. Lingeman, K.–P. Hupe and U.A.Th. Brinkman, J. Chromatogr. A, 608 (1992) 151.
[37] P.J.M. Hendriks, H.A. Claessens, J.T.H. Verhoeven, T.H.M. Noij and C.A. Cramers, J. Chromatogr., 655 (1993) 325.
[38] T. Tsuda, T. Mizuno, J. Akiyama, Anal. Chem., 59 (1987) 799.
[39] I. Morita and J.I. Sawada, J. Chromatogr., 641 (1993) 375.
[40] J. Cai and Z. El Rassi, J. Liq. Chromatogr., 15 (1992) 1179.
[41] J. Cai and Z. El Rassi, J. Liq. Chromatogr., 16 (1993) 2007.
[42] A.J. Tomlinson, L.M. Benson, R.P. Oda, W.D. Braddock, B.L. Riggs, J.A. Katzmann and S. Naylor, J. Cap. Elec., 2 (1995) 97.
[43] A.J. Tomlinson, L.M. Benson, S. Jameson, D.H. Johnson and S. Naylor, J. Am. Soc. Mass Spectrom., 8 (1997) 15.
[44] S. Naylor, L.M. Benson and A.J. Tomlinson, J. Chromatogr. A, 735 (1996) 415.
[45] L.M. Benson, A.F. Tomlinson and S. Naylor, J. High Resol. Chromatogr., 17 (1994) 671.
[46] M.A. Strausbauch, B.J. Madden, P.J. Wettstein and J.P Landers, Electrophoresis, 16 (1995) 541.
[47] M.A. Stausbauch, J.P. Landers and P.J. Wettstein, Anal. Chem., 68 (1996) 306.
[48] M.A. Strausbauch, S.J. Xu, J.E. Ferguson, M.E. Nunez, D. Machacek, G.M. Lawson, P.J. Wettstein and J.P. Landers, J. Chromatogr. A, 717 (1995) 279.
[49] S. Li and S.G. Weber, Anal. Chem., 69 (1997) 1217.
[50] J.J. Cole and R.T. Kennedy, Electrophoresis, 16 (1995) 549.
[51] M.B. Bushey and J.W. Jorgenson, Anal. Chem., 62 (1990) 978.
[52] A.V. Lemmo and J.W. Jorgenson, J. Chromatogr., 633 (1993) 213.
[53] M.B. Bushey and J.W. Jorgenson, J. Microcol. Sep., 2 (1990) 293.
[54] C.A. Monning and J.W. Jorgenson, Anal. Chem., 63 (1991) 802.
[55] A.W. Moore Jr., and J.W. Jorgenson, Anal. Chem., 65 (1993) 3550.

[56] J.P. Larmann Jr., A.V. Lemmo, A.W. Moore Jr., J.W. Jorgenson, Electrophoresis, 14 (1993) 439.
[57] A.W. Moore Jr. and J.W. Jorgenson, Anal. Chem., 67 (1995) 3448.
[58] A.V. Lemmo and J.W. Jorgenson, Anal. Chem., 65 (1993) 1576.
[59] P.S. Janssen, J.W. van Nispen, M.J.M. Zeeland and P.A.T.A. Melgers, J. Chromatogr., 470 (1989) 171.
[60] A.W. Moore Jr. and J.W. Jorgenson, Anal. Chem., 67 (1995) 3456.
[61] D. Kaniansky, I. Zelenský, A. Hybenová and F.I. Onuska, Anal. Chem., 66 (1994) 4258.
[62] M. Deml, F. Foret and P. Bocek, J. Chromatogr., 320 (1985) 159.
[63] D. Kaniansky, J. Marák, V. Madajová and E. Simunicová, J. Chromatogr., 638 (1993) 137.
[64] D. Kaniansky and J. Marák, J. Chromatogr., 498 (1990) 191.
[65] N.J. Reinhoud, A.P. Tinke, U.R. Tjaden, W.M.A. Niessen and J. van der Greef, J. Chromatogr., 627 (1992) 263.
[66] F. Foret, E. Szoko and B.L. Karger, J. Chromatogr., 608 (1992) 3.
[67] D.G. Stegenhuis, U.R. Tjaden and J. van der Greef, J. Chromatogr., 591 (1992) 341.
[68] C.–C. Wang, W.P. McCann and S.C. Beale, J. Chromatogr. B, 676 (1996) 19.
[69] D.T. Witte, S. Någård and M. Larsson, J. Chromatogr., 687 (1994) 155.
[70] M. Larsson and S. Någård, J. Microcol. Sep., 6 (1994) 107.
[71] N.J. Reinhoud, U.R. Tjaden and J. van der Greef, J. Chromatogr., 653 (1993) 303.
[72] S.J. Locke and P. Thibault, Anal. Chem., 66 (1994) 3436.
[73] M.J. van der Schans, J.L. Beckers, M.C. Molling and F.M. Everaerts, J. Chromatogr. A, 717 (1995) 139.
[74] S. Auriola, I. Jääskeläinen, M. Regina and A. Urtti, Anal. Chem., 68 (1996) 3907.
[75] N.J. Reinhoud, U.R. Tjaden and J. van der Greef, J. Chromatogr., 641 (1993) 155.
[76] T.J. Thompson, F. Foret, P. Vouros and B.L. Karger, Anal. Chem., 65 (1995) 900.
[77] M.H. Lamoree, N.J. Reinhoud, U.R. Tjaden, W.M.A. Niessen and J. van der Greef, Biol. Mass Spectrom., 23 (1994) 339.
[78] N.J. Reinhoud, U.R. Tjaden and J. van der Greef, J. Chromatogr., 673 (1994) 255.
[79] J.–L. Liao, R. Zhang and S. Hjertén, J. Chromatogr., 676 (1994) 421.
[80] J. Bergmann, U. Jaehde, M. Mazereeuw, U.R. Tjaden and W. Schunack, J. Chromatogr. A, 734 (1996) 381.
[81] P. Gebauer, W. Thormann and P. Bocek, Electrophoresis, 16 (1996) 2039.
[82] P. Gebauer, W. Thormann and P. Bocek, J. Chromatogr., 608 (1992) 47.
[83] R. Szücs, J. Vindevogel, P. Sandra, L.C. Verhagen, Chromatographia, 36 (1993) 323.
[84] D.S. Burgi and R.–L. Chien, Anal. Chem., 63 (1991) 2042.
[85] D.S. Burgi and R.–L. Chien, J. Microcol. Sep. 2 (1991) 199.
[86] J.L. Beckers and M.T. Ackermans, J. Chromatogr., 629 (1993) 371.
[87] M.A. Friedberg, M.E. Hinsdale and Z.K. Shihabi, J. Chromatogr. A, 781 (1997) 491.
[88] M. Friedberg and Z.K. Shihabi, J. Chromatogr. B, 695 (1997) 193.
[89] Z.K. Shihabi, M.V. Rocco and M.E. Hinsdale, J. Liq. Chromatogr., 18 (1995) 3825.
[90] R.–L. Chien and D.S. Burgi, Anal. Chem., 64 (1992) 1046.
[91] K. Bächmann, G Göttlicher, I. Haag, M. Hannina and W. Hensel, Fresenius J. Anal. Chem., 350 (1994) 368.
[92] G.B. Harland, G. McGrath, S. McClean and W.F. Smyth, Anal. Commun., 34 (1997) 9.
[93] S.C.C. Chiang and S.F.Y. Li, Biomed. Chromatogr., 11 (1997) 366.
[94] E. van der Vlis, M. Mazereeuw, U.R. Tjaden, H. Irth and J. van der Greef, J. Chromatogr. A, 712 (1995) 227.

[95] J. Bergquist, M.J. Vona, C.–O. Stiller, W.T. O'Conner, T. Falkenberg and R. Ekamn, J. Neurosc. Meth., 65 (1996) 33.
[96] T.J. O'Shea, P.L. Weber, B.P. Bammel, C.E. Lunte and S.M. Lunte, J. Chromatogr., 608 (1992) 189.
[97] M.W. Lada and R.T. Kennedy, Anal. Chem., 68 (1996) 2790.
[98] B.A.P. Buscher, U.R. Tjaden and J.van der Greef, J. Chromatogr. A, 764 (1997) 135.
[99] E.L. Gardner, J. Chen and W. Paredes, J. Neurosc. Meth., 48 (1993) 173.
[100] C.E. Lunte, D.O. Scott and P.T. Kissinger, Anal. Chem., 63 (1991) 773A.
[101] S. Pálmardóttir, E. Thordarson, L.–E. Edholm, J.Å. Jönsson and L. Mathiasson, Anal. Chem., 69 (1997) 1732.
[102] J.F. Staden, Fresenius J. Anal. Chem., 352 (1995) 271.
[103] R.E. Majors, LC–GC Intern., 8 (1995) 375.
[104] J.R. Veraart, J. van Hekezen, M.C.E. Groot, C. Gooijer, H. Lingeman, N.H. Velthorst and U.A.Th. Brinkman, Electrophoresis, 19 (1998) 2944.
[105] T. Hu, H. Zuo, C.M. Riley, J.F. Stobaugh and S.M. Lunte. J. Chromatogr. A, 716 (1995) 318.
[106] M.W. Lada and R.T. Kennedy, Anal. Chem., 68 (1996) 2790.
[107] M.W. Lada, G. Schaller, M.H. Carriger, T.W. Vickroy and R.T. Kennedy, Anal. Chim. Acta, 307 (1995) 217.
[108] B.L. Hogan, S.M. Lunte, J.F. Stobaugh and C.E. Lunte, Anal. Chem., 66 (1994) 596.
[109] M.W. Lada and T. Kennedy, J. Neurosc. Meth., 63 (1995) 147.
[110] M.A. Malone, H. Zuo, S.M. Lunte and M.R. Smyth, J. Chromatogr. A, 700 (1995) 73.
[111] L. Hernandez, J. Escalona, P. Verdeguer and N.A. Guzman, J. Liq. Chromatogr., 16 (1993) 2149.
[112] S. Palmarsdottir, L. Mathiasson J.A. Jonsson and L.E. Edholm, J. Chromatogr. B, 688 (1997) 127.
[113] S. Palmarsdottir, L. Mathiasson, J.A. Jonsson and L.-E. Edholm, J. Cap. Elec., 3 (1996) 255.
[114] S. Palmarsdottir, B. Lindegard, L.-E. Edholm, L. Mathiasson and J.A. Jonsson, J. Cap. Elec., 2 (1995) 185.
[115] B.A.P. Buscher, U.R. Tjaden and J. van der Greef, J. Chromatogr. A, 788 (1997) 165.
[116] M.H. Lamoree, U.R. Tjaden and J. van der Greef, J. Chromatogr. A, 777 (1997) 31.
[117] S. Tellez, N. Forges, A. Roussin and L. Hernandez, J. Chromatogr. B, 581 (1992) 257.
[118] B.L. Lee, A.L. New and C.N. Ong, J. Chromatogr. B, 792 (1997) 463.
[119] C.P. Chen and G.M. Pollack, J. Chromatogr. B, 681 (1996) 363.
[120] M. Siluveru and J.T. Stewart, J. Chromatogr. B, 693 (1997) 205.
[121] M. Siluveru and J.T. Stewart, J. Chromatogr. B, 691 (1997) 217.
[122] M.I. Maguregui, R.M. Alonso and R.M. Jimenez, J. Liq. Chromatogr. & Rel. Techn., 20 (1997) 3377.
[123] M. Walshe, J. Howarth, M.T. Kelly, R. Okennedy and M.R. Smyth, J. Pharma. Biomed. Anal., 16 (1997) 319.
[124] M. Lanz, R. Brenneisen and W. Thormann, Electrophoresis, 18 (1997) 1035.
[125] M. Lanz and W. Thormann, Electrophoresis, 17 (1996) 1945.
[126] M.R. Taylor, S.A. Westwood and D. Perrett, J. Chromatogr. A, 745 (1996) 155.
[127] A.J. Tomlinson, W.D. Braddock, L.M. Benson, R.P. Oda and S. Naylor, J. Chromatogr. B, 669 (1995) 67.
[128] A.J. Tomlinson, L.M. Benson, W.D. Braddock, R.P. Oda and S. Naylor, J. High Resol. Chromatogr., 17 (1994) 729.

[129] M.A. Strausbauch, S.J. Xu, J.E. Ferfuson, M.E. Nunez, D. Machacek, G.M. Lawson, P.J. Wettstein and J.P. Landers, J. Chromatogr. A, 717 (1995) 279.
[130] J.H. Beattie, R. Self and M.P Richards, Electrophoresis, 16 (1995) 322.
[131] A.M. Hoyt, S.C. Beale, J.P Larmann Jr. and J.W. Jorgenson, J. Microcol. Sep., 5 (1993) 325.
[132] D. Figeys, A. Decret and R. Aebersold, J. Chromatrogr. A, 763 (1997) 295.
[133] A.J. Tomlinson, L.M. Benson, W.D. Braddock, R.P. Oda and S. Naylor, J. High Resol. Chromatogr., 18 (1995) 381.
[134] A.J. Tomlinson, S. Jameson and S. Naylor, J. Chromatogr. A, 744 (1996) 273.
[135] A.J. Tomlinson and S. Naylor, J. Liq. Chromatogr., 18 (1995) 3591.
[136] H. Yamamoto, T. Manabe and T. Okuyama, J. Chromatogr., 480 (1989) 277.
[137] L.M. Benson, A.J. Tomlinson, A.N. Mayeno, G.J. Gleich, D. Wells and S. Naylor, J. High Resol. Chromatogr., 19 (1996) 291.
[138] M. Mazereeuw, U.R. Tjaden and J. van der Greef, J. Chromatogr., 677 (1994) 151.
[139] C. Schwer and F. Lottspeich, J. Chromatogr., 623 (1992) 345.
[140] F. Foret, E. Szoko and B.L. Karger, Electrophoresis, 14 (1993) 417.
[141] Y. Tanaka, M. Yoshida, M. Sakairi and H. Koizumi, Rapid Commun. Mass Spectrom., 9 (1995) 895.
[142] S.M. Lunte and T.J. O'Shea, Electrophoresis, 15 (1994) 79.
[143] D.K. Hansen and S.M. Lunte, Amer. Lab., (1996) 26.
[144] S.M. Lunte, M.A. Malone, H. Zuo and M.R. Smyth, Current Sep., 13 (1994) 75.
[145] X. Páez, P. Rada, S. Tucci, N. Rodríguez and L. Hernández, J. Chromatogr. A, 735 (1996) 263.
[146] M.E. Hadwiger, S.R. Torchia, S. Park, M.E. Biggin and C.E. Lunte, J. Chromatogr. A, 681 (1996) 241.
[147] T.J. Oshea, M.W. Telting–Diaz, S.M. Lunte, C.E. Lunte and M.R. Smyth, Electroanalysis, 4 (1992) 463.
[148] A. Fujinami, T. Miyazawa, N. Tagawa and Y. Kobayashi, Biol. Pharm. Bull., 21 (1998) 1207.
[149] D. Figeys, Y. Zhang and R. Aebersold, Electrophoresis, 19 (1998) 2338.
[150] E. Rohde, A.J. Tomlinson, D.H. Johnson and S. Naylor, Electrophoresis, 19 (1998) 2361.
[151] E. Rohde, A.J. Tomlinson, D.H. Johnson and S. Naylor, J. Chromatogr. A, 713 (1998) 301.
[152] J.Z. Song, H.F. Chen, S.J. Tian and Z.P. Sun, J. Chromatogr. B, 708 (1998) 277.
[153] E. Ameyibor and J.T. Stewart, J. Liq. Chromatogr. & Rel. Techn., 21(1998) 953.
[154] J.P. Quirino and S. Terabe, J. Chromatogr. A, 798 (1998) 251.
[155] J.P. Quirino and S. Terabe, J. Chromatogr. A, 781 (1997) 119.
[156] J.P. Quirino and S. Terabe, J. Chromatogr. A, 791 (1997) 255.
[157] J.P. Quirino and S. Terabe, Anal. Chem., 70 (1998) 149.
[158] K. Jinno, Y. Han, H. Sawada and M. Taniguchi, Chromatographia, 46 (1997) 309.
[159] N.A. Guzman, S.S. Park, D. Schaufelberger, L. Hernandez, X. Paez, P. Rada, A.J. Tomlinson and S. Naylor, J. Chromatogr. B, 697 (1997) 37.
[160] A.J. Tomlinson, L.M. Benson and S. Naylor, J. Chromatogr. 744 (1996) 3.
[161] D. Figeys, Y.B. Ning and R. Aebersold, Anal. Chem., 69 (1997) 3153.
[162] D. Figeys, A. Ducret, J.R. Yates and R. Aebersold, Nature Biotech., 14 (1996) 1579.
[163] J.R. Veraart M.C.E. Reinders, H. Lingeman and U.A.Th Brinkman, Chromatographia. (in press).
[164] L.L. Shultz-Lockyear, C.C. Colyer, Z.H. Fan, K.I. Roy and D.J. Harrison, Electrophoresis, 20 (1999) 529.
[165] S. Pedersen-Bjergaard and K.E. Rasmussen, Electrophoresis, 21 (2000) 579.
[166] S.H. Hansen, M.E. Jensen and I. Bjornsdottir, J. Pharm. Biomed. Anal., 17 (1998) 1160.

[167] C. Mardones, A. Rios and M. Valcarcel, Electrophoresis, 20 (1999) 2922.
[168] H.W. Chen and Z.-L. Fang, Anal. Chim. Acta, 394 (1999) 13.
[177] W. Zhan, T. Wang and S.F.Y. Li, Electrophoresis 21 (2000) 573.
[170] E. Bonneil and K.C., Chan, J. Chromatorgr. B. 736 (1999) 273.
[171] H.J. Issaq, K.C. Chan, G.M. Janini and G.M. Muschik, Electrophoresis, 20 (1999) 1533.
[172] T. Adam, J. Sevcik, Z. Svagera, L.D. Fairbanks and P. Bartak, Electrophoresis 20 (1999) 564.
[173] A.B. Wey and W. Thormann, Chromatographia Suppl. I, 49 (1999) S12.
[174] J.P. Quirino and S. Terabe, Electrophoresis, 21 (2000) 355.
[175] F. Hempel, Electrophoresis, 21 (2000) 691.
[176] Z.K. Shihabi, Electrophoresis, 21 (2000) 3008.
[177] J.R. Veraart, M.C.E. Groot, C. Gooijer, H. Lingeman, N.H. Velthorst and U.A.Th. Brinkman, Analyst, 124 (1999) 115.
[178] J.R. Veraart and U.A.Th. Brinkman, J. Chromatogr. A, submitted.
[179] M. Petersson, K.-G. Wahlund and S. Nilsson, J. Chromatogr. A, 841 (1999) 249.
[180] A.B. Wey, C.-X. Zhang and W. Thormann, J. Chromatogr. A, 853 (1999) 95.

Chapter 3

The Tools

3.1

Thermostating in capillary electrophoresis

Summary

The use of high voltages across a electrophoresis capillary will increase the temperature of the buffer due to Joule heating. As a result temperature control in CE is rather important since variations in the buffer temperature will result in changes in the pH of the buffer, peak shape, migration time, reproducibility, efficiency, 3-D structure of macromolecular analytes, etc.

Six different thermostating systems have been evaluated: (i) natural convection, (ii) fan, (iii) home-made and (iv and v) two commercial available high-speed air and a (vi) liquid thermostated device. In all cases the temperature of the buffer in the capillary is calculated according to the temperature-conductivity relationship. For this goal two parameters are introduced describing temperature control: the temperature onset (δT) and the temperature rise factor (α). From these results, it can be concluded that high speed air thermostating can be as efficient as liquid thermostating.

Introduction

During the past ten years the number of commercially available capillary electrophoresis (CE) systems has rapidly increased. Comparison of these systems shows important differences such as automation potential, maximum number of samples that can be analyzed, thermostating method of the capillary, thermostating range and detection unit. One of the most important features, with respect to reproducibility, is temperature control of the capillary.

In CE a relatively high power (up to 9 W) is produced in a rather small volume (about 1–10 µl), which corresponds to a generated heat of up to 900 J/cm^3/s. Therefore, accurate control of the buffer temperature is needed to avoid excessive temperature effects. An increase of the temperature (which can be over 70 °C [1]) in the capillary can have the following effects: (i) a change in the pH of the buffer because the dissociation constants strongly depend on the temperature [2], a part of the sample can be purged out of the capillary due to buffer expansion when a voltage is applied across the capillary resulting in sample losses [3], (iii) the radial temperature gradient of the buffer in the capillary is proportional to the increase of the temperature of the buffer compared with the temperature of the surrounding medium. The viscosity is also temperature-dependent. This results in a radial dependence of the ion mobility, which affects band broadening [4, 5], (iv) when the voltage is increased the system reproducibility is decreased in the order of migration time > peak height > peak area [6], (v) as a result of the current and voltage limitations of the power supply. When the current increases faster than expected according to Ohm's law - due to Joule heating - the current limitation may be reached at lower voltages resulting in longer analysis times, (vi) the concentration profile can change on boundary surfaces due to axial temperature fluctuations [7], (vii) because of the temperature-dependence of the refractive index in the capillary, the fluctuations of the baseline can be increased [8], (viii) the mobility of (analyte) ions can be changed because of the higher temperature in the capillary [9, 10] because of the shifting equilibrium constants of micellar processes, protein orientations and chiral processes are changing with temperature [11].

Thermostating can be performed in two ways: by using convection or thermal conductivity. In the latter approach, denoted as solid-state thermostating, the capillary is placed in a medium with high thermal conductivity (i.e. aluminium). This possibility is not frequently used in CE, although it is rather efficient [12].

With respect to convection thermostating, two modes can be distinguished: natural and forced convection. Natural, or non-controlled, convection implies cooling of the capillary by heat transfer to a still-standing medium. When a voltage is applied across the capillary, the buffer and capillary temperature will increase, leading to an increase of the temperature of the surrounding medium and to a decrease of its density.

Therefore, the surrounding medium will start moving by convection. Forced convection implies that the outside wall of the capillary is heated/cooled by a moving thermostating medium. Definition of at least two parameters seems to be relevant to ensure efficient thermostating: the heat capacity and the velocity of the surrounding medium. Two media are generally used in CE: liquids (i.e. halogenated alkanes) or air. Liquids have the advantage to posses a larger heat absorption capacity [1], but air has the advantage of being safe (in case of a broken capillary), easy-to-use and cheap. In natural convection the velocity of the medium is related to the temperature difference between the medium and the capillary; with forced convection this difference is related to the medium velocity. Air cooling is generally performed either by means of a fan (typical air velocities are 1.5 m/s [1]) or by using a high-speed gas flow (> 10 m/s). In the latter approach air thermostating can be as adequate as liquid thermostating [13].

Because the influence of temperature and thermostating on a CE separation has been discussed in detail [2–11], the emphasis in this study is on the thermostating process itself. To quantify this effect, first of all, the theoretical background will be outlined.

In CE the resistance (expressed in Ω/m) is constant for all voltages during increase of the electric field (E in V/m) across the capillary, provided that all heat generated in the buffer is transported out of the capillary. The electric current (i in A) is directly proportional to the electric field.

When the transport of heat out of the capillary is not sufficient, the temperature will increase and the resistance will decrease. The effect is a faster increase of the current than expected on the basis of Ohm's law. This information can be used to quantify the thermostating behaviour of CE systems by measuring the current during a stepwise increase of the voltage and plotting the current versus the applied field [14]. The lower the resistance of the buffer, the more critical thermostating of the system is. However, it should be emphasized that even in a properly thermostated capillary, a temperature increase of the buffer can appear if a low resistance is combined with a high E and a large I.D. of the capillary [1]. For a properly thermostated capillary with an I.D. of 100 µm the radial difference in temperature between the buffer in the capillary, and the capillary wall is about 1° C when a field of 7 W/m is applied [15].

Various researchers have addressed the problem of measuring the temperature inside the capillary and several approaches have been followed:

Wätzig added thermochromic molecules to the buffer [16]. The absorbance of these molecules is a function of the temperature. By measuring the increase of the background absorption, caused by these molecules, the temperature of the buffer can be calculated. The disadvantage of the method is that solutes have to be added to the

buffer which can interfere with the analysis. Another disadvantage is that the thermochromic behaviour of the added solutes depends on the pH which requires calibration for each individual pH.

Burgi et al. used the (inter)relation between the electro-osmotic flow and the temperature [17]. This approach has the disadvantage that a quantitative relation has to be determined for each individual pH and each ionic strength. Another disadvantage is that in the same CE system, using the same buffer and capillary, a highly fluctuating electric-osmotic flow can be found [18].

Terabe et al. followed a straightforward approach, i.e. measurement of the temperature with a thermocouple [19]. Unfortunately, the exact temperature in the capillary is difficult to measure because of a relatively large surface of the thermocouple compared with the O.D. of the capillary, resulting in disturbance of the temperature measurement by the surrounding medium (i.e. air).

Hjerten calculated the buffer temperature for a non-thermostated as well as a thermostated system [20]. For the first system this is based on the difference between the current immediately after applying the voltage and the current found at the end of the run. In this approach a higher current is correlated with an increase of the temperature of the buffer. This method can only be applied when the voltage is applied instantaneously. This is in contradiction with the normally used procedure, where the voltage is applied rather gradually (in at least a few seconds). For the second system the temperature inside the capillary is calculated from the geometry of the capillary and the thermal conductivities of quartz and the polyimide coating. In this case the velocity and the type of the surrounding medium are not taken into consideration. However the limitation of both procedures is that they are not based on experimental data, but are only using theoretically derived equations.

Bello et al. correlated the change of the electric conductivity of the buffer (κ in $1/(\Omega \cdot cm)$) with the temperature (T in °C) using experimentally determined data, as described below [10, 21].

Only by using this method, the temperature of the buffer can be determined accurately during the analysis without adding solutes to the buffer or modifying the CE system. The method introduced by Bello et al., will be used in this paper.

The temperature(T)–conductivity(κ) relation of a buffer can be written as:

$$T = c + d\kappa + e\kappa^2 \tag{1}$$

where c, d and e are empirical parameters. If the values of these parameters are determined for a buffer (using a thermostated box and conductivity meter), the temperature in the capillary (T_{cal}) during an electrophoretic process can be calculated from the current, length of the capillary (L in m) and cross-sectional area (A in m^2) of the capillary [22]. The values of T_{cal} obtained at different fields can be plotted as a

function of the power (P in W/m). According to the literature, T_{cal} is linear with the generated power [10, 15, 17, 18]:

$$T_{cal} = T_{0\,Watt} + \alpha P \tag{2}$$

In this equation $T_{0\,Watt}$ is the temperature of the buffer in the capillary (in °C) in case no power is generated. The temperature rise factor (in °C m/W) is called α. The difference between $T_{0\,Watt}$ and the temperature set (T_{set} in °C) is called the temperature onset (δT in °C). Including this term in Eq. 2 results in:

$$T_{cal} = T_{set} + \delta t + \alpha P \tag{3}$$

Eq. 3 indicates that the thermostating quality of a CE system can be characterized by the parameters α and δT, which can be determined by plotting the temperature versus the generated power. The parameter δT provides information on the systematic bias in the temperature control of the system due to incomplete thermostating of the capillary. The second parameter α provides the ability of the CE system to remove the generated heat. In an ideal situation the temperature is independent of the generated power ($\alpha = 0$) and the complete capillary is thermostated ($\delta T = 0$). Only under these conditions, the temperature of the buffer is really independent of the generated power ($T_{cal} = T_{set}$).

The thermostating behaviour of CE systems can be characterized by the following procedure: (i) a test buffer, with high conductivity, is selected, (ii) the temperature-conductivity relation and the parameters of Eq. 1 are determined from the test buffer, (iii) the current is measured in different CE systems, by using a stepwise increase of the field across capillaries filled with the test buffer, and at different temperatures, (iv) the temperatures at different fields strengths are calculated from Eq. 1 and finally, (v) the CE systems are characterized by calculating δT and α from Eq. 2.

When using the procedure described above, it should be kept in mind that the radial temperature profile is neglected and the temperature in the whole capillary is assumed to be constant. Therefore, the calculated temperature is not the local temperature of the buffer in the capillary but a length- and radial-averaged temperature.

Experimental

Materials

Sodium chloride, pro analyse, was purchased from Riedel-de Haën (Seelze, Germany), boric acid, pro analyse, and sodium hydroxide came from J.T. Baker (Deventer, The Netherlands). Water was demineralized and distilled before use.

Capillary electrophoresis

The six CE configurations used to compare the thermostating capabilities were:

(i) The non-thermostated configuration: The system consisted of a PrinCE unit (Lauer Labs, Emmen, the Netherlands) and was equipped with an UV detector, model 759A (Applied Biosystems, Foster City, CA). The UV detector was set at 210 nm. The device consisted of three compartments: the sample/injection, the UV-detector and the thermostating compartment. In this set-up the fan was in the off position resulting in a non thermostated capillary.

(ii) The fan thermostated configuration: The system set-up was the same as in (i) but in this set-up the fan was switched on. Only 25% of the capillary was thermostated.

(iii) The home-made high-speed thermostated configuration: The system set-up used was the same as in (i) except that the capillary was placed for 70% in a tube with a 6 mm I.D. through which temperature controlled air was blown (35 m/s). The air was thermostated in a copper tube, situated in a thermostated bath, and transported via an isolated tube with an I.D. of 8 mm. The bath consisted of a cryostat and a temperature controlled heater with a water jet.

(iv) The commercial high-speed thermostated configuration A: The CE system was an HPCE 3D system (Hewlett Packard, Waldbronn, Germany). The capillary was placed in a cassette, in which a fast, temperature controlled flow of air (10 m/s) thermostats the capillary. About 78% of the capillary was temperature-controlled. The UV/VIS detector was set on, to include heating of the capillary by the lamp. The sample tray temperature was 25°C.

(v) The commercial high-speed thermostated configuration B: A SpectroPhoresis 2000 system (TSP, San Jose, CA) was used. The capillary was placed in a cassette through which a thermostated gas was blown at high speed. As the result 77% of the capillary was thermostated. The UV lamp was set on.

(vi) The liquid thermostated configuration: The BioFocus 3000 (Bio-Rad, Hercules, CA) was used. The capillary was placed in a tube, 1.5 mm I.D., through which thermostated liquid flows (0.29 m/s). The tube was placed in a cassette and 87% of the capillary was temperature controlled. Also in this set-up the UV/VIS-detector was set on. The sample tray temperature was 20°C.

All CE experiments were performed with the same fused silica capillary of 75 µm I.D. and 375 µm O.D. The length of the capillary was 860 mm in the experiments with configuration i to iv, 669 mm in configuration v and 858 mm in configuration vi.

The capillary was obtained from LC-Service (Emmen, The Netherlands). No window was burned off because only the electric current was measured. The capillary was conditioned by flushing with 1 M NaOH (5 min), water (15 min) and finally the test buffer (30 min) using a pressure of 2 bar. After the experiments the capillary was washed with water (5 min).

The test buffer (100 mM sodium chloride, 40 mM boric acid, pH=8.0) was filtered through a 0.2 µm filter, purchased from Schleicher & Schuell (Dassel, Germany). The voltage was increased every 2 min with 3 kV starting from 0 kV until the voltage was 30 kV. During the run the current was measured.

Conductivity measurements

A conductivity meter, model PW 9501/01, equipped with a conductivity electrode, type PW 9550/60 (both Philips, Eindhoven, The Netherlands) was used. The samples were thermostated as described above.

Results and discussion

Ohms law plots

Values of the electric current against the electric field applied in the different set-ups using temperatures of about 30 °C (except for the experiments performed at room temperature) are given in figure 3.1.1. It can be seen that the current depends on the set-up conceived. For fields below 10 kV/m the differences are rather small. This contrary to the experiments performed at higher fields, where it significantly depends on the set-up used.

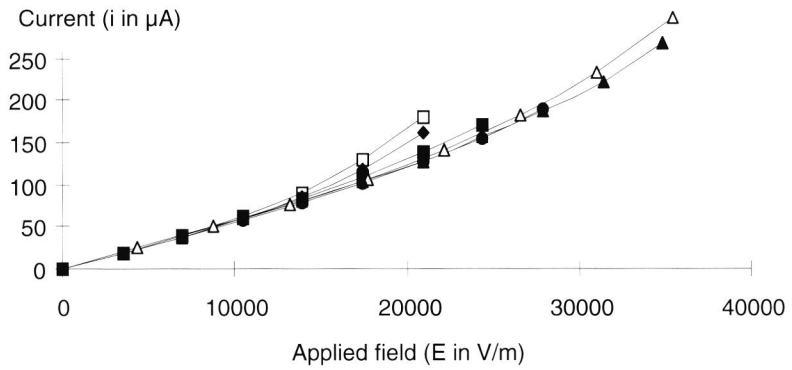


Figure 3.1.1 Ohm's plot for the configuration i (❑), ii (◆), iii (■), iv (●), v (Δ) and vi (▲). The set temperature was 21°C (i), 33.1°C (iii) and 30.0°C (ii, iv-vi).

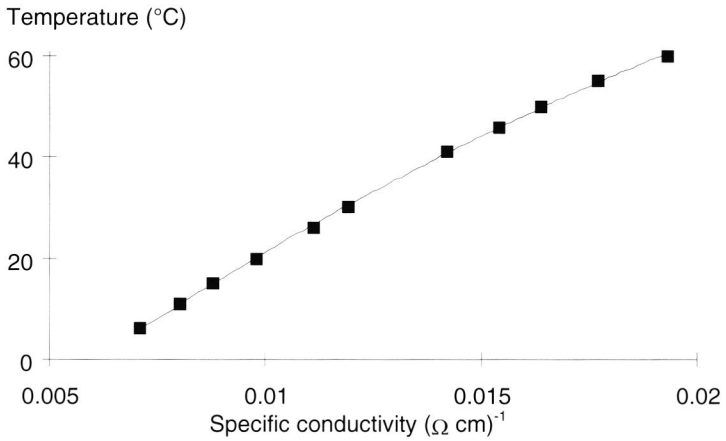


Figure 3.1.2 Plot of the temperature versus the specific conductivity measured with the conductivity electrode.

The highest currents are found for the natural convection device, despite the fact that for this system the T_{set} value is the lowest. With the fan temperature controlled capillary a smaller current is measured; while significantly lower currents are found for the high speed air and the liquid thermostated devices which indicates better thermostating capabilities.

Temperature-conductivity relationship

The relationship observed between the measured temperature and conductivity of the test buffer, is depicted in figure 3.1.2. The parameters of Eq. 1, calculated in the temperature range of 6 – 60 °C, were c = -37.834, d = 6777 and e = -87480. The maximum error between the calculated and measured temperatures is ± 0.4 °C in this range.

Calculated temperatures

As anticipated, the buffer temperatures calculated on the basis of the Ohms law (table 3.1.1), strongly depend on the generated power. This is shown in figure 3.1.2. These temperatures significantly change with the applied field. This is rather pronounced for configuration i where the temperature was set on 21°C and the actual temperature inside the capillary was about 60 °C when 4 W/m was generated. The buffer temperature of the fan thermostated device increased rather rapidly with the field. The other four devices behaved quite similarly. The temperature was set on 33.1 °C for configuration iii and 30.0 °C for the configurations ii and iv-vi.

Table 3.1.1 Comparison of the calculated temperatures according to the conductivity method and the method according to Hjerten (for details see text).

V	Current	*Tcal* (°C)	
(kV)	(mA)	Conductivity method	Hjerten method
0	0		
3	19	32.3	
6	39.1	34.1	33.4
9	62.4	37.7	35.8
12	91.1	43.2	39.7
15	128.5	50.9	45.4
18	180.6	61.1	53.9

The temperature can also be calculated according to Hjerten [21]. In order to do this the current should be measured immediately after applying the voltage and after equilibration of the system. The current just after the voltage has been applied is not known in this study, because the voltage is applied stepwise. This value can be estimated assuming that at a low voltage (i.e. 3 kV) Joule heating is minimal and extrapolation to a higher voltage, according to Ohms law, is allowed. In table 3.1.1 the the two calculated temperatures are compared. As can be seen, the temperature calculated with the procedure used in this study, is higher than calculated with the method proposed by Hjerten. This difference will be taken in account when comparing α values.

Temperature rise factor and temperature onset

The temperature of the CE buffer was calculated for several powers applied using the different CE systems as is shown in figure 3.1.3. From this figure the parameters δT and α can be derived as is explained above.

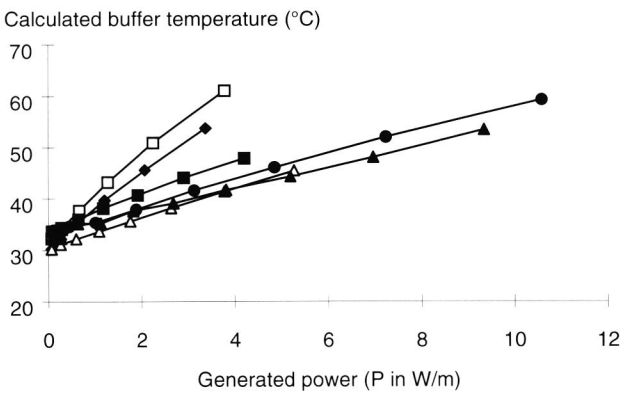


Figure 3.1.3 Calculated buffer temperatures in the capillary versus the generated power (symbols and temperatures as in figure 3.1.1).

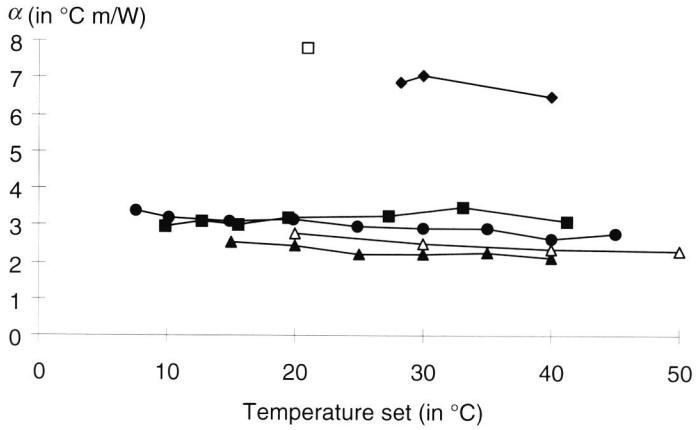


Figure 3.1.4 Temperature rise factors plotted against the temperature set (symbols as in figure 3.1.1).

The δT values mainly depend on T_{set}. The smaller the difference between T_{set} and room temperature the smaller δT as can be seen in figure 3.1.5. A value of T_{set} below the temperature in the CE capillary (which is somewhat higher than room temperature) results in a positive δT; the capillary is heated. A value of T_{set} higher than the temperature in the CE capillary results in a negative δT, the capillary is cooled by the surrounding medium. The smallest deviations of zero for δT are found for the commercial high-speed air thermostated configuration A (iv).

For the α values the following results are found: natural convection has the highest value, the values for fan thermostating system are slightly smaller and much lower values are found with forced air thermostated devices and the liquid thermostated device (figure 3.1.5). The difference between the three high speed gas thermostating

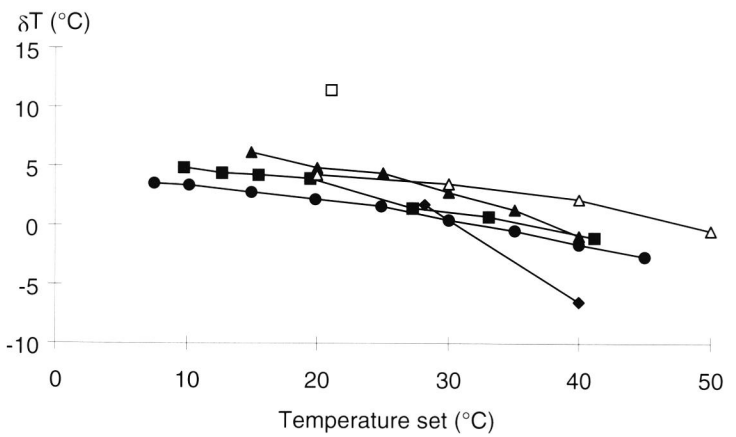


Figure 3.1.5 The temperature onset plotted against the temperature set (Symbols as in figure 3.1.1).

systems is larger than the difference between the high speed gas thermostating systems (iii–v) and the liquid thermostating device (vi). Because of this, it can be concluded that the α value is about the same for liquid and high-speed thermostating methods with a small benefit for the liquid thermostating method. Although liquid thermostating will become more efficient using a power higher than 6 W/m, this is not realistic because this will negatively influence peak widths in the electropherogram.

The thermostating potential is characterized by the combination of α and δT. When these two parameters are taken into account it is difficult to decide which one (liquid or high speed gas thermostating) is better. However, both are significantly better than the free convection and the fan thermostated devices.

Evaluation of the results

CE systems can be compared by using the temperature rise factors as shown in table 3.1.2. The results are calculated with data that can be found in the literature [8, 12, 16, 17]. The results of the present study are in agreement with those found in the literature, except for two α values which are greater than presented before [15]. Except these two, there is a constant factor (ca.1.5–2) between the literature data and

Table 3.1.2 Comparison of the determined temperature rise factors with the calculated ones from the literature.

Thermostating type	Source	average α (°C m/W)	Temperature (°C)
Natural convection	PS	7.8	21
	[17]	11.1	25
	[17]	12.5	25
	[17]	11	25
	[12]	12.4	?
	[21]	5.8	21
Fan thermostating	PS	6.8	28–40
	[8]	10	27
Forced air thermostating	PS	3.2	10–41
	PS	3.0	8–45
	PS	2.5	20–50
	[17]	5	25
	[17]	6	25
	[12]	5	?
Liquid thermostating	[15]	0.35	25
	PS	2.3	15–40
Solid state thermostating	[12]	0.6	?

PS = present study

the values presented above. The α value for the liquid thermostated system is a factor 7 higher than found in the literature [16]. The latter value is rather small, and implies an increase of only 6 °C when a power of 10 W/m is applied. This is unlikely given the data mentioned in figure 3.1.3.

The value for the non-thermostated device calculated according to Hjerten is smaller than the values found in this study and given in the literature. The most important conclusion is that accurate calculation of α and δT values, from existing data in the literature, is rather difficult because in most studies quite a number of assumptions have been made, this probably explains the differences found between the present and former studies.

Conclusions

The present method can be used to determine the buffer temperature in the capillary, even for experiments that have been performed in the past.

By using the temperature rise factor and temperature onset the thermostating quality of a CE device can be characterized in a simple way. From the results presented it can concluded that high speed air thermostating can be as efficient as liquid thermostating .

In addition it is shown that using the PrinCE with a high-speed air thermostating unit significantly improves its thermostating quality.

Acknowledgements

We want to acknowledge E. Majoor of Hewlett-Packard, C. Benoy of Bio-Rad, and W. Duinkerken of TSP for the use and their help of the HP3D CE, the BioFocus 3000 CE and SpectraPhoresis 2000 system, respectively.

References

[1] J.H. Knox, Chromatographia, 26 (1988) 329.

[2] J. Vindevogel, P. Sandra, "Introduction of micellar electrokinetic chromatography", Hüthig, Heidelberg, 1992.

[3] J.H. Knox, K.A. McCormack, Chromatographia, 38 (1994) 279.

[4] E. Grushka, R.M. McCormick, J.J. Kirkland, Anal. Chem., 61 (1989) 241.

[5] S. Hjerten, Chromatogr. Rev., 9 (1967) 122.

[6] J.P. Landers, R.P. Oda, B. Madden, T.P. Sismelich,T.C. Spelsberg, J. High Resolut. Chromatogr., 15 (1992) 517.

[7] B. Gas, J. Chromatogr., 644 (1993) 161.
[8] M.S. Bello, P. de Besi, P.G. Righetti, J. Chromatogr., 652 (1993) 317.
[9] S.L. Petersen, N.E. Ballou, Anal. Chem., 64 (1992) 1676.
[10] A. Vinther, H. Søeberg, J. Chromatogr., 559 (1991) 27.
[11] R.S. Rush, A.S. Cohen, B.L. Karger, Anal. Chem., 63 (1991) 1346.
[12] R.J. Nelson, A. Paulus, A.S. Cohen, A. Guttman, B.L. Karger, J. Chromatogr., 480 (1989) 111.
[13] D.N. Heiger, "High performance capillary electrophoresis - an introduction", Hewlett Packard (1992) 30.
[14] Y. Kurosu, K. Hibi, T. Sasaki, M. Saito, J. High Resolut. Chromatogr., 14 (1991) 200.
[15] A.E. Jones, E. Grushka, J. Chromatogr., 466 (1989) 219.
[16] H. Wätzig, Chromatographia, 33 (1992) 445.
[17] D.S. Burgi, K. Salomon, R.-L. Chien, J. Liq. Chromatogr., 14 (1991) 847.
[18] S.Terabe, K. Otsuka, T. Ando, Anal. Chem., 57 (1985) 834.
[19] P. Coufal, K. Stlík, H.A. Claessens, C.A. Cramers, J. High Resolut. Chromatogr., 17 (1994) 325.
[20] S. Hjerten, Electrophoresis, 11 (1990) 665.
[21] M.S, Bello, M. Chiari, M. Nesi, P.G. Righetti, M. Saracchi, J. Chromatogr., 625 (1992) 323.
[22] J.J. Lingane, "Electroanalytical chemistry", second edition revised and enlarged, Interscience publishers, London (1958).

3.2

On-line chromatographic pretreatment of samples with varying salt concentrations for capillary electrophoresis

Summary

Various methods are currently under investigation to improve concentration detection limits in capillary electrophoresis (CE). Stacking and isotachophoresis coupled in-line with free-solution electrophoresis are the techniques most frequently used. Samples containing a high and/or varying salt concentration, such as urine and serum, are difficult to handle: usually a significant loss in electrophoretic efficiency is observed compared with samples possessing a low electric conductivity.

In the present paper an alternative approach is developed, i.e. a liquid chromatographic (LC)-type of sample pretreatment is coupled on-line with CE. To demonstrate the feasibility of this approach, the separation of three model compounds (benzoates) in water containing up to 400 mM of sodium chloride is studied using a 50 mM borate CE buffer of pH 9.5.

The direct injection of samples with high salt concentrations in CE results in peak splitting and/or serious band broadening. These problems are not encountered when using the present LC–CE system. In addition, the detection limits are hardly influenced by the salt concentration of the sample; this underlines the robustness of the system.

Introduction

The applicability of capillary electrophoresis (CE) in biomedical analysis is mainly limited by two problems. One is related to the unfavourable concentration detection limits in CE compared with techniques like liquid chromatography (LC). This is due to the low sample loadability and, if optical detectors are used, to the small optical path length of the capillary which normally is used as the flow cell. The second problem is disturbance of the separation process caused by matrix compounds. Biomedical matrices like urine and plasma [1] usually contain variable and high salt concentrations, which severely reduce the robustness of the method. Thus, to extend the applicability of CE, attention should be devoted to the development of appropriate sample pretreatment procedures, such as special CE techniques (field-amplified polarity switching, stacking or in-line isotachophoresis–CE) or the at-line or on-line combination with solid-phase extraction (SPE), or alternatively, to the enhancement of detection sensitivity and/or selectivity.

Until recently, research has been mainly focused on the improvement of detection techniques, for instance by using (diode) laser-induced fluorescence [2–6], electrochemical [7] or chemiluminescence [8] detection instead of UV absorbance detection. Even though a significant increase in both sensitivity and selectivity can be obtained, an important limitation of such advanced detection methods, unfortunately, is that only a limited number of compounds show native fluorescence or are electrochemically active, while chemiluminescence is only suitable for an even more limited group of analytes. Of course, these problems can (partly) be solved by introducing chemical derivatization procedures either prior to [2], during [3] or after [4, 5] the separation or by using indirect detection methods [6]. However, when following one of these approaches new problems may be encountered because trace amounts of analytes often cannot be derivatized quantitatively and additional chemical noise will result in an increased background. As far as UV absorbance detection is concerned, the optical path length can be extended by using bubble [8], rectangular [9] and Z- or U-shaped cell [10] configurations. With the bubble cell, the signal-to-noise ratio can be increased by a factor of 5 when the proper geometry is applied. The rectangular cells undoubtedly have a higher potential, but they cannot easily be purchased and their installation in existing detection devices is rather difficult. The use of Z- and U-shaped cells can improve the signal-to-noise ratio up to 60-fold, but this is accompanied by a reduction of resolution [10].

The three main sample matrix problems are: (i) the presence of large particles which can clog the capillary, (ii) high electric conductivity because of a high salt concentration, which will cause band broadening and (iii) the presence of interfering matrix compounds (proteins) which can be bound to the capillary wall. Sample preparation procedures which can handle these problems include one or more of the following manipulations: centrifugation [11], SPE [11], addition of ion-exchange

material [12], dilution [12] and filtration [13]. All these steps are executed manually and off-line, which is time consuming and easily can decrease the reproducibility. Sample dilution will of course reduce the problem of salt interferences, but simultaneously increase the detection limit.

Another procedure that can be followed is the use of an in-line sample pretreatment procedure to improve the concentration detection limits and to remove interfering compounds, either by applying a chromatographic column to preconcentrate the analytes or by implementing sophisticated buffer/run programmes. The latter option can be divided into field-amplified polarity-switching [14], stacking [14] and in-line isotachophoretic (ITP)–CE [15] injection techniques. With these techniques, the concentration and separation is performed in a single capillary. They are suitable for samples with matrices containing small and constant amounts of salt. High salt concentrations result in band broadening and rather unfavourable detection limits. In the in-line micro-column approach, a small amount of packing material is brought into the CE capillary or, alternatively, a coated capillary is connected with the separation capillary [16–19]. Thus, in the ideal situation, the injected analytes are quantitatively concentrated. However, in the next step the analytes have to be desorbed in the separation capillary in a small volume (a few nanolitres) to prevent band broadening, which is rather difficult. Furthermore, matrix compounds such as proteins can be irreversibly bound to the capillary wall, which will detract the efficiency of the separation procedure.

The problems mentioned above can in principle be circumvented by using on-line techniques such as the coupling of ITP or LC with CE. In both cases two capillaries are coupled; the first capillary is used for the preconcentration process performed by ITP or LC, and the second one for the separation. Unfortunately, on-line ITP–CE cannot deal with varying and/or high salt concentrations [20–22]. On-line LC–CE combinations seem to be more promising [23–25]. The use of a rotary-type of injector [25], in which the preconcentration is performed off-line and the elution is executed in the capillary, is not particularly successful. The main problem is the same as encountered with in-line systems; desorption of the analytes from the small column in the CE system. Interestingly, Jorgensson et al. have described an on-line LC–CE system, which involved the combination of a reversed-phase (RP) [24] or size exclusion [25] column and a CE capillary. However, the main aim in that study was to develop a multi-dimensional separation system, to increase the separation power for relatively clean samples, i.e. tryptic digest of proteins.

In the present paper the feasibility of an on-line LC–CE system is explored with emphasis on sample handling, to remove interfering solutes, and so to improve the robustness of the analysis of samples with varying salt concentrations. Three benzoic acids were used as model compounds and sodium chloride was chosen as salt because it is present in high amounts in biological samples where salt concentrations typically are about 100 mM for plasma and 60–500 mM for urine depending on sex, person,

day and hour [1]. For a robust CE system the effect of a varying electric conductivity (simulated by a varying sodium chloride concentration) on the separation should be small.

Experimental

Materials

Acetonitrile (HPLC gradient grade) was purchased from Biosolve (Barneveld, The Netherlands), phosphoric acid, sodium hydroxide and boric acid from J.T. Baker (Deventer, The Netherlands), benzoic acid from Merck (Darmstadt, Germany), 2-chlorobenzoic acid from Acros Organics (Geel, Belgium) and 2,4-dichlorobenzoic acid from Janssen Chimica (Beerse, Belgium). Water was demineralized and distilled before use.

A fused silica capillary of 75 µm I.D. and 375 µm O.D. (Composite Metal Services, Hallow, UK) was conditioned by flushing with, subsequently, 1 M sodium hydroxide (5 min), water (15 min) and CE buffer (30 min) at a pressure of 2000 mbar.

Samples and buffers

Benzoic acid, 2-chlorobenzoic acid and 2,4-dichlorobenzoic acid with concentrations of 5.4, 3.1 and 2.4 mg/l, respectively, were dissolved in a 4 mM phosphoric acid solution containing 0 to 400 mM of sodium chloride. A 4 mM phosphoric solution was used as the LC loading solvent, the LC eluent was acetonitrile-7 mM phosphoric acid (40/60, v/v) and the CE buffer was a 50 mM borate buffer (pH 9.5).

CE analysis with direct injection

A PrinCE CE system (Lauerlabs, Emmen, The Netherlands) was equipped with an additional thermostating device to avoid Joule heating effects, set at 20 °C as described before [26]. The total length of the capillary was 86 cm and the injection detection length, 20 cm. A Model 795A UV absorption detector, (Applied Biosystems, Foster City, CA, USA) was used at 200 nm with a rise time of 0.5 s. The analyses were performed with the time schedule given in table 3.2.1.

Table 3.2.1 Time schedule for direct CE analysis of benzoic acids.

Step	Pressure (mbar)	Voltage (kV)	Time (min)	Vial	Description
1	2000	0	2.0	Buffer	Rinsing capillary
2	-10	0	inj. time sample	Sample	Injection
3	0	30	5.0	Buffer	CE run

CE analysis with LC pretreatment

The LC–CE system is schematically shown in figure 3.2.1. The LC part contains three valves: V1 is a manual six-port valve, V2 and V3 are automated six-port valves (Must, Spark Holland, Emmen, The Netherlands). The system was fully automated and valves V2 and V3 were controlled by the PrinCE CE system. The eluent and the loading solvent were delivered by two Model 2150 pumps (LKB, Bromma, Sweden) and the CE buffer by a Model 305 pump equipped with a Module 805 manometer (both from Gilson Medical Electronics, Villiers-le-Bel, France). The flow rate was 0.5 ml/min for all three pumps. A 100 x 4.6 mm I.D. stainless-steel LC column slurry-packed with 5 µm ODS-Hypersil (Shandon, Runcorn, UK) was used to pretreat the samples: V1 controlled the injection of the sample on the LC column and was equipped with a 434 µl loop, V2 was used for the elution of the compounds retained on the column and V3 for the transfer of either the CE buffer or the LC eluent to the interface.

The LC and the CE system were connected via a home-made interface, which is depicted in the insert of figure 3.1.1. It was constructed from 7 mm of green PEEK tubing (0.75 mm I.D., 1/16" O.D.) positioned above a 30 cm piece of long red PEEK tubing (0.13 mm I.D., 1/16" O.D.) which were both placed inside a piece of PTFE tubing with a length of 37 mm (1/16" I.D., 1/8" O.D.). 18 mm of the red PEEK tubing were inserted in the PTFE tubing, while its other end was connected with valve V3. The CE capillary was inserted in, and the electrode on top of, the green PEEK tubing (see insert of figure 3.2.1). The injection of liquid from the interface into the CE capillary was performed by applying a pressure of -10 mbar at the other end of the capillary (by the PrinCE). The eluent or CE buffer only entered the capillary when at least some pressure was applied by the CE system. Only part of the LC eluate was introduced and the remaining liquid was flushed to waste (excess of eluent or CE buffer overflows the interface). A detailed schedule for the analysis is given in table 3.2.2.

Table 3.2.2 Time schedule for the LC–CE system for the analysis of benzoic acids.

Step	Pressure (mbar)	Applied voltage (kV)	Valve position* V1	V2	V3	Time (min)	Description
1	0	0	1	0	0	1.0	Inj. sample on column
2	2000	0	0	1	0	2.0	Rinsing capillary/column
3	0	0	0	0	0	0.2	Start elution from column
4	-10	0	0	0	0	1.0	Injection on capillary
5	0	0	0	0	1	1.5	Rinsing interface
6	0	-30	0	0	1	5.0	CE analysis

* For the valve positions, see figure 3.2.1.

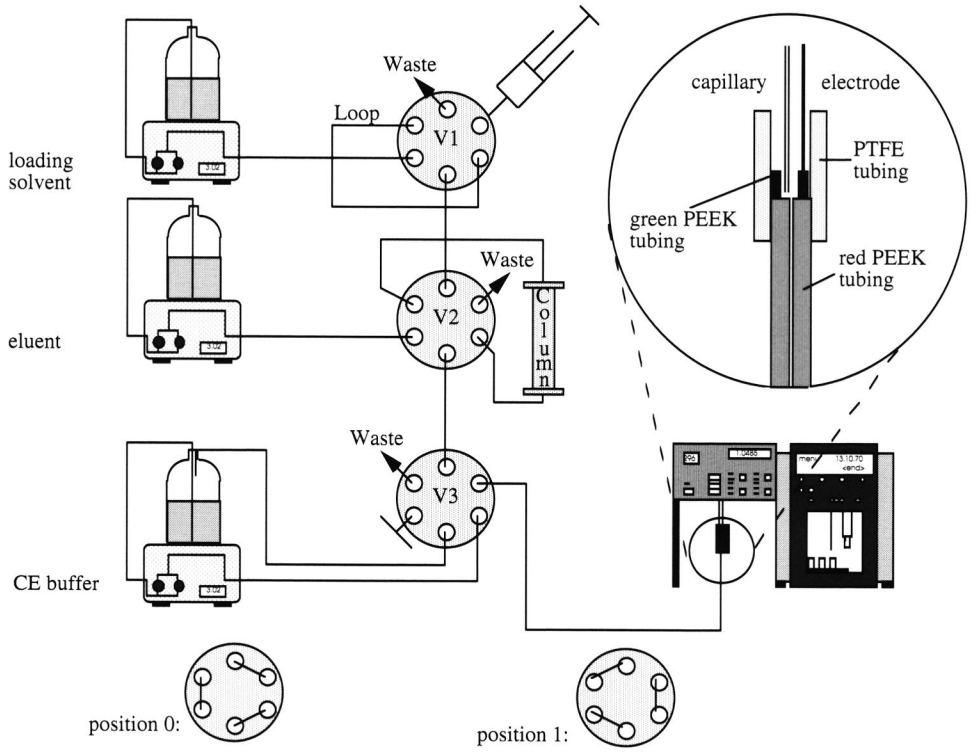


Figure 3.2.1 Schematical representation of the LC–CE configuration. For details, see text.

Results and Discussion

The migration order for the buffer chosen was 2,4-dichlorobenzoic acid (140 s), chlorobenzoic acid (155 s) and benzoic acid (165 s), while the electric-osmotic flow (EOF) peak was observed at 85 s. This migration order is to be expected on the basis of the charge/size ratios of the three analytes at the pH used.

In a first set of experiments the samples containing different salt concentrations were analysed by CE using direct injection, this to study the effect of the salt concentration on the number of theoretical plates and analyte detectability. Next, the on-line LC pretreatment was introduced to improve the robustness of the CE system. The breakthrough volumes of the analytes on the LC column as well as the corresponding elution profiles were determined. The elution profile was used to enable a proper timing of the underpressure during injection of the sample. Finally the samples were analysed with the optimized system and the number of theoretical plates and the detection limits obtained were compared with those of the direct injection system.

Direct injection

The influence of the electrical conductance on the peak shape was studied by direct injection of different volumes of the benzoate solutions. Three injection times were arbitrarily selected corresponding with 60, 180 and 600 mbar.s. Figure 3.2.2A shows typical electropherograms for the benzoic acids at different salt concentrations. As can be seen, the peak shapes are completely disturbed for samples containing 100 mM of salt. Negative effects start to occur at about 10 mM. The explanation is that when the salt concentration is too high, compared with that of the CE buffer, the electric field over the sample plug is too low. As a result, the analyte ions in the sample plug migrate significantly slower than the analyte ions in the buffer solution. The velocity of the fraction of the analyte molecules, which has escaped from the sample plug, is higher than of those in the sample plug. These observations are in agreement with data described in the literature where the conductivity of the sample plug is high due to a high analyte concentration compared with the buffer conductivity [27].

The influence of the sodium chloride concentration and the injection volume on the number of theoretical plates is shown in figure 3.2.3. Salt concentrations higher than 40 mM were not taken into consideration because of the peak disturbances mentioned above. Two effects are obvious. Firstly, the larger the injection volume i.e. the higher the injection pressure, the lower is the number of theoretical plates. Apparently, the focusing effect is not efficient enough to reconcentrate the injection plugs to the same peak width as obtained with a small injection volume. Secondly, there is a distinct influence of the amount of salt in the injection plug on the number of theoretical plates. Though the relationships are complicated and different for the three analytes concerned, the general trend is that, especially for the large injection volumes, an increase of the salt concentration causes a decrease of the number of theoretical plates. This phenomenon can be attributed to stacking effects. At low salt concentrations the field over the sample plug is higher than the field over the buffer so that the migration rates in the sample plug are higher than in the sample plug, consequently the peak widths are reduced due to the stacking effect. If the conductance within the sample plug is increased by the sodium chloride ions, the peak sharpening effect is less effective. This is in line with the observation that the influence of the sodium chloride concentration is greater for larger sample plugs.

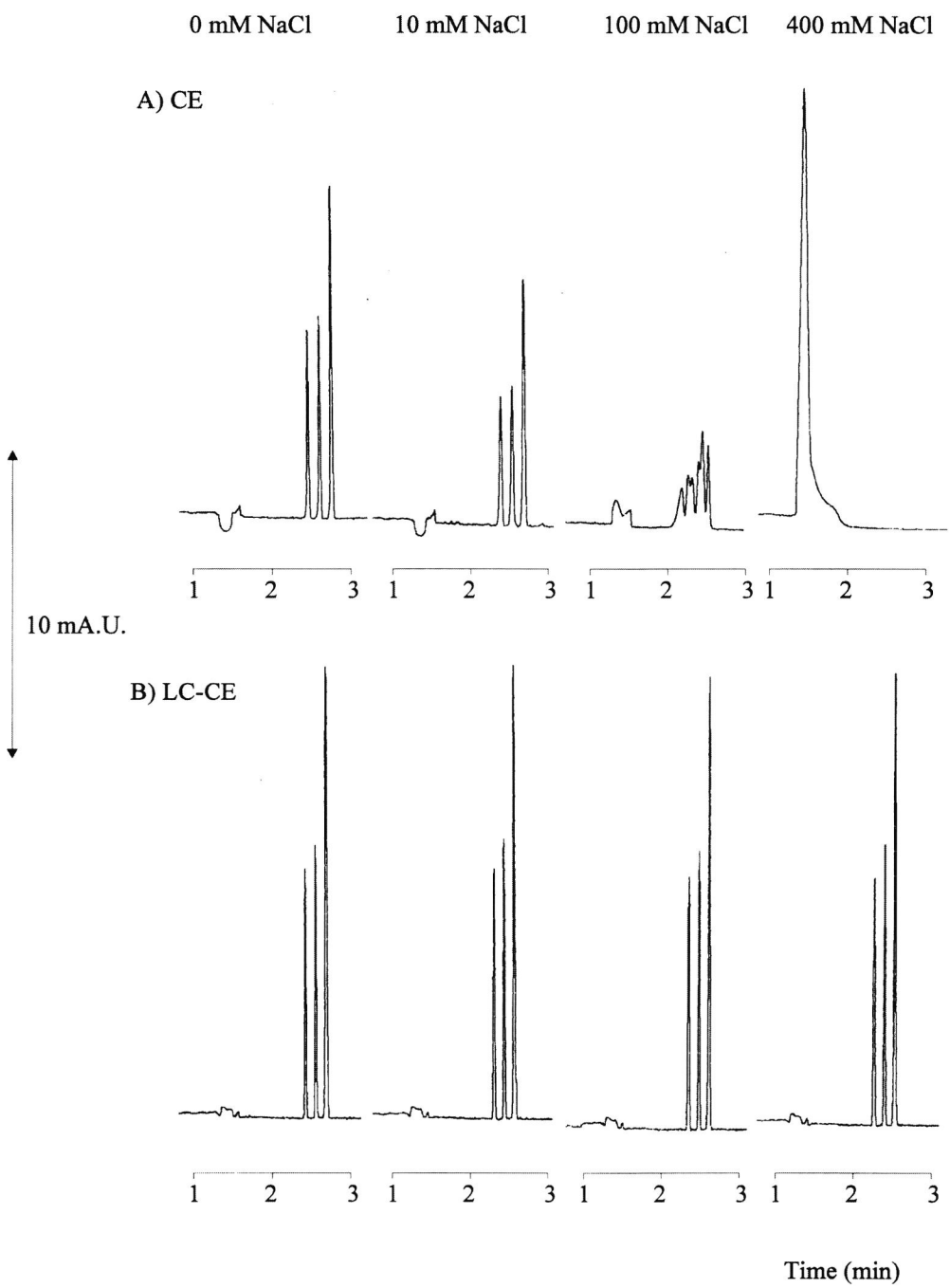


Figure 3.2.2 Electropherograms of a mixture of 2,4-dichlorobenzoic acid, 2-chlorobenzoic acid and benzoic acid at four different sodium chloride concentrations, analysed with (A) the direct CE-system and (B) the LC–CE system.

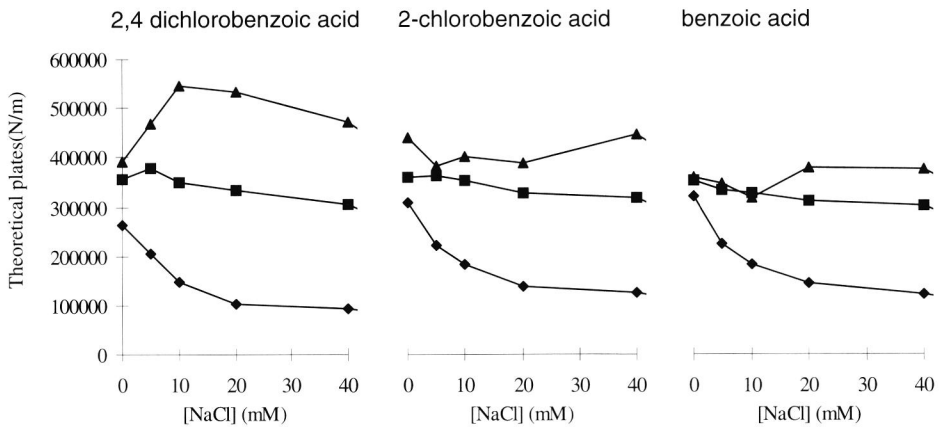


Figure 3.2.3 Dependence of the number of theoretical plates of the benzoic acids on the sodium chloride concentration in direct CE. Amounts injected: 60 mbar.s (triangles), 180 mbar.s (boxes) and 600 mbar.s (diamonds).

Detection limits as a function of the salt concentration for different injection volumes are shown in figure 3.2.4. As expected, an increase of the injection time results in a decrease of the detection limits. The influence of the salt concentration is most pronounced for the smallest injection plugs. The influence of the salt concentration is not very pronounced: irrespective of the injection time and, there is a less than 2-fold difference over the range 5–40 mM sodium chloride.

On the basis of the above results, the most important conclusion is that the salt concentration has a distinct influence on the peak shapes of the analytes. Samples with a salt concentration of over 40 mM cannot be analysed using direct injections. At lower sodium chloride concentrations, direct injection can be performed, but even

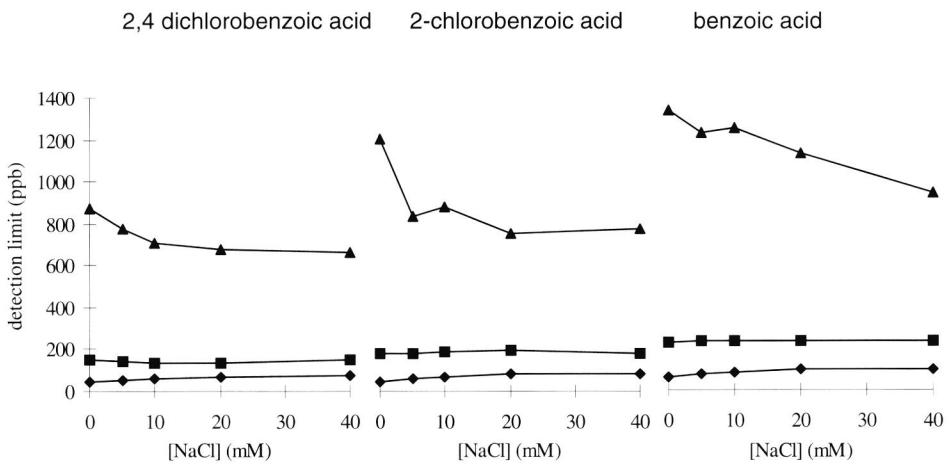


Figure 3.2.4 Dependence of the detection limits of the benzoic acids on the salt concentrations in direct CE. Amounts injected: 60 mbar.s (triangles), 180 mbar.s (boxes) and 600 mbar.s (diamonds).

then there is a concentration dependence of the peak shapes. In other words, the electrophoretic performance depends on the sample matrix. Furthermore, large injection plugs result in low detection limits but also in low efficiencies. For small plugs the reverse is observed. In other words, with direct CE analysis, unfortunately, a compromise always has to be made between narrow peaks and low detection limits.

LC pretreatment

In the coupled LC–CE system, the analytes have to be trapped on the LC column. Therefore, the breakthrough volumes of the benzoic acids have to be determined and their elution profiles should be recorded in order to programme exactly the injection of benzoic acids onto the CE capillary. In fact, the internal volume of the green PEEK tubing in the interface should be considered as a buffer or sample vial with a rather small volume (3 µl), which minimizes liquid mixing effects and allows the liquid to flow through the tubing without pressure build-up. Finally, because of the limited injection volume which can be handled in CE, the maximum underpressure should be determined.

Breakthrough volumes were measured according to Nielen et al. [28]. For the C18 column and with 4 mM phosphoric acid as the sample solvent, the breakthrough volumes were over 10 ml for the three benzoic acids. The elution profile for the mixture of the benzoic acids in water recorded by a UV absorbance detector is shown as trace A in figure 3.2.5, while trace B represents the blank. The first two minutes were used to flush the loop and to trap the compounds on the LC column and to wash the salts from the column. Then, elution with a 40% acetonitrile solution was started by switching valve V2 (figure 3.2.1). As can be seen from trace A in Figure 3.2.5, the elution starts 0.2 min after switching valve V2 and takes ca. 1 min. The elution profiles were identical irrespective of the salt concentration of the sample. Identification of the two peaks was performed by injecting the analytes separately. The first main peak results from co-eluting benzoic acid and 2-chlorobenzoic acid, and the second one is due to 2,4-dichlorobenzoic acid.

From the above results it can be concluded that the injection onto the CE capillary has to be performed by applying an underpressure of -10 mbar between 0.2–1.2 min after switching valve V2. From the eluted plug a volume of 53 nl was injected in the capillary during this minute. Next, the interface was flushed with CE-buffer and, subsequently, the CE analysis was started. Using this procedure, electropherograms such as shown in figure 3.2.2B were recorded. In other words, it is clear that when using the LC–CE system the peak shape of the analytes are the same for all salt concentrations, which is a distinct improvement compared with the results obtained by direct injection (figure 3.2.2A). Apparently, the sample cleaning effected on the LC column is considerable.

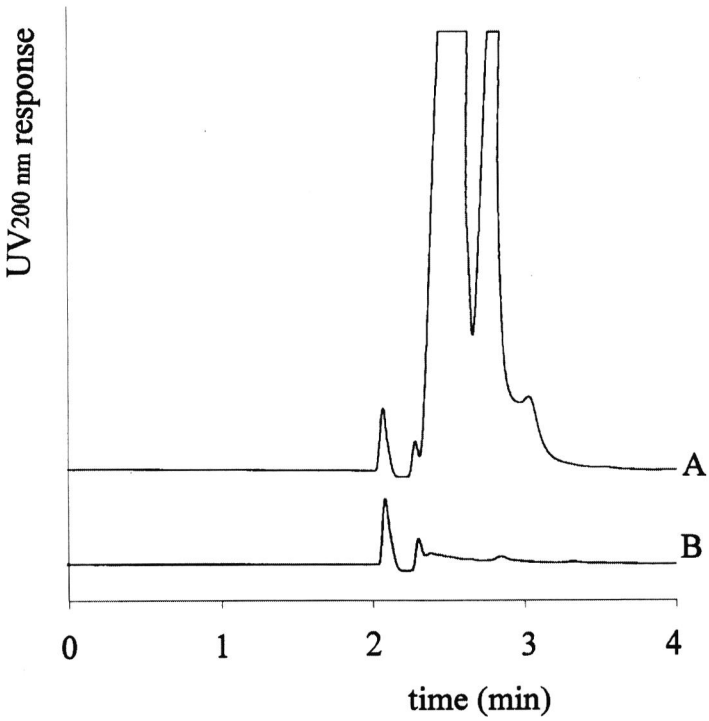


Figure 3.2.5 Elution profile of the LC column loaded with (A) benzoic acids and (B) blank with elution buffer.

The dependence of the number of theoretical plates on the salt concentration of the sample in LC–CE, is shown in figure 3.2.6. The volume of the sample injected after pretreatment with LC, was the same as with direct CE analysis using 600 mbar.s. The number of theoretical plates is seen to be constant over the whole salt concentration range studied, which is in sharp contrast with the results of direct CE analysis. In addition, the plate numbers achieved in LC–CE are similar to the maximum values obtained for direct CE analysis. A similar conclusion holds true for the detection limits (figure 3.2.6): in LC–CE they are the same as those found for direct injection at 600 mbar.s.

In preliminary experiments the linearity was tested for benzoic acid (0.1–10 mg/l) using 5 samples in duplicate. The R^2 was at least 0.99.

It should be noted that under the experimental conditions applied, the plug eluting from the LC column has about the same size as the sample which is introduced onto the LC column, 434 µl vs 500 µl. This implies that the sample is hardly diluted during the pretreatment process. Since, in principle, much larger sample volumes can be introduced on the LC column, it will be possible to improve the detection limits in the future.

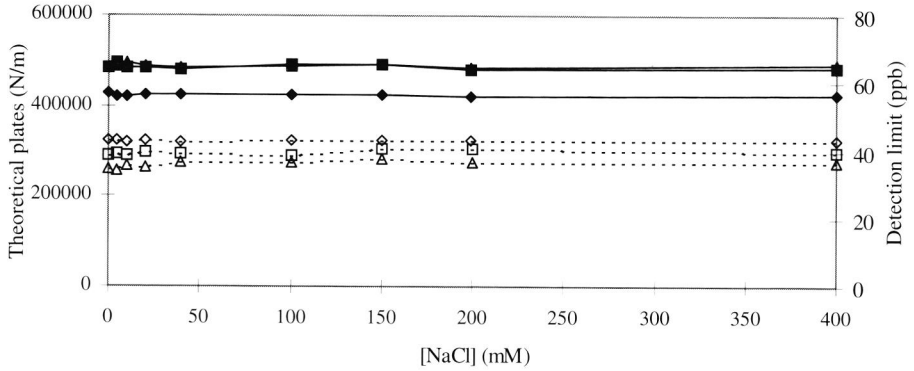


Figure 3.2.6 Dependence of the number of theoretical plates (solid lines) and detection limits (dashed lines) of 2,4-dichlorobenzoic acid (triangles), 2-chlorobenzoic acid (boxes) and benzoic acid (diamonds) on salt concentration in LC-CE.

Table 3.2.3 Comparison of peak areas, migration times and relative standard deviations of the three benzoic acids with direct CE and LC–CE*.

Compound	Method of analysis	Injection volume (mbar.s)	Area (mV.s) (RSD)	Migration time (s) (RSD)
2,4-Dichlorobenzoic acid	Direct CE	60	0.5 (6)	148 (0.7)
		180	2.9 (4)	153 (0.5)
		600	10.7 (4)	141 (1.2)
	LC–CE	600	9.0 (3)	143 (0.2)
2-Chlorobenzoic acid	Direct CE	60	0.6 (19)	160 (0.6)
		180	3.1 (4)	161 (0.9)
		600	11.0 (4)	154 (1.3)
	LC–CE	600	11.4 (2)	152 (0.3)
Benzoic acid	Direct CE	60	1.0 (12)	171 (0.3)
		180	5.1 (3)	170 (0.7)
		600	18.3 (4)	164 (0.9)
	LC–CE	600	20.6 (0.7)	159 (0.4)

*For the direct CE only the samples containing less than 40 mM sodium chloride were considered (n=5), whereas for LC–CE all the experimental results were taken into account (n=9).

Finally, the performance of the LC–CE and direct CE systems was compared by determining the standard deviations of the peak areas and migration times of the three analytes at various salt concentrations. The data are collected in table 3.2.3. For reasons given above, for direct CE only the five samples containing the lowest salt concentrations were considered, whereas for the LC–CE method all nine samples were included. It is evident that the relative standard deviations of the migration times and of the peak areas are smaller in LC–CE than in direct CE. Or, in other words, in contrast with direct CE, the results obtained with LC–CE are independent of the salt concentration.

Conclusions

On-line LC–CE can be applied successfully to the analysis of samples with high and/or varying salt concentrations. In principle, the system can also be used for sample preconcentration by loading larger sample volumes on the LC column. Furthermore, it seems possible to achieve full automation without the problem of significantly longer analyses times, because the sample pretreatment of the $(n+1)^{th}$ and the CE analysis of the n^{th} sample can be performed simultaneously.

Of course, instead of the reversed-phase column used in the present set-up, other types such as an ion-exchange or a size-exclusion column, can also be coupled to the CE capillary. In other words, the on-line LC–CE combination can probably be used for a wider variety of analytes.

In addition, a sample pretreatment procedure such as dialysis can be included in the system in prior to the LC separation to allow an even more efficient clean-up. Current research is directed at the development of a dialysis–LC–CE system and a SPE–CE system, using disposable cartridges. Both systems will be fully automated and on-line. The systems will be tested with pharmaceuticals in biological matrices.

The present set-up is mainly used for desalting and removal of organic interferences. In the near future, a preconcentration step will be incorporated by decreasing the desorption volume of the LC column by using, for example, particle-loaded membranes.

References

[1] K. Diem, C. Lentner, "Scientific tables", Ciba-Geigy, Basle, Switzerland, pp 561 and 663.

[2] A.J.G. Mank, H. Lingeman, C. Gooijer, Trends Anal. Chem., 11 (1992) 210.

[3] B.D. Clark, M.J. Sepaniak, J. Microcol. Sep., 5 (1993) 275.

[4] S.D. Gilman, J.J. Pietron, A.G. Ewing, J. Microcol. Sep., 6 (1994) 373.

[5] B. Nickerson, J.W. Jorgenson, J. Chromatogr., 480 (1989) 157.

[6] W.G. Kuhr, E.S. Yeung, Anal. Chem., 60 (1988) 1832.

[7] I-C. Chen, C.-W. Whang, J. Chromatogr., 644 (1993) 208.

[8] D.N. Heiger, P. Kaltenbach, H.-J. P. Sievert, Electrophoresis, 15 (1994) 1234.

[9] T. Tsuda, J.V. Sweedler, R.N. Zare, Anal. Chem., 64 (1992) 2149.

[10] S.E. Morning, R.T. Reel, R.E.J. van Soest, Anal. Chem., 65 (1993) 3454.

[11] M. Shirao, R. Furuta, S. Suzuki, H. Nakazawa, S. Fujita, T. Maruyama, J. Chromatogr., 680 (1994) 247.

[12] M.A. Jenkins, T.D. O'Leary, M.D. Guerin, J. Chromatogr B., 662 (1994) 108.

[13] T. Nakagawa, Y. Oda, A. Shibukawa, H. Fukud, H. Tanaka, Chem. Pharm. Bull., 37 (1989) 707.

[14] R.L. Chien, J.C. Helmer, Anal. Chem., 63 (1991) 1354.

[15] C. Schwer, LC-GC Int., 6 (1993) 630.

[16] M.A. Strausback, B.J. Madden, P.J. Wttstein, J.P. Landers, Electrophoresis, 16 (1995) 534.

[17] M.E. Schwarz, M. Merion, J. Chromatogr., 632 (1993) 209.

[18] A.M. Hoyt, S.C. Beale, J.P. Larmann, J.W. Jorgenson, J. Microcol. Sep., 5 (1993) 325.

[19] J.H. Beattie, R. Self, M.P. Richards, Electrophoresis, 16 (1995) 322.

[20] D. Kaniansky, J. Marák, J. Chromatogr., 498 (1990) 191.

[21] D. Kaniansky, J. Marák, V. Madajová, J. Chromatogr., 638 (1993) 137.

[22] T. Hirowaka, A. Ohmori, Y. Kiso, J. Chromatogr., 634 (1993) 101.

[23] A.J.J. Debets, M. Mazereeuw, W.H. Voogt, D.J. van Iperen, H. Lingeman, K.-P. Hupe, U.A.Th. Brinkman, J. Chromatogr., 608 (1992) 151.

[24] J.P. Larmann, A.V. Lemmo, A.W. Moore, J.W. Jorgenson, Electrophoresis, 14 (1993) 439.

[25] A.V. Lemmo, J.W. Jorgenson, J. Chromatogr. 633 (1993) 213.

[26] J.R. Veraart, H. Lingeman, C. Gooijer, Biomed. Chromatogr., 9 (1995) 271.

[27] S.V. Ermakov, M.Y. Zhukov, L. Capelli, P.G. Righetti, Anal. Chem, 67 (1995) 2957.

[28] M.W.F. Nielen, R.W. Frei, U.A.Th. Brinkman in R.W. Frei, K. Zech (editors), "Selective sample handling and detection in liquid chromatography", Part A, Elsevier, Amsterdam, The Netherlands (1988).

Chapter 4

Solid-phase Extraction Coupled at-line to Capillary Electrophoresis

4.1

At-line solid-phase extraction for capillary electrophoresis: application to negatively charged solutes

Summary

The analysis of complex biological samples with capillary electrophoresis (CE) requires proper sample pretreatment. In this paper the applicability of solid-phase extraction (SPE) coupled at-line with CE is studied, by using a home-made interface. A fresh (disposable) SPE cartridge is used for each sample to prevent carry-over effects. The sample handling procedure is performed parallel with the analysis of the previous sample, to improve sample throughput. Using this set-up, negatively charged test compounds (some non-steroid anti-inflammatory drugs) can be determined in serum and urine. The method is linear over at least two decades and detection limits are around 40 μg/l.

A single capillary, flushed only once a week with a sodium hydroxide solution, was used without problems for the analysis of ca. 900 samples during one year. The robustness of the system was very good: no blocking of loop, interface or capillary was found during this period. Furthermore, the system was successfully used for overnight runs.

Introduction

Because of its high efficiency, capillary electrophoresis (CE) is very suitable for the analysis of complex biological samples. There are, however, several distinct problems to be solved. Large amounts of salts, as present in urine (50–500 mM of sodium chloride) and serum (ca. 150 mM of sodium chloride), will disturb the analysis because of the high electrical conductivity of the sample which may result in peak splitting or even in a complete loss of resolution [1]. Furthermore, proteins, as present in serum (ca. 75 g/l), can bind irreversibly to the silanols of the capillary wall, which can cause dramatic changes of the migration times. Proteins can also bind with drug molecules and therefore reduce their recovery. Finally, particulate matter can cause clogging of the CE capillary. In addition, it should be noted that the analyte concentrations usually dealt with, are rather low compared with the detectability that can be obtained in CE. Obviously, the development of appropriate sample treatment procedures for biological matrices is urgently required.

Most sample treatment procedures available today can be performed either manually (off-line) or in an automated fashion (at-line or on-line). If large numbers of samples have to be analysed, the latter alternative is to be preferred. Sample dilution [2] (to lower the salt concentration), filtration [3] and centrifugation [4] (to remove particulate matter), liquid–liquid extraction (LLE) [5, 6] (to remove salts and other interferences) and the addition of acetonitrile [6] (to precipitate proteins) are generally carried out off-line. Isotachophoresis is one of the electrophoretic techniques considered for sample concentration [7, 8]. Unfortunately, it can only be used for samples with a stable matrix.

Solid-phase extraction (SPE) is a more promising option. Two different modes for the coupling of SPE and CE have been described in the literature, the off-line use of disposable cartridges [4, 9, 10] and the use of an in-line (in-capillary) microcolumn [11, 12] or particle-loaded membranes [13, 14]. Off-line disposable SPE cartridges do not create any technical problems. However, the inherent disadvantages that it requires much time and is a rather laborious technique, are well-known. In-line SPE–CE systems can be fully automated, but also have some disadvantages. Desorption of the analytes as a narrow plug is difficult, the sample may contaminate the capillary or the SPE device, and finally the choice of CE buffer is limited. And furthermore, only relatively clean samples (no proteins or particulate matter and low concentration of interferences) can be analysed. Therefore, urine and serum are pretreated with off-line sample preparation methods, such as SPE, LLE or precipitation.

The purpose of the present study is to develop a fully automated sample treatment procedure coupled at-line with CE, which is able to analyse urine and serum directly without additional sample pretreatment, to overcome the above problems. In an at-line system the SPE unit is connected with the CE unit by means of a home-made interface, which was also used for the coupling of an LC system with a CE unit [1].

Non-steroid anti-inflammatory drugs (NSAIDs) were used as test compounds and urine and serum as biological matrices. NSAIDs are weak acids with pKa values of about 4.5 [15]. The NSAIDs were selected because, so far, mainly manual pretreatment procedures were used for their CE determination in biological fluids.

Experimental

Materials

Ibuprofen, ketoprofen, naproxen and flurbiprofen were purchased from Sigma (St. Louis, MO, USA), acetic acid (>99.8%) and sodium acetate came from Riedel-de Haën (Seelze, Germany) and formamide (99.8%), methanol (99.8%) and acetonitrile (>99%) were from J.T. Baker (Deventer, The Netherlands). Water was demineralized and distilled before use.

Samples

Urine was collected from five healthy volunteers on three subsequent days. The samples were pooled and divided into 100-ml portions and frozen at –18 °C. Bovine serum of untreated objects was purchased from Sigma; it was divided into 10-ml portions and frozen at –18 °C. Urine and serum samples were stored at –18 °C for, at maximum, six months.

Off-line CE

Optimization of the CE buffer conditions, which led to using a 20 mM acetate CE buffer (pH 4–5), was performed using a HPCE system (Hewlett Packard, Waldbronn, Germany) with a capillary (Hewlett-Packard) of 48.5 cm in length (effective length, 40 cm), an I.D. of 50 μm and an O.D. of 375 μm. The capillary was conditioned by flushing (at 900 mbar) with, successively, 1 M sodium hydroxide aqueous (5 min), water (15 min) and CE buffer (30 min). Before each analysis, the CE buffer in the electrophoresis vials was replaced and the capillary was flushed with CE buffer for 2 min. Sample injection was performed for 20 s at a pressure of 10 mbar. Electrophoresis was performed by applying a voltage of -30 kV for 10 min. The CE runs were performed at 20 °C, using absorbance UV detection at 200 nm (band width, 10 nm).

At-line SPE–CE

At-line SPE–CE experiments were performed using an automated SPE (ASPEC XL) system (Gilson, Villiers-le-Bel, France) which automates all solvent handling procedures. The desorption liquid from the SPE cartridge was injected into a

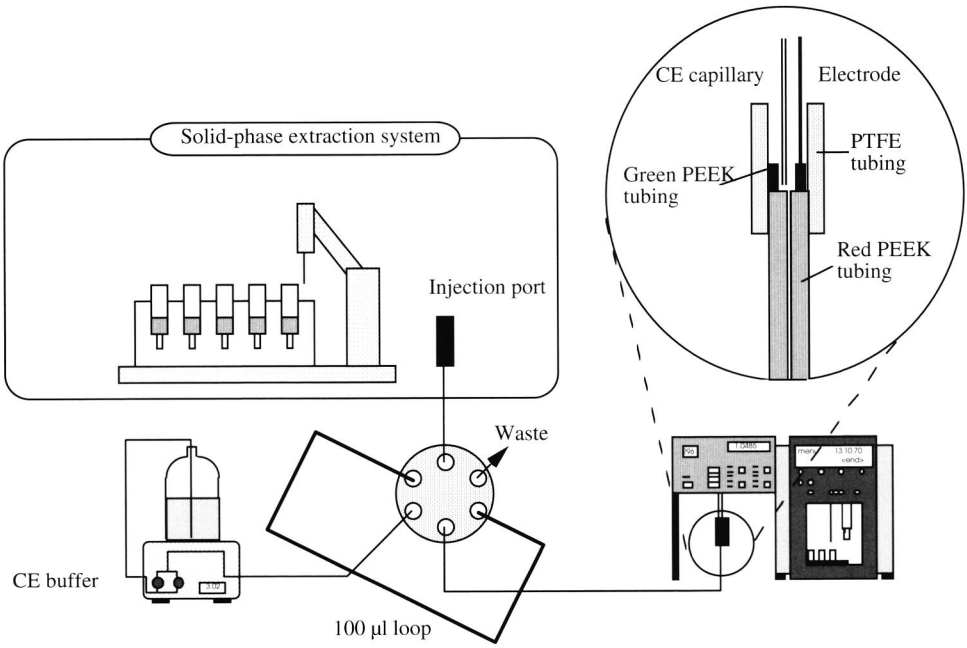


Figure 4.1.1 At-line SPE–CE configuration. For more details, see text.

100 µl loop via the injection port, which was connected to the six-port valve of the ASPEC system. The six-port valve was connected with the interface as shown in figure 4.1.1; it has been discussed in detail before [1]. The interface constructed from 7 mm of green PEEK tubing (0.75 mm I.D., 1/16" O.D.) was positioned on top of 30 cm of red PEEK tubing (0.13 mm I.D., 1/16" O.D.). Both pieces of PEEK were placed inside a piece of PTFE tubing with a length of 37 mm (1/16" I.D., 1/8" O.D.). The red PEEK tubing was inserted into the PTFE tubing over a length of 18 mm, while the other end was connected to the valve. The CE capillary was inserted into, and the electrode placed on top of, the green PEEK tubing (see insert of figure 4.1.1).

The CE part of the SPE–CE experiments was performed using a PrinCE CE system (Prince Technologies, Emmen, The Netherlands). After completing the SPE procedure by the ASPEC, an 'SPE ready' signal is sent to the PrinCE system, while the ASPEC waits in its turn for the 'CE ready' signal. In the next step, the six-port valve is switched and the content of the loop is flushed to the interface by the CE buffer. When the effluent plug containing the analytes, passes the tip of the capillary (between 0.2 and 0.5 min after switching of the valve), injection is performed by applying an underpressure of -40 mbar at the other end of the capillary. It should be realized that only a minor part of the desorbed SPE effluent is introduced into the CE capillary; by far the largest fraction is flushed to waste. The CE capillary (50 µm I.D., 375 µm O.D.), total length of 96 cm and effective length of 40 cm (LC-service, Emmen, The Netherlands), was equipped with a thermostating device, set at 20 °C,

[16] to avoid Joule heating effects. The capillary was conditioned at a pressure of 2000 mbar by means of the same procedure as described for the HPCE system. Before injection, the capillary was flushed with the optimized CE buffer. The analysis was performed using a voltage of -30 kV (the electrode in the interface was grounded) and detection was performed using a Model 759A UV/VIS detector (Applied Biosystems, Foster City, CA, USA), set at 200 nm.

SPE procedure

Disposable LC-18 cartridges (Supelco, Bellefonte, PA, USA), packed with 100 mg of C-18-bonded silica, were conditioned with 2 ml of methanol and, subsequently, 2 ml of 10 mM phosphate buffer (pH 2). Next, the cartridges were loaded with urine (8 ml) or serum (1 ml). In the latter case 1 vol. % of formic acid was added to release the drugs from the proteins. Then for the urine samples the cartridges were washed with 3 ml of wash buffer (10 mM phosphate buffer (pH 4.5)/acetonitrile – 80/20, v/v); with serum, the cartridges were washed first with 1 ml of 10 mM phosphate buffer (pH 2), followed by 3 ml of wash buffer and, again, 1 ml of 10 mM phosphate buffer (pH 2). Finally, the analytes were desorbed with 400 µl of desorption solvent (10 mM phosphate buffer (pH 7.8)/acetonitrile – 25/75, v/v).

Results and Discussion

In the present study, after optimization of the CE separation of the test compounds, the SPE procedure was optimized and, next, the conditions for the at-line SPE–CE coupling were determined. Finally, the total SPE–CE set-up was used for the analysis of biological samples.

Optimization of CE analysis

However, the CE analysis of the test compounds was optimized again [17–21], because a buffer system without micelles and chiral additives had to be developed to avoid interfacing disturbances. Analyte separation in CE is based on differences in effective mobilities, which mainly depend on the size/charge ratios. All test compounds (pKa around 4.5) have about the same size but, at pH values of 4 – 5, their charges are slightly different. The resolution data of figure 4.1.2 indeed show that optimum conditions are obtained at a pH of 4.5 – 4.7. A pH value of 4.6 was chosen for all further experiments. The resolution is less at lower pH values because of decreasing charge of all analytes which results in smaller differences of the charge/size ratios.

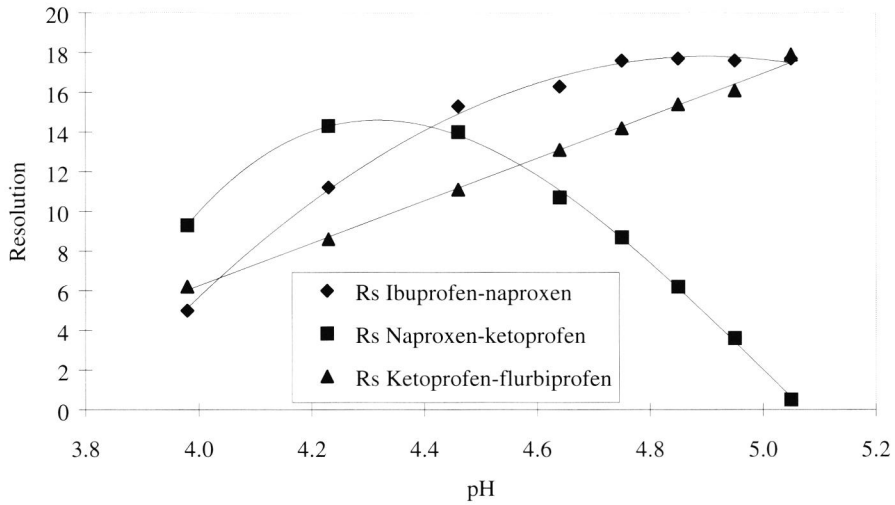


Figure 4.1.2 Dependence of NSAID resolution on pH of CE buffer.

Interfacing SPE with CE

The SPE module, connected to the CE by means of the PEEK-tubing interface (figure 4.1.1) allows a continuous flow of CE buffer through the interface. At the end of the SPE procedure the analytes are desorbed and the solvent is flushed through the interface via a loop. The dimensions of the loop, the loop filling and injection of the sample into the CE capillary had to be optimized.

Loop dimensions.

Since the loop is filled by flushing it with solvent and the loop content is subsequently flushed to the interface, mixing effects may occur which will depend on the loop diameter and volume applied. Loops with a volume of 100 µl (constructed of PEEK tubing) and internal diameters of 0.25, 0.50 and 0.75 mm were used. Since the syringe pump of the ASPEC can only handle pressures less than 4 bar, diameters smaller than 0.25 mm cannot be used because of pressure build-up or unacceptable washing and loading times. The loops were flushed with a 1 vol. % formamide solution and flushed by switching the six-port valve in the solvent stream (flow, 0.2 ml/min). A 50 cm x 75 µm I.D. fused silica capillary was used instead of the interface. The tested loop diameters resulted in peak widths of 1.2, 1.4 and 1.7 min, respectively. Therefore, the peak tubing with the smallest diameter (0.25 mm) was applied in all further experiments.

To monitor the influence of the loop volume, the loops were flushed with 1 ml of solvent (1 vol. % formamide). After switching the six-port valve the contents of the loop were flushed to the interface and the elution profile recorded. The dilution effect can be visualised by plotting the maximum detector response (i.e. peak height) versus

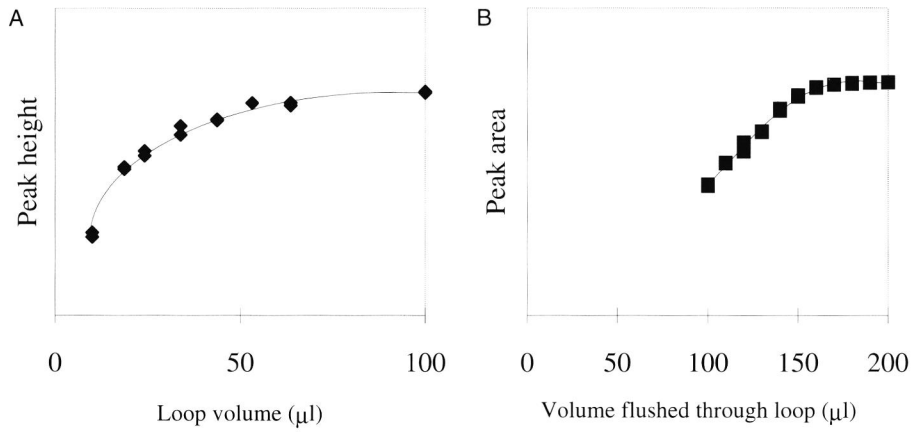


Figure 4.1.3. Influence of (A) loop volume on height of eluted peak and (B) volume flushed into a 100-µl loop on area of peak.

the loop volume. Figure 4.1.3A shows that the peak height increases with larger loop volumes, indicating reduced mixing effects. Plateau conditions are reached for a loop volume of 70–80 µl. A loop volume of 100 µl in combination with a diameter of 0.25 mm I.D. was used in all further experiments.

Loading of the loop.

In the above experiments the loops were flushed with 1 ml of solvent. Because of the limited sample extract volume available after desorption of the analytes from an SPE cartridge, it is necessary to reduce this volume significantly. In order to study this aspect, a 100-µl loop was flushed with different volumes of 1 vol. % of formamide and the peak area was recorded. The area, which indicates the total amount of sample loaded on the loop, was plotted versus the volume loaded. Figure 4.1.3B shows that the peak area becomes constant after at least 180–200 µl are loaded. To be on the safe side, in all further experiments the loop was flushed with 190 µl of desorption solvent. The flow rate of the CE buffer did not affect the plug profile in the range from 0.1 to 0.4 ml/min (data not shown). The time period during which the underpressure has to be applied should not be too short, because this will adversely affect the precision of the injection. A flow rate of 0.2 ml/min was chosen as a compromise.

Injection parameters.

Injection of an aliquot of the SPE desorption plug into the CE capillary is started by applying an underpressure at the detection side of the CE capillary. The parameters of interest are the timing of the injection and the injection pressure. Two approaches can be used. In the first method the injection starts before, and ends after all of the eluted compounds have passed the interface (between 0 and 1.2 min after switching

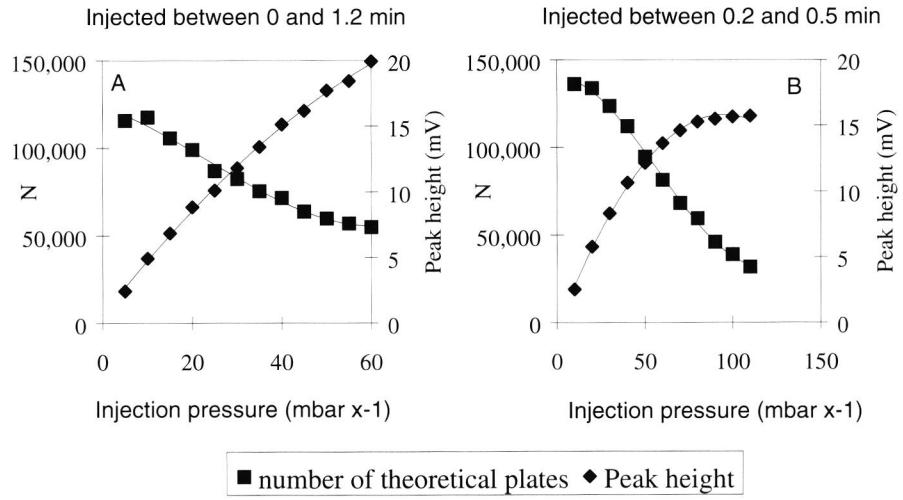


Figure 4.1.4 Dependence of the number of theoretical plates and peak height, on injection procedure used, (left) 0-1.2 min and (right) 0.2-0.5 min. Test compound is ibuprofen. For further details, see text.

the valve). The other method is to inject an aliquot out of the middle of the elution plug, i.e. when the maximum analyte concentrations in the plug passing the tip of the capillary are maximal, i.e. between 0.2 and 0.5 min after switching the valve. The injection pressure was varied between -5 and -60 mbar for the former, and between -10 and -110 mbar for the latter approach. The criteria used to select the optimum time and pressure were: (i) the efficiency, expressed as the number of theoretical plates and (ii) the detectability, expressed as the peak height (since the noise was about the same during all experiments).

The loop was filled with a solution containing 30 µg/ml of each of the test compounds in acetonitrile/phosphate buffer pH 7.8 (75/25, v/v). In figure 4.1.4 the results for ibuprofen are shown; the other compounds showed similar behaviour. If 100,000 plates are arbitrarily taken as the minimum value for providing sufficient resolution, the second injection procedure (figure 4.1.4B) is seen to be more favourable: the peak height is 12 mV which is about two times higher than when using the first approach (figure 4.1.4A). For this reason the 0.2–0.5 min injection procedure, applying a pressure of -40 mbar, was selected.

Optimizing the SPE procedure

The breakthrough of the analytes on the SPE cartridge was studied using a test solution of the NSAIDs in water (10 µg/ml each). Even after loading of 8 ml of the standard solutions on the cartridge, no breakthrough was observed. Larger volumes were not tested because of the limited availability of biological samples. Because of this it can be concluded that this type of cartridge is able to retain the of analytes sufficiently, and therefore, this cartridge is used in further experiments. After loading of urine (8 ml) or serum (1 ml) no breakthrough of the analytes was observed.

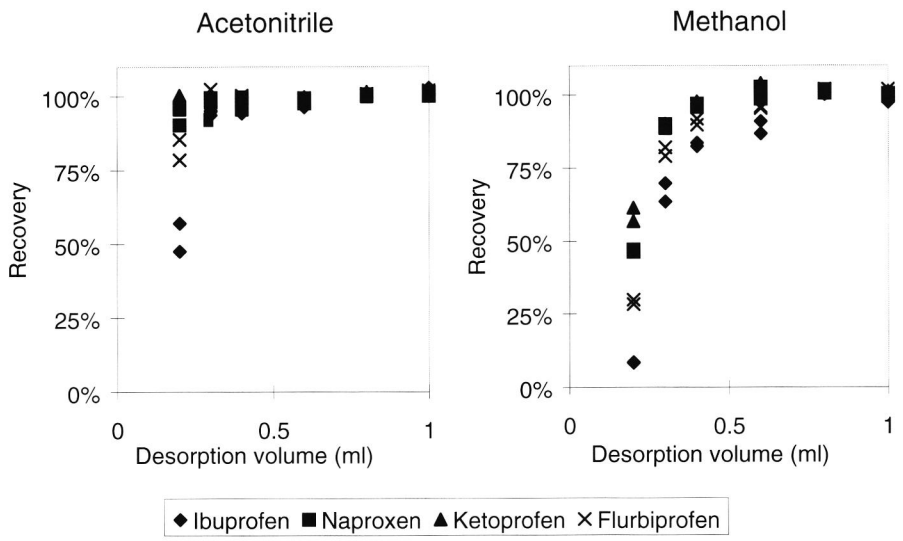


Figure 4.1.5 Influence of desorption volume on NSAID recovery during SPE, when using (left) acetonitrile or (right) methanol in modifier/40 mM phosphate pH 7.8 (75/25, v/v) mixture.

In order to obtain low detection limits, the analytes should be desorbed in a volume as small as possible. To achieve this, a high modifier concentration (elution strength) and pH ≥ pK_a + 2 (fully charged analytes) were used. Two organic solvents were used as a modifier, methanol and acetonitrile. The concentration of organic modifier was limited to 75 vol. % to avoid precipitation of (in)organic salts. The buffer concentration (40 mM phosphate, pH 7.8) was high enough to ensure sufficient buffer capacity; higher concentrations should not be used since they will make the stacking procedure less efficient.

After loading of the sample, the cartridges were desorbed with different volumes (i.e. 0.2–1.0 ml) of either methanol/40 mM phosphate buffer, pH 7.8 (75/25 v/v) or acetonitrile/40 mM phosphate buffer, pH 7.8 (75/25 v/v). Figure 4.1.5 shows that with methanol the required desorption volume is about 800 µl, while with acetonitrile ca. 400 µl will suffice. For this reason desorption with 400 µl acetonitrile/40 mM phosphate buffer, pH 7.8 (75/25 v/v) was selected.

At-line SPE–CE of urine and serum samples

Spiked urine samples were analysed using both direct CE and the present at-line SPE–CE procedure. Figure 4.1.6A shows that direct CE, i.e. without sample pretreatment, is not possible. Because of the high salt concentration in the sample, the plug containing a part of the analytes migrates together with the EOF at about 8 min [1]. Incorporating the SPE pretreatment step using 1 ml of 10 mM phosphate buffer (pH 2.5) to wash the cartridge and the desorption procedure described above, resulted in a significant decrease of the interferences in the electropherogram. Still, the

Table 4.1.1 Influence of volume, pH and percentage of acetonitrile of the washing buffer on at-line SPE–CE of urine samples.

Acetonitrile	*pH*								
(%)	2.5			4.5			6.5		
	1 ml	*2 ml*	*3 ml*	*1 ml*	*2 ml*	*3 ml*	*1 ml*	*2 ml*	*3 ml*
0	4(0)	4(0)	4(0)	4(0)	4(0)	4(0)	4(0)	4(0)	4(1)
10	4(0)	4(0)	4(0)	4(1)	4(2)	4(2)	4(1)	2(1)	1(1)
20	-	-	-	4(2)	4(3)	4(4)	-	-	-

Results are given in the format *a(b)*, with *a*, number of analyte peaks observed and *b*, number of analyte peaks baseline-separated from the interferences.

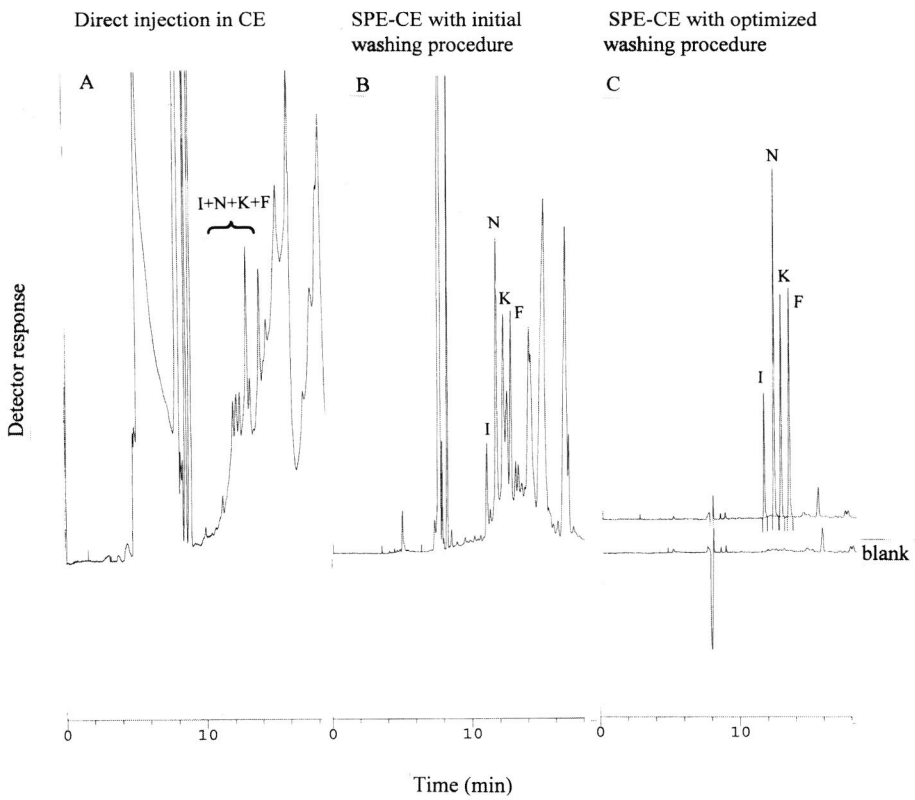


Figure 4.1.6 Electropherograms of spiked urine (8 ml sample; 10 µg/ml of each NSAID) with (A) direct CE analysis, (B) SPE–CE using 1 ml phosphate buffer (pH 2) in washing procedure and (C) SPE–CE using 3 ml of acetonitrile/phosphate buffer pH 4.5 (20/80, v/v) in washing procedure; blank, blank urine.

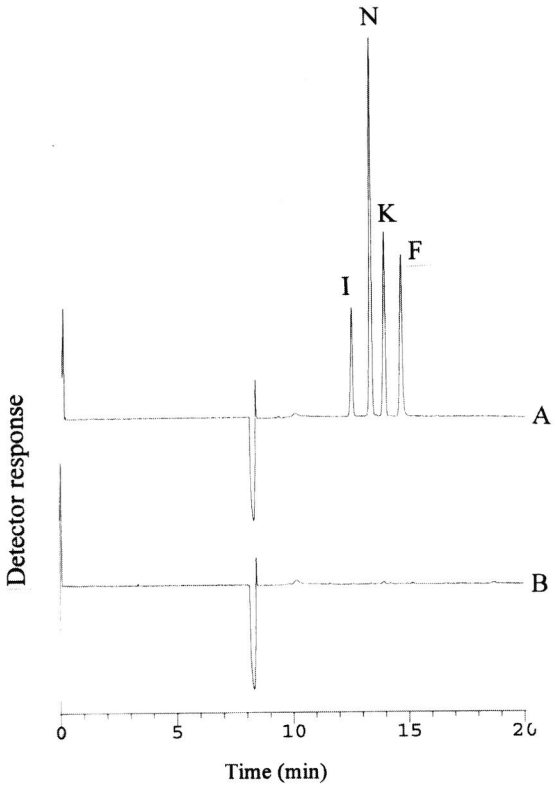


Figure 4.1.7 SPE–CE analysis of (top) 1 ml serum spiked with 10 μg/ml of each NSAID and (bottom) blank. In both cases 1 vol. % of formic acid was added to the sample prior to sample preparation.

number of interfering peaks was quite large (figure 4.1.6B); therefore, a more effective washing procedure had to be developed. This aspect was studied varying the pH (2.5–6.5), percentage of modifier (0–20 vol. % acetonitrile) and volume (1–3 ml) of the washing buffer, as is shown in table 4.1.1. The results for pH 2.5 solutions are in most cases rather poor. The better results found at higher pH values can be explained by the increased charge of the interferences, which are therefore more easily removed under these conditions. However, a too large increase of the pH results in desorption of the analytes (compare pH 6.5 and pH 4.5 with 10 vol. % acetonitrile). The optimum was found by using 3 ml of acetonitrile/10 mM phosphate buffer, pH 4.5 (20/80 v/v). The fully satisfactory result then obtained is shown in figure 4.1.6C for the analysis of a spiked and a blank urine sample.

Serum protein binding of analytes can be a serious problem during the analysis of serum samples. The test compounds are known to be bound for over 99% to serum proteins and indeed no analyte peaks showed up in SPE–CE electropherograms for serum spiked with 10 μg/ml of each of the NSAIDs [22, 23]. To disrupt the analyte–protein bonds, 1 vol. % of formic acid was added to the sample before loading it on

Table 4.1.2 Calibration plots of the test compounds in urine (1 ml loaded) and serum (8 ml loaded) recorded from the detection limit up to 10 µg/ml.

	Compound	Intercept (std)	Slope (std)	Correlation coefficient (r^2)	LOD (µg/ml)
Serum	Ibuprofen	-0.01 (0.03)	0.30 (0.01)	0.9934	0.2
(n=7)	Ketoprofen	-0.01 (0.06)	1.12 (0.01)	0.9983	0.4
	Naproxen	-0.01 (0.03)	0.52 (0.01)	0.9983	0.4
	Flurbiprofen	-0.02 (0.04)	0.49 (0.04)	0.9969	0.2
Urine	Ibuprofen	-0.2 (0.1)	2.01 (0.02)	0.9984	0.08
(n=8)	Ketoprofen	0.5 (0.1)	6.61 (0.03)	0.9997	0.04
	Naproxen	1.4 (0.2)	4.01 (0.05)	0.9978	0.04
	Flurbiprofen	-0.2 (0.1)	4.11 (0.02)	0.9997	0.04

the SPE cartridge [22, 23]. An almost quantitative release (>99%) of the test compounds was obtained as is shown in figure 4.1.7, trace A. The optimized washing procedure described above efficiently removed all low molecular-weight interferences (figure 4.1.7, trace B). Two additional washing steps with 1 ml of water before and after removal of these interferences by the washing buffer, were necessary to remove the bulk of endogenous high-molecular-weight interferences.

Quantitative data

Calibration plots were constructed both for serum and urine using analyte concentrations from the detection limit (signal-to-noise ratio = 3) up to 10 µg/ml. The data of table 4.1.2 show that the plots are linear ($r^2 > 0.99$) for all test compounds in both matrices. The detection limits are 0.04–0.08 µg/ml in urine, and 0.2–0.4 µg/ml in serum. The differences in the detection limits for the various test compounds can be explained by their differences in molar absorptivity at the detection wavelength, 200 nm. The somewhat higher detection limits obtained for serum can be attributed to the smaller sample volumes loaded on the SPE cartridges, 1 ml for serum and 8 ml for urine. Nevertheless, the analyte detectability is sufficient for therapeutic drug monitoring.

The within-day and day-to-day precision were determined using solutions of the test compounds in water and the biological matrices containing high (10 µg/ml) and low (five times the detection limit) concentrations of the NSAIDs. The results are summarized in table 4.1.3. As is to be expected, in most cases a better precision is obtained for aqueous samples. However, the differences with the real samples - which are of course primarily due the presence of residual endogenous interferences - are not too large, and sometimes even surprisingly small. In any case, the rsd values of the precision are lower than 15%, which is the often used acceptance level for quantitative data in biomedical analysis [24].

Table 4.1.3 Day-to-day and within-day reproducibility for SPE–CE of NSAIDs in urine, serum and water (n=5).

Volume loaded on cartridge:		8 ml			1 ml		
Matrix:		Urine	Water		Serum	Water	
Concentration:		5xLOD[1]	5xLOD[1]	10 µg/ml	5xLOD[1]	5xLOD[1]	10 µg/ml
Day-to-day reproducibility (%)	Ibuprofen	11.0	7.6	5.8	5.5	2.2	3.5
	Ketoprofen	6.6	6.8	4.6	4.7	3.7	3.7
	Naproxen	8.6	4.6	4.8	4.2	1.2	3.5
	Flurbiprofen	8.3	3.3	2.8	7.6	3.5	3.8
Within-day reproducibility (%)	Ibuprofen	8.2	3.0	3.1	6.7	3.7	1.0
	Ketoprofen	7.9	3.3	2.8	2.8	2.8	1.1
	Naproxen	7.7	3.9	3.3	2.9	3.4	2.5
	Flurbiprofen	6.0	3.0	1.6	2.6	3.2	1.2

[1] Concentrations 5 times LOD for urine and serum, respectively (see table 4.2.2).

Conclusions

The present study shows that SPE can be coupled at-line to CE using a home-made interface. The construction of the interface and the SPE-to-CE transfer procedure is performed in such a way that the only parameter to be adapted if analytes other than the present test compounds have to be determined, will be the injection pressure.

Our set-up was used to determine NSAIDs in serum and urine. The optimized sample pretreatment procedure causes the release of analytes from protein binding, their preconcentration, and the removal of essentially all interfering compounds. The SPE–CE method is linear over two decades and detection limits down to 0.04 µg/ml can be obtained. The detection limits are sufficiently low to perform therapeutic drug monitoring studies.

A single CE capillary was used for the analysis of at least 150 serum, 300 urine and 400 standard samples over a period of one year. The capillary was flushed once a week with a sodium hydroxide solution. To avoid carry-over effects and clogging of the interface and CE system, the SPE cartridges were used only once when biological samples were analysed. The robustness of the total SPE–CE system was enough to perform unattended analyses.

References

[1] J.R. Veraart, C. Gooijer, H. Lingeman, N.H. Velthorst and U.A.Th. Brinkman, Chromatographia, 44 (1997) 581.

[2] M.A. Jenkins, T.D. O'Leary and M.D. Guerin, J. Chromatogr. B, 662 (1994) 108.

[3] T. Nakagawa, Y. Oda, A. Shibukawa, H. Fukuda and H. Tanaka, Chem. Pharm. Bull., 37 (1989) 707.

[4] M. Shirao, R. Furuta, S. Suzuki, H. Nakazawa, S. Fujita, and T. Maruyama, J. Chromatogr., 680 (1994) 247.

[5] H. Soini, M.V. Novotny and M.L. Riekkola, J. Microcol. Sep., 4 (1992) 313.

[6] Z.K. Shihabi and M.E. Hinsdale, J. Chromatogr. B., 683 (1996) 115.

[7] D. Kaniansky and J. Marák, J. Chromatogr., 498 (1990) 191.

[8] D.G. Stegenhuis, H. Irth, R. Tjaden and J. van der Greef, J. Chromatogr., 538 (1991) 393.

[9] W.C. Brumley, C.M. Brownrigg and A.H. Grange, J. Chromatogr., 680 (1994) 635.

[10] M.C. Roach, P.Gozel and R.N. Zare, J. Chromatogr., 426 (1988) 129.

[11] M.A. Strausbauch, S.J. Xu, J.E. Ferfuson, M.E. Nunez, D. Machacek, G.M. Lawson, P.J. Wettstein, J.P. Landers, J. Chromatogr., 717 (1995) 279.

[12] M.A. Stausbauch, J.P. Landers and P.J. Wettstein, Anal. Chem., 68 (1996), 306.

[13] A.J. Tomlinson, L.M. Benson, W.D. Braddock, R.P. Oda and S. Naylor, J. High Resol Chromatogr., 18 (1995) 381.

[14] L.M. Benson, A.J. Tomlinson, A.N. Mayeno, G.J. Gleich, D. Wells and S. Naylor, J. High Resol Chromatogr., 19 (1996) 291.

[15] A. Ffini, P. De Maria, A. Guarnieri and L. Varoli, J. Pharm. Sci., 76 (1987) 48.

[16] J.R. Veraart, C. Gooijer and H. Lingeman, Chromatographia, 44 (1997) 129.

[17] H. Nishi, T. Fukuyama,, M. Matsuo and S. Terabe, J. Chromatogr., 513 (1990) 279.

[18] H. Nishi and M. Matsuo, J. Liq. Chromatogr., 14 (1991) 973.

[19] H. Nishi and S. Terabe, Electrophoresis, 11 (1990) 691.

[20] R. Weinberger and M. Albin, J. Liq. Chromatogr., 14 (1991) 953.

[21] A. Wainright, J. Microcol. Sep., 2 (1990) 166.

[22] I.D. Wilson and J.K. Nicholson, Anal. Chem., 59 (1987) 2830.

[23] I.D. Wilson and I.M. Ismail, J. Pharm. Biomed. Anal., 5 (1986) 663.

[24] V.P Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman and S. Spector, J. Pharm. Sci., 309 (1992) 309.

4.2

At-line solid-phase extraction coupled to capillary electrophoresis: Determination of amphoteric compounds in biological samples

Summary

An at-line SPE–CE method was presented to determine sulphonamides in urine and serum samples. It was shown that using the set-up that was presented before for acidic compounds can be adapted with a few relatively limited number of optimisation steps to amphoteric compounds, or in other words, the at-line SPE–CE method is a quite universal analysis tool for analytical determination of compounds in biological matrices. The effect of the cartridge choice, effect of desorption solvents, washing procedure on the analyte determination was described. The linearity, precision and LOD obtained for the analytes are satisfactory.

Introduction

Capillary electrophoresis (CE) can be successfully used for the determination of analytes in biological samples provided that a proper sample preparation procedure is available. Different sample preparation techniques have been presented in the literature [1]. These include chromatographic, electrophoretic and dialysis-based procedures. One approach is to use disposable solid-phase extraction (SPE) columns for the clean up of samples. When this procedure is coupled at-line to the CE system the analysis can be fully automated. Such an at-line SPE–CE method in which coupling was done via a homemade interface [2], has been described for the determination of negatively charged compounds [3].

In the present study the same set-up is used to determine amphoteric compounds, i.e. sulphonamides, in serum and urine which emphasis on fully automated sample preparation, which has not been described so far. Because of their amphoteric character, sulphonamides are charged over a wide pH range. Therefore, their SPE–CE determination is much more complicated than the determination of weak acids [2]. Papers directed at the determination of sulphonamides using CE mostly deal with optimization of the CE buffer used for the analysis of aqueous samples [4–7]. For sulphonamides in pork meat [5], detection limits of 2–9 mg/ml were obtained after liquid–liquid extraction (LLE) with acetonitrile. The determination of sulphonamides in milk [6] was performed after LLE and centrifugation (20 min). No detection limits were reported but the electropherograms of sulphonamides in milk (62.5–77.5 μg/ml) resulted in signal-to-noise ratios of 15–60. The resolution of the analytes in the milk extracts was less good than in standard solutions.

Experimental

Chemicals and samples

Sulphadiazine (SDZ), sulphamethizole (SMI), sulphamethoxazole (SMO), sulphamethoxypyridazine (SMP), sulphamerize (SMA) and sulphaquinoxaline (SQ) were obtained from Sigma (St. Louis, MO, USA). Acetic acid (>99.8%), phosphoric acid, sodium acetate and disodium monohydrogen phosphate-12-hydrate (>99.5%) came from Riedel-de Haën (Seelze, Germany). Methanol (>99.8%), tetrahydrofuran (THF) (>99%) and acetonitrile (>99.8%) were purchased from J.T. Baker (Deventer, The Netherlands). Decanoic acid and sodium dihydrogen phosphate monohydrate (>99.0%) were obtained from Merck (Darmstadt, Germany). All chemicals were of the highest quality available. Water was demineralized and distilled prior to use.

Urine was obtained from four healthy volunteers, pooled and divided into 100-ml portions, and frozen at -18 °C. Bovine serum of untreated objects, purchased from Sigma, was divided into 10-ml portions and frozen at -18 °C.

SPE–CE set-up

All experiments were performed using an SPE (ASPEC XL) system (Gilson, Villiers-le-Bel, France), which performs all solvent-handling procedures automatically. The desorption fluid is injected, via an injection port, into a 100-ml loop (figure 4.1.1). By switching the 6-port valve the desorption fluid is flushed into a homemade interface [2, 3]. The electrode in the interface was grounded.

A bare fused silica capillary of 375 μm O.D. and 50 μm I.D. was used for the separation (LC-service, Emmen, The Netherlands). This capillary had a total length of 106 cm and an injection-to-detection length of 40 cm. Fresh capillaries were conditioned by flushing with water (5 min), 1 M sodium hydroxide (2 min) and water (5 min), using a pressure of 2000 mbar. All CE experiments were performed using a PrinCE CE system (PrinCE Technologies, Emmen, The Netherlands) equipped with an automatic 4-tray and an additional thermostating device as described before [8]. A Model 759A UV/VIS detector (Applied Biosystems, Foster City, CA, USA) was used for detection at 260 nm.

SPE–CE procedures

Disposable cartridges packed with 50 mg of styrene–divinylbenzene copolymer (SDB) purchased from J.T. Baker were conditioned with, subsequently, 2 ml of methanol, 2 ml of acetonitrile and 2 ml of 20 mM phosphate buffer (pH 3). Then the activated cartridges were loaded with either 8 ml of urine or 1 ml of serum. The cartridges were washed with, subsequently, 1 ml of water and 5 ml of acetonitrile–10 mM phosphate buffer, pH 3 (20/80, v/v). Desorption was performed with 0.7 ml of acetonitrile.

After the SPE procedure, the 100-μl loop was loaded with desorption fluid from the SPE cartridge (figure 4.1.1). Next, the six-port valve was switched and the eluted compounds were flushed to the interface. The plug containing the analytes passed the interface between 0.2 and 0.5 min after switching the six-port valve. An underpressure of 20 mbar was applied by the CE system. The valve was switched to its original position and after flushing of the interface with CE buffer (2 min), the CE analysis was performed using a voltage of -30 kV (12 min). A solution containing 20 mM phosphate buffer (pH 7) was used as CE buffer. Before each injection the capillary was flushed with CE buffer (2 min), which was filtered before use.

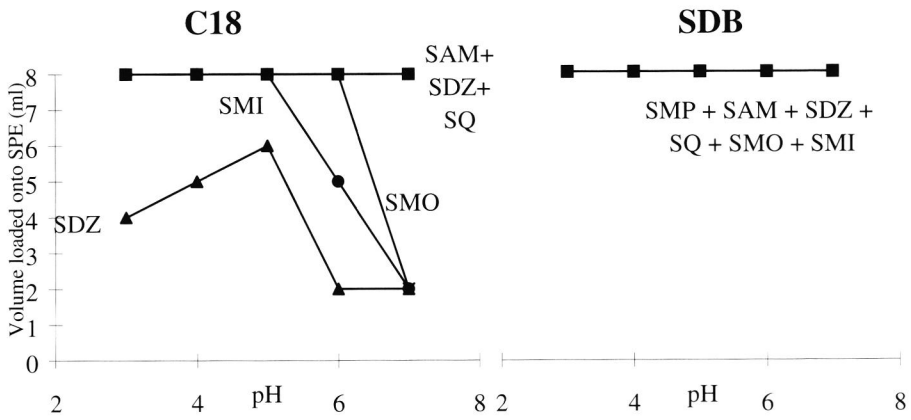


Figure 4.2.1 The maximum volume of test solution, containing 10 μg/ml of each sulphonamide, that can be loaded onto a cartridge containing C18 or SDB sorbent, without any breakthrough of the analytes, as a function of the pH of this test solution.

Results and discussion

Optimization of SPE procedure

The SPE sorbent should retain sufficiently all the analytes of interest. Therefore, rather hydrophobic sorbents were selected even though their use, will result in an increased number of interferences in the final electropherogram. Cartridges packed with C18-bonded silica and SDB were tested at pH values between 3 and 7. The cartridges were loaded with increasing volumes of a 10 μg/ml solution of the test compounds in pH buffer when using the C18 phase, analyte breakthrough was observed for pH ≥ 5 (figure 4.2.1). This can be explained by the negative charge of the test compounds at pH > 4 [9]. On the other hand, up to 8 ml of a solution of the analytes in a 10 mM phosphate buffer with pH varying from 3 to 8 could be loaded on the SDB cartridge without any breakthrough. This copolymer sorbent was, therefore, used in all further experiments. After loading of urine (8 ml) or serum (1 ml), no breakthrough of the analytes was observed either. Volumes larger than 1 ml of serum are not relevant in most cases, and were therefore, not tested.

Three desorption solvents were tested: THF–water (75/25 v/v), pure methanol and pure acetonitrile. Desorption of the test compounds required 0.8 ml of methanol, while 0.7 ml of the other two solvents was needed. Such desorption volumes are quite large and smaller volumes are preferable to improve detection limits.

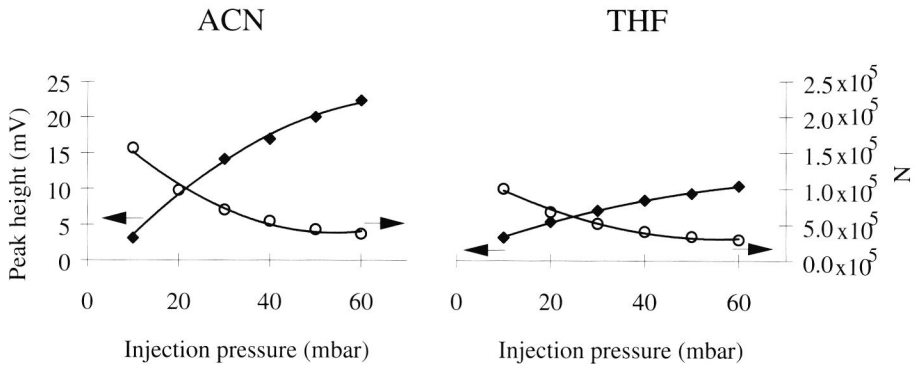


Figure 4.2.2 Peak height and number of theoretical plates for SMO versus injection pressure (mbar) when using acetonitrile or THF–water (75/25 v/v) as desorption fluid.

As regards injection, the injection of larger volumes of desorption fluid into the CE capillary will, on the one hand, result in an improved sensitivity but, on the other hand, in broader bands [3]. Figure 4.2.2 shows, with SMO as an example and when using 100,000 theoretical plates as an arbitrary minimum, that peak heights of 10 mV are obtained with acetonitrile as desorption fluid as against a mere 2.5 mV in case of THF–water (75/25 v/v). Similar results were obtained for the other sulphonamides. Acetonitrile combined with an underpressure of 20 mbar was, therefore, selected during the injection into the CE capillary.

Analysis of biological samples

The direct injection of urine samples containing sulphonamides into the SPE–CE system, resulted in a large number of interferences in the corresponding electropherograms. Therefore, the sample preparation step had to be improved and the wash procedure had to be optimized. To this end, SPE cartridges were loaded with 8 ml of urine spiked with 10 mg/ml of the sulphonamides and washed with up to 5 ml of wash buffers of different composition, i.e. containing 0–20% of acetonitrile and having different pH values (2–8). The best result was obtained with 5 ml of acetonitrile–10 mM phosphate buffer, pH 7 (20/80 v/v). Using a larger volume of washing solvent or a higher percentage of acetonitrile resulted in loss of analytes, while a smaller volume of wash buffer or the use of less acetonitrile, and also a lower pH led to insufficient removal of interferences. The electropherograms of figure 4.2.3 show that the test compounds can be determined in urine with this procedure. At the 10 μg/ml level, a recovery of 100 ± 2% was obtained for all test compounds.

When analysing serum samples, the protein binding of drugs can create serious problems. For the analytes under investigation, the drug–protein binding is in the range of 40–90% [10, 11]. Nevertheless, the SPE–CE analysis of serum samples without the addition of a displacer resulted in recoveries of 70–100%, and the

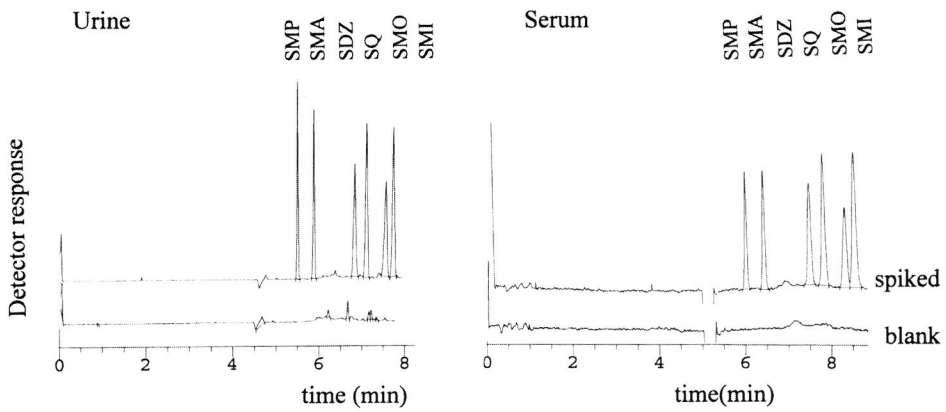


Figure 4.2.3 SPE–CE analysis of 8 ml of blank urine, 1 ml of blank serum and urine and serum spiked with 10 μg/ml each of the sulphonamides. Detector response: urine, 35 mV full scale; serum, 3 mV full scale. Migration order: SMP, SMA, SDZ, SQ, SMO and SMI. For details, see text.

addition of octanoic or decanoic acid to the sample did not result in a noticeable increase. Probably, the SPE procedure itself causes a disruption of the drug–protein bonds. Therefore, no modification of the procedure for urine analysis was necessary and direct injection of serum onto the SPE cartridge was used in all further experiments. A typical result is included in figure 4.2.3.

Analytical data and performance

Linear calibration curves were obtained from the detection limits up to 10 and 25 mg/ml for urine and serum, respectively. The results are shown in table 4.2.1. As can be seen from this table, the LOD values for the analytes in serum are considerably higher than with urine. This is at least partly due to the much lower volume of urine loaded onto the cartridge.

The RSD values of the within-day precision were 4–7% and 5–9% for urine and serum, respectively, while the results for the day-to-day precision were 6–9% and 7–10%, respectively. Obviously, the performance of the present procedure makes it fully acceptable for quantitative in drug analysis [12].

Over a period of one year a single CE capillary was used for the analysis of over 100 serum and 200 urine samples and 200 aqueous standards. To avoid carry-over effects and for clogging of the interface and CE system, the SPE cartridges were used only once when biological samples were analysed. The total SPE–CE system turned out to be robust and many unattended analyses of biological samples were performed with the fully automated system.

Table 4.2.1 Analytical data for at-line SPE–CE of sulphonamides.

Analyte	Slope (std)	Intercept (std)	Correlation coefficient (r^2)	LOD (ng/ml)
Urine				
Sulphamethoxypyridazine	0.608 (0.002)	0.04 (0.02)	0.9998	10
Sulphamerazine	0.569 (0.002)	0.04 (0.02)	0.9999	10
Sulphadiazine	0.507 (0.010)	-0.10 (0.06)	0.9978	30
Sulphaquinoxaline	0.657 (0.002)	0.04 (0.02)	0.9999	10
Sulphamethoxazole	0.451 (0.002)	-0.02 (0.02)	0.9998	10
Sulphamethizole	0.586 (0.002)	-0.03 (0.02)	0.9998	10
Serum				
Sulphamethoxypyridazine	0.101 (0.004)	-0.02 (0.05)	0.9923	500
Sulphamerazine	0.106 (0.003)	-0.02 (0.04)	0.9960	500
Sulphadiazine	0.099 (0.003)	0.01 (0.03)	0.9961	500
Sulphaquinoxaline	0.139 (0.004)	-0.05 (0.05)	0.9966	500
Sulphamethoxazole	0.084 (0.003)	-0.03 (0.03)	0.9964	500
Sulphamethizole	0.138 (0.005)	-0.01 (0.05)	0.9954	500

Calibration curves taken between detection limit and 25 µg/ml using 5 and 7 data points in duplicate for serum and urine, respectively.

Conclusions

At-line SPE–CE can be used to determine amphoteric compounds in biological samples. The total set-up, i.e. SPE procedure, the at-line SPE–CE coupling, the injection into the CE column and the CE analysis, is fully automated and controlled by the SPE unit and CE device. Both urine and serum can be injected directly into the SPE–CE system, provided that a proper wash step with an acetonitrile–phosphate buffer mixture is used to remove interfering compounds.

The analytical data such as linearity, precision and LODs are fully satisfactory. The procedure can therefore be recommended for, e.g. clinical analyses and metabolic studies.

A further improvement of analyte detectability is required decreasing the desorption volume by using particle-loaded membranes combined with 96-well plates, rather than conventional SPE cartridges, probably is a promising option. This aspect will be the topic of a future study.

References

[1] J.R. Veraart, C. Gooijer, H. Lingeman, N.H. Velthorst and U.A.Th. Brinkman, J. Chromatogr. A, 856 (1999) 483.

[2] J.R. Veraart, C. Gooijer, H. Lingeman, N.H. Velthorst and U.A.Th. Brinkman, Chromatographia, 44 (1997) 581.

[3] J.R. Veraart, C. Gooijer, H. Lingeman, N.H. Velthorst and U.A.Th. Brinkman, J. Pharm. Biomed. Anal., 17 (1998) 1161.

[4] C.L. Ng, H.K. Lee and S.F.Y. Li, J. Chromatogr., 632 (1993) 165.

[5] M.T. Ackermans, H.L. Beckers, F. M. Everaerts, H. Hoogland and M.J.H. Tomassen, J. Chromatogr., 596 (1992) 101.

[6] C.-E. Lin, W.-C. Lin, W.C. Chiou, E.C. Lin and C.-C. Chang, J. Chromatogr., 755 (1996) 261.

[7] M.E.P. Hows, D. Perrett and J. Kay, J. Chromatogr. A, 768 (1994) 97.

[8] J.R. Veraart, C. Gooijer and H. Lingeman, Chromatographia, 44 (1997) 129.

[9] M.C. Ricci and R.F. Cross, J. Microcol. Sep., 5 (1993) 207.

[10] Informatorium Medicatorium, Kon. Ned. Maatsch. Bevord. Pharm., 's-Gravenhage, The Netherlands, Part I 1990, pp. 139.

[11] W. Lammers, F.A. Nelemans, Th.J. Bouwman, J. van Noordwijk, L. Offerhaus, W.M. Rosinga and A. Vermeulen, Algemene Farmacotherapie, Stafleu's wetenschappelijke uitgevers-maatschappij, Brussel, 1980, pp. 672.

[12] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman and S. Spector, J. Pharm. Sci., 309 (1992) 306.

4.3

Non-aqueous capillary electrophoresis of biological samples after at-line solid-phase extraction

Summary

Solid-phase extraction (SPE) was coupled at-line to capillary electrophoresis (CE) for the determination of a series of basic test compounds (i.e. tricyclic antidepressants). The analysis was performed using a non-aqueous CE buffer, which resulted in baseline separation of all test compounds. This is in marked contrast with CE using aqueous buffers where hardly any separation was obtained either with or without micelles. The SPE procedure was used to remove simultaneously most of the water from the sample, because no direct analysis of aqueous samples is possible when a non-aqueous CE buffer is used. With the present method the antidepressants can be determined in both urine and serum. Analyte detectability is increased up to 10-fold due to trace enrichment during the extraction process; the limits of detection (LODs; UV 214 nm) are 30–300 ng/ml in urine and 300–1000 ng /ml in serum. The RSD values (n=5) of the within-day and between-day precision are below 9% and 11%, respectively. Therefore, the present procedure can be used for drug monitoring.

Introduction

Capillary electrophoresis (CE) can be used for the determination of analytes in biological samples. A wide variety of buffer systems is available to obtain a proper separation in CE. A large majority of these are aqueous in nature but in some cases, such as with the separation of tricyclic antidepressants (TCADs), this approach (with or without the addition of micelles) was not successful [1]. The addition of Tween 20 resulted in a baseline separation but, unfortunately, the total analysis time was over 50 min. Better results were obtained using a non-aqueous buffer [1]. From the different solvents tested acetonitrile gave the best results. Ammonium acetate (25 mM) and acetic acid (1 M) were added to create a certain degree of conductivity [1]. Next to the increased separation power, the volatility of the buffer system makes non-aqueous CE highly suitable for mass spectrometric detection. To avoid band broadening only samples with a resistance equal or higher than that of the buffer can be injected. In other words, no water-containing samples can be analysed, and certainly no biological samples with their high salt concentrations.

One solution to the above problem is the combination of non-aqueous CE and a proper sample preparation procedure such as at-line solid-phase extraction (SPE). Although at-line SPE–CE, using a home-made interface, is closely similar to earlier work described for aqueous buffers [2 – 4] there is an important difference: next to removal of salts and proteins, the removal of the water is essential.

Experimental

Chemicals and Materials

Amitriptyline and imipramine were purchased from Aldrich (Milwaukee., Wl, USA), and nortriptyline, desipramine and maprotyline from Sigma (St. Louis, MO, USA). Acetic acid (> 99.8%), phosphoric acid, sodium acetate and disodium monohydrogen phosphate-12-hydrate (> 99.5%) were obtained from Riedel-de Haën (Seelze, Germany), and methanol (> 99.8%), tetrahydrofuran (> 99%), acetonitrile (> 99.8%), and ammonium acetate (> 97%) from J.T. Baker (Deventer, The Netherlands). Sodium dihydrogen phosphate monohydrate (> 99.0%) was obtained from E. Merck (Darmstadt, Germany). All chemicals were of the highest purity available. Water was demineralized and distilled prior to use.

Urine samples obtained from four healthy volunteers were pooled, filtered and divided into 100-ml portions and frozen at -18°C.

Bovine serum of untreated animals was purchased from Sigma. It was divided into 10-ml portions and also frozen at -18°C.

SPE–CE set-up

All experiments were performed using an ASPEC XL sample-handling system (Gilson, Villiers-le-Bel, France), which performs all solvent handling procedures in an automated way. A home-made interface was used to couple the SPE system and the CE unit [2 – 4]. The electrode in the interface was grounded.

Bare fused-silica capillaries of 375 μm O.D. and 50 μm I.D. (LC-service, Emmen, The Netherlands) were used. The total length of the capillaries was 106 cm and the injection-to-detection length was 40 cm. A new capillary was conditioned by flushing subsequently for 5 min with water, 1 M sodium hydroxide and, again water, using a pressure of 2000 mbar. Before each injection, the CE capillary was flushed with a fresh CE buffer solution, which was filtered before use. All CE experiments were performed at a voltage of -30 kV using a PrinCE CE system (PrinCE Technologies, Emmen, The Netherlands) equipped with an automated four-tray autosampler and an additional thermostating device [5]. A Model 759A UV/VIS detector set at 214 nm (Applied Biosystems, Foster City, CA, USA) was used for detection.

SPE–CE procedure

Disposable alkyl-bonded silica cartridges (IST, Hengoed, Mid Glamorgan, UK) packed with 100 mg of C2-bonded silica or C2-bonded end-capped silica were conditioned with, subsequently, 1 ml of methanol, 1 ml of water and 1 ml of a 20 mM phosphate buffer of pH 7. Next, the cartridges were loaded with either 8 ml of urine or 1 ml of serum. The cartridges were washed with, subsequently, 2 ml of a 20 mM phosphate buffer of pH 7, 2 ml of acetonitrile/water (20/80 v/v) and 2 ml of a 20 mM phosphate buffer of pH 7. The TCADs were eluted with 200 μl of methanol followed by 400 μl of glacial acetic acid/acetonitrile (1/99 v/v). Both solvents were collected in a tube and the mixed desorption solvent was analysed hereafter. Because the two solvents used during desorption, have to be mixed before analysis, and therefore, the use of an at-line system is preferred above an on-line system.

After the SPE procedure, the 100-μl loop was loaded, via the injection port, with the desorption solvent from the SPE cartridge, using the set-up as described in section 4.1 [2, 3]. Next, the valve was switched and, when the eluted compounds passed the interface (0.2–0.5 min after switching), the injection was performed by applying an underpressure of 70 mbar at the far end of the CE capillary. Finally, the interface was flushed with CE buffer by the HPLC pump and the valve switched again.

A solution of 25 mM ammonium acetate and 1 M acetic acid in acetonitrile was used as the non-aqueous CE buffer [1]. The CE procedure was performed in 12 min. The CE analysis is performed between the end of the capillary that is normally the detection side and the detector, because this end is grounded and better accessible. Overnight, the capillary was kept in water because the polyimide coating of the CE capillary slowly dissolved in acetonitrile, which made the capillary rather fragile and decreases its lifetime. A typical lifetime of the capillary was 6 – 8 weeks.

Results and Discussion

CE Analysis and SPE–CE Interfacing

CE using non-aqueous buffers is gaining more and more interest because it offers a distinct advantage compared with aqueous buffers: analytes that do not or slightly dissolve in aqueous buffers can be determined. When micelles are added to dissolve such solutes in water, a combined effect of pure electrophoretic migration by mass/charge ratio and a solvent–micelle interaction is obtained. When using a non-aqueous buffer only the charge/mass ratio is taken into account. Furthermore, analytes which are attracted by the capillary wall and, therefore, show band broadening when using aqueous buffers will give a better peak shape when using a non-aqueous buffer. As was shown before, TCADs cannot be separated using aqueous buffers, but the separation is successful when applying a non-aqueous buffer. However, acetonitrile has then to be used as the sample solvent. Unfortunately, the presence of even a small amount of water in the injection plug already severely disturbs the baseline of the CE electropherogram, as is demonstrated in figure 4.3.1 where a direct injection of a TCAD standard in water is compared with that of a TCAD standard in acetonitrile. Obviously, no water should be present in the injection plug in CE and the sample preparation procedure should be designed accordingly.

Because of the nature of potentially interfering salts and/or proteins, an alkyl-bonded silica sorbent was preferred. However, since the interaction between TCADs and C18-bonded silica is quite strong, a less hydrophobic phase was chosen. Two types of sorbent were tested, a non-end-capped C2 phase (C2) and an end-capped C2 phase (C2EC). Standard solutions containing the TCADs in water adjusted to pH values of 3–7 were loaded, eluted with 0.2 ml of methanol and analysed by the CE system. A strong pH effect was observed for the C2 cartridge: breakthrough values of less than 8 ml occur with a pH of 6 or lower. On the other hand, up to 8 ml could be loaded onto the C2EC cartridge without any pH effect being observed. Therefore, the latter cartridge was used for further experiments. This SPE optimization approach is very similar to the optimization of the acidic or amphoteric compounds presented before [2, 3]. The only additional requirement is the removal of the water.

Although acetonitrile/acetic acid (99/1 v/v) gives a good separation of the test analytes, it could not be used to elute the test analytes from the C2EC cartridge. However, the TCADs could be eluted with a small volume of an organic solvent, *i.e.* 0.2 ml of methanol. Unfortunately, injection of a methanol plug causes severe band broadening and the current decreases to zero due to the low conductivity of the desorption solvent. This problem could be solved by adding various amounts of acetonitrile/acetic acid (99/1 v/v), after the elution with methanol, as becomes evident

from the dramatically improved peak shape of maprotyline shown in figure 4.3.2. If the acetonitrile/acetic acid mixture was added to the methanol, the peak width of the TCADs registered upon the addition of from 0.1 up to 0.4 ml of acetonitrile/acetic acid decreased still further. However, a larger addition resulted in decreased peak heights of the test analytes, because of the dilution effect. Therefore, the combination of 0.2 ml of methanol and 0.4 ml of acetonitrile/acetic acid (99/1 v/v) was used in all further experiments.

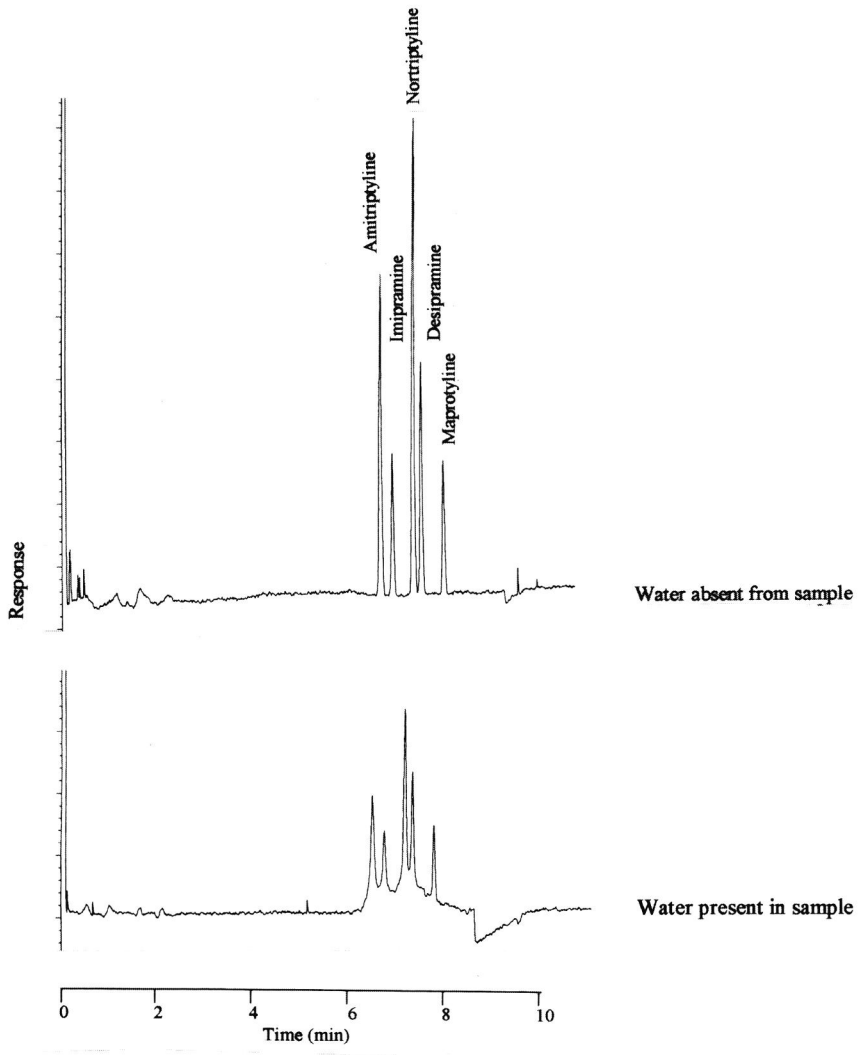


Figure 4.3.1 Analysis of a standard solution of five TCADs (10 µg/ml each) dissolved in acetonitrile/acetic acid and water using SPE–CE. For further details, see text.

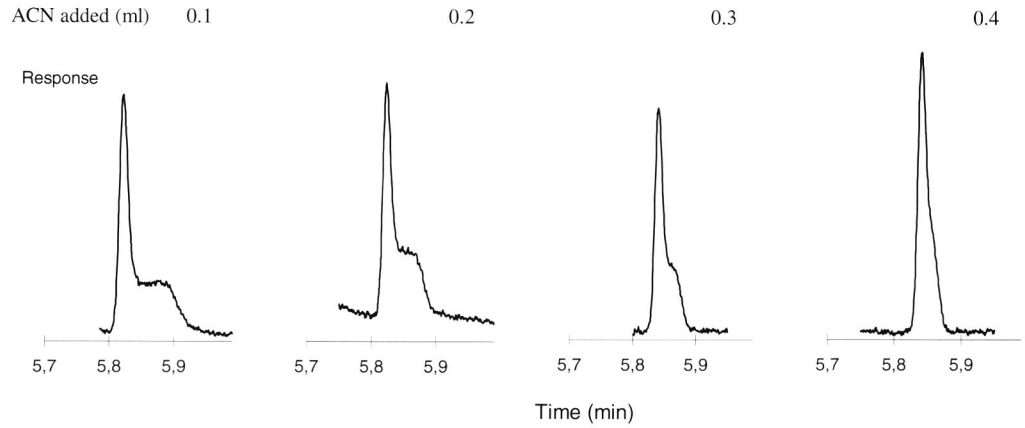


Figure 4.3.2 Effect of amount of acetonitrile/acetic acid (99/1) added to 0.2 ml of methanol desorbate on peak shape of maprotyline. For details, see text.

Analysis of Biological Samples

The direct analysis of urine with SPE–CE according to the above protocol resulted in the presence of many interfering compounds in the electropherograms. Previous experiments with acidic and amphoteric buffers showed that the best results (no breakthrough and elimination of interferences) were obtained by using a buffer containing a small amount of organic solvent (typically 20%) and a pH close to the pK_a value (acids) or the iso-electric point (amphoteric compounds) of the analyte. To achieve this pH values of 9–11 should be applied for the TCADs. However, because silica will dissolve at these high pH values, a lower pH had to be selected. The procedure was further improved by washing the loaded cartridge with 2 ml of a 20 mM phosphate buffer (pH 7), and next, with different volumes (0–3 ml) of the acetonitrile/20 mM phosphate buffer (pH 7) (20/80 v/v). The best results were obtained with 2 ml of the latter buffer. This volume resulted in the removal of all interferences, without the loss of analytes. As can be seen from figure 4.3.3a, the optimized procedure can be applied to determine the five analytes in urine.

The determination of the TCADs of these analytes in serum (1 ml sample) is complicated by of the strong protein binding of the drugs (> 95%) [6]. Loading the serum samples onto the cartridges without any pretreatment (and applying the same washing and desorption procedure as for urine), resulted in recoveries of 70–80%. Probably, during the loading process and/or the washing procedure the protein–drug equilibrium of the TCADs is shifted because the TCADs become bonded on the stationary phase, which results in a lower concentration of analyte in solution. Various methods were reported in the literature to obtain a further increase of the recovery, such as mixing of the serum with an equal volume of water, a 10 mM phosphate buffer of pH 9, or pH 7 [7]. However, in our hands, neither of these methods caused a noticeable increase of the recovery. Therefore, loading of serum

samples without further pretreatment was preferred. A correction can be made for quantitation purposes of the compounds in serum. Figure 4.3.3b shows that the final result is fully satisfactory.

Quantitation data

Calibration curves of the analytes in spiked urine and serum samples were constructed from the detection limits up to 10 µg/ml. The results, reported in table 4.3.1, show that the linearity is satisfactory. As can be seen in this table the LOD values in serum are higher than those in urine. This can be explained by the different volumes loaded onto the SPE cartridge, viz 1 ml vs. 8 ml. The LOD values of the TCADs with immunoassays are in the 300 ng/ml range [8, 9] and using SPE–LC, in the 10 ng/ml range [7, 10 – 14].

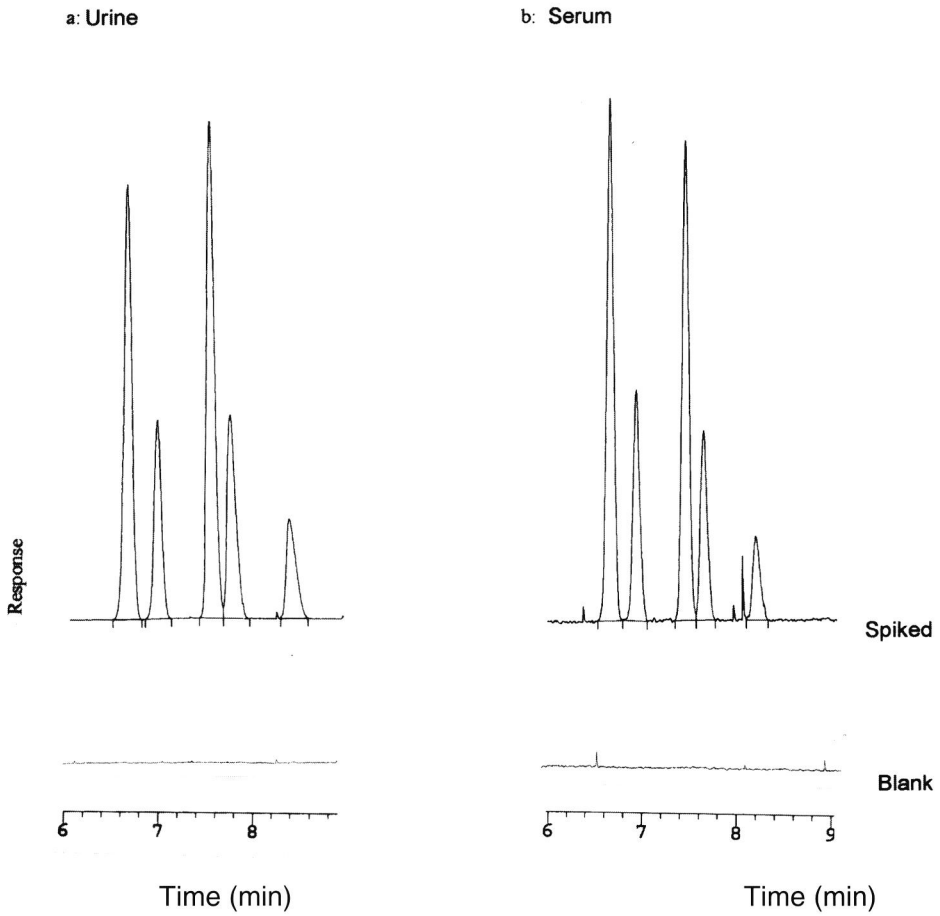


Figure 4.3.3 SPE–CE of (a) urine and (b) serum spiked with 10 µg/ml of each TCAD. For details, see text. Peak order as in figure 4.3.1.

Table 4.3.1 Analytical data of the SPE–CE $UV_{214\,nm}$ of TCADs in serum and urine[a].

Analyte	Slope (std)	Intercept (std)	Correlation coefficient (r^2)	LOD (ng/ml)
Urine				
Amitriptyline	3.14 (0.04)	0.06 (0.1)	0.998	30
Imipramine	1.31 (0.02)	0.02 (0.06)	0.998	60
Nortriptyline	3.03 (0.03)	-0.3 (0.1)	0.998	30
Desipramine	1.35 (0.02)	-0.31 (0.08)	0.998	100
Maprotyline	0.65 (0.02)	-0.21 (0.08)	0.992	300
Serum				
Amitriptyline	0.378 (0.003)	-0.004 (0.01)	0.999	300
Imipramine	0.158 (0.002)	-0.002 (0.01)	0.999	600
Nortriptyline	0.308 (0.003)	-0.03 (0.01)	0.999	300
Desipramine	0.123 (0.004)	-0.02 (0.02)	0.993	600
Maprotyline	0.054 (0.003)	-0.01 (0.01)	0.988	1000

[a] Calibration curves taken between detection limit and 10 µg/ml.

The values of the within-day precision were 5–9% (n=5) for urine and 4–7% (n=5) for serum. The day-to-day precisions were 6–11% (n=5) for serum and 4–7% (n=5) for serum. Such RSD values are low enough to make the procedure acceptable for quantitative drug analysis studies [15].

Conclusions

The present SPE–CE procedure enables the quantification of basic drugs in biological fluids in a fully automated fashion. The sample procedure on an end-capped C2-bonded silica is used to (i) preconcentrate the analytes, (ii) remove the bulk of interfering salts and proteins, and also (iii) to remove the water, thereby allowing a final non-aqueous CE analysis. The analytical performance, which was tested for about 3 months, was fully acceptable and the method is well suited for drug monitoring.With the fully automated at-line SPE–CE and dialysis–SPE–CE procedures now having been established, basic, acidic and amphoteric compounds can be determined in urine and serum using a rapid sample preparation and aqueous or non-aqueous buffers. The set-up of a novel procedure can be performed rapidly because a similar approach can be used in all cases irrespective of the nature of the analytes.

References

[1] I. Bjønsdottir and S.H.J. Hanssen, Chromatogr. A, 711 (1995) 313.

[2] J.R. Veraart, C. Gooijer, H. Lingeman, N.H. Velthorst, and U.A.Th. Brinkman, J. Chromatogr. B, 719 (1998) 199.

[3] J.R. Veraart, C. Gooijer, H. Lingeman, N.H. Velthorst, and U.A.Th. Brinkman, J. Pharm. Biomed. Anal., 17 (1998) 1161.

[4] J.R. Veraart, C. Gooijer, H. Lingeman, N.H. Velthorst, and U.A.Th. Brinkman., Chromatographia, 44 (1997) 581.

[5] J.R. Veraart, C. Gooijer and H. Lingeman, Chromatographia, 44 (1997) 129.

[6] J.R. Veraart, H. Lingeman and U.A.Th. Brinkman, J. Chromatogr. A. 856 (1999) 483.

[7] M.Mazkar and S.R. Binder, J. Chromatogr. B., 497 (1989) 201.

[8] G.M. Meenan, S. Barlotta and M. Lehrer, J. Anal. Tox., 14 (1990) 273.

[9] T.J. Schroeder, J.J. Tasset, E.J. Otten and J.R. Hedges, J. Anal. Tox., 10 (1990) 221.

[10] K. Ensing, J.P. Franke, A. Temmink, X.H. Chen and R.A. de Zeeuw, J. For. Sci., 37 (1992) 460.

[11] G.L. Lensmeyer, D.A. Wiebe and B.A. Darcey, J. Chromatogr. Sci., 29 (1991) 444 .

[12] G.L. Lensmeyer and M.A. Evenson, Clin. Chem., 30 (1984) 1774.

[13] S.H. Preskorn, K. Leonard and C.J. Hignite, Chromatogr., 197 (1980) 246.

[14] S.H.Y. Wong, T. McCauley and C.J. Hignite, Chromatogr. B. 226 (1981)147.

[15] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman and S. Spector, J. Pharm. Sci., 309 (1992) 306.

4.4

Determination of phenprocoumon in plasma and urine using at-line solid-phase extraction – capillary electrophoresis

Summary

The use of capillary electrophoresis (CE) for the analysis of biological samples is rather problematic because of the large number of interferences present in the matrix. One of the possibilities to solve such problems is to couple solid-phase extraction (SPE) at-line with CE, a technique developed in our laboratory. In this study at-line SPE–CE is performed for the determination of the anticoagulant phenprocoumon in biological fluids. Plasma samples are injected after the addition of 1 vol. % of formic acid to release the drug from binding proteins, while urine samples can be directly injected. The procedure is linear between 0.2 and 30 μg/ml with a correlation coefficient, r^2, of 0.9996. The detection limit in plasma is 0.1 μg/ml, which is fully adequate in view of the concentrations, that have to be dealt with in practice. The phenprocoumon concentration in a plasma sample of a patient treated with the anticoagulant was 3.8 μg/ml.

Introduction

Capillary electrophoresis (CE) is rapidly gaining acceptance for routine analyses even though sample handling in e.g. bioanalyses still creates problems. Biological samples frequently contain high concentrations of organic and inorganic salts, proteins, particulate matter and many low-molecular-weight compounds. Moreover, the concentrations of the analytes of interest are usually low. Various approaches can be used for the clean-up of a sample and trace enrichment of the analytes, which are based on dialysis, chromatographic and/or electrophoretic principles. In this study, solid-phase extraction (SPE) coupled at-line to CE was the preferred option. To this end, an ASPEC sampling-handling device was used [1]. A fresh (disposable) cartridge was used for every run to avoid carry-over effects. The present study dealts with the determination of the anticoagulant phenprocoumon (for the chemical structure see figure 4.4.2), after oral administration, in plasma and urine. The total concentration of phenprocoumon in plasma of patients, who typically use a daily dose of 1.5–7.5 mg, ranges from 0.3 to 5.1 μg/ml [2]. Its therapeutic window in human plasma is between 0.2 and 5 μg/ml; at concentrations exceeding 5 μg/ml toxic effects are observed. Because of a rapid metabolism in the body, the parent compound is normally not found in urine [2]. The aim of the present study is to demonstrate the advantages of a fully automated procedure over current off-line procedures such as those reported in refs. [2–4].

Experimental

Chemicals and samples

Methanol, acetic acid, phosphoric acid, formic acid and acetonitrile were obtained from J.T. Baker (Deventer, The Netherlands). All chemicals used were of HPLC quality. Water was demineralized and distilled before use. Phenprocoumon was a gift of Roche Nederland (Mijdrecht, The Netherlands).

Urine was collected during three consecutive days from five healthy male volunteers, pooled, and frozen in small quantities at -18 °C. The plasma of healthy volunteers was a gift from the Academic Hospital of the Vrije Universiteit and was stored at -18 °C. The patient was treated orally with 5.0 mg of phenprocoumon a day and the plasma collected in heparinized tubes. The biological samples were stored for a maximum of 6 months at a temperature of -18 °C.

An acetonitrile/12.5 mM phosphate buffer of pH 4.5 (20/80 v/v) was used as SPE wash buffer. The desorption buffer consisted of acetonitrile/40 mM phosphate buffer of pH 7.8 (75/25 v/v). A 20 mM acetate buffer, pH 4.6/methanol (90/10 v/v) solution was used as CE buffer.

SPE equipment and procedures

SPE experiments were performed fully automated using an ASPEC system (Gilson, Villiers-le-Bel, France). LC-18 disposable cartridges (Supelco, Bellefonte, PA, USA) packed with 100 mg C-18 bonded silica material were used. The cartridges were washed with, subsequently, 2 ml of methanol and 2 ml of 10 mM phosphoric acid at a flow of 3 ml/min, prior the sample loading. In case of urine, 8 ml of sample were loaded without any pretreatment (at flow of 3 ml/min). For plasma samples, 1 ml was loaded at 1 ml/min. Before loading, the plasma samples were acidified with 1 vol. % of formic acid and after vortex mixing (1 min) the interferences were removed with 3 ml of wash buffer purged through the SPE column with 1 ml of air. For plasma samples the SPE cartridge was also flushed with 1 ml of 10 mM phosphoric acid solution before and after the washing procedure. For both urine and plasma, the analytes were desorbed with 400 µl of desorption buffer at 400 µl/min.

Interfacing SPE and CE

After completion of the SPE procedure, the ASPEC injected a volume of 190 µl out of 400 µl of desorption fluid into a 100 µl loop, constructed of PEEK tubing (0.25 mm I.D.), see figure 4.4.1. After switching the six-port valve, the loop content was flushed to the interface, which was essentially the same as that given for the at-line SPE–CE was described in an earlier paper [1]. The interface was constructed of 7 mm of green PEEK tubing (0.75 mm I.D., 1/16" O.D.) and 30 cm of red PEEK tubing (0.13 mm I.D., 1/16" O.D.), which were placed inside a piece of PTFE tubing with a length of 37 mm (1/16" I.D., 1/8" O.D.); details of the positioning can be seen in figure 4.4.1. One end of the red PEEK tubing was inserted over a length of 18 mm in the PTFE tubing; the other end was connected to the valve. The CE capillary was inserted into the green PEEK tubing and the electrode was placed on top of this tubing, (see insert of figure 4.4.1). Since, only a minor part of the contents of the loop can be injected into the CE capillary. Injection was performed during the period that the plug containing the highest concentration of analytes passed the tip of the capillary, which is between 0.2 and 0.5 min after switching of the 6-port valve. During the injection an underpressure of -40 mbar at the other end of the CE capillary was applied. The PrinCE and the ASPEC were electronically connected in order to synchronise the sample preparation, injection and CE procedures.

CE set-up

A PrinCE (Prince Technologies, Deventer, The Netherlands) CE system was used. The CE capillary (50 µm I.D., 375 µm O.D.), with a total length of 96 cm and an effective length of 40 cm (LC-Service, Emmen, The Netherlands). The CE system was equipped with a thermostating device set at 20 °C by blowing air around the capillary (35 m/s) [5]. The capillary was conditioned by flushing it with, successively,

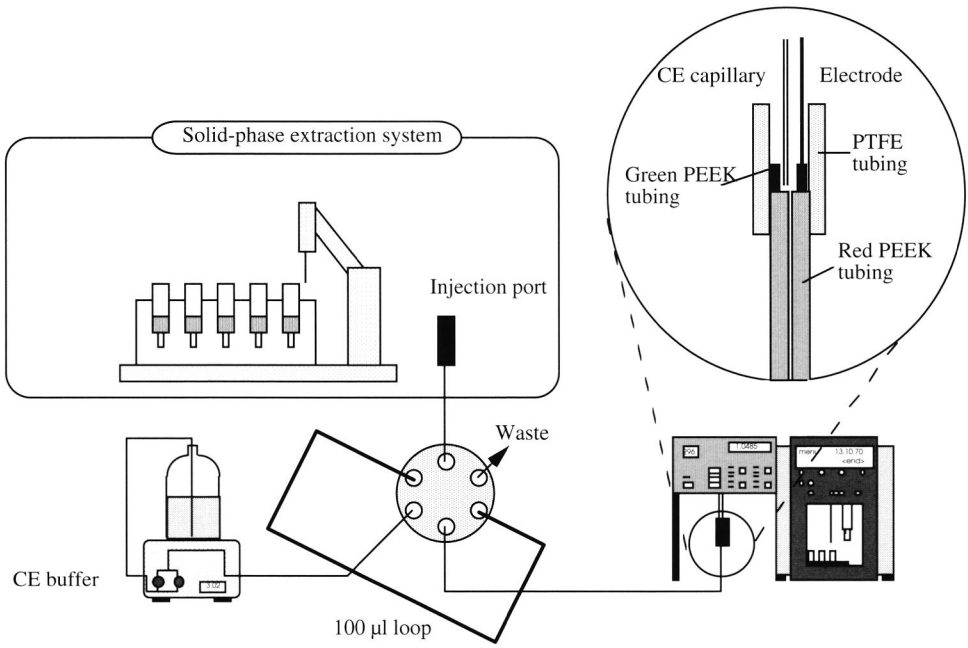


Figure 4.4.1 Schematic representation of the SPE–CE set-up.

1 M aqueous sodium hydroxide (5 min), water (15 min) and CE buffer (30 min); in all cases a pressure of 2000 mbar was used. Prior to each injection, the capillary was rinsed for 2 min with CE buffer. Analyses were performed using a voltage of -30 kV (the electrode in the interface was grounded) and detection was performed using a Model 759A UV/VIS absorbance detector, (Applied Biosystems, Foster City, CA USA), at 200 nm.

Results and discussion

Analysis of plasma samples

For the determination of the total phenprocoumon concentration in plasma 1 vol. % of formic acid has to be added to disrupt the drug-protein binding [1]. After vortex mixing, the SPE–CE procedure was used to determine phenprocoumon in the plasma of a healthy volunteer (blank and spiked with 3 µg/ml phenprocoumon) and of a patient treated orally with 5.0 mg of phenprocoumon a day. The electropherograms of figure 4.4.2 show a fully satisfactory separation of phenprocoumon from their sample constituents (traces I and II), and the absence of interfering compounds in the blank sample (trace III). The calibration curves were constructed in the range of 0.2–30 µg/ml, using eight data points (n=2). Linearity was good with a slope of 0.386 (std = 0.003), an intercept of -0.01 (std = 0.04) and correlation coefficient, r^2, of 0.9996. The

Figure 4.4.2 At-line SPE–CE electropherograms of plasma samples after addition of 1 vol. % of formic acid of plasma from I) a patient treated with 5.0 mg phenprocoumon a day, II) a healthy volunteer, spiked at 3 μg/ml and III) healthy volunteer.

linear dynamic range covers both the therapeutic range of phenprocoumon, which is between 0.25 and 5 μg/ml [2], and the toxic range, which is between 5 and 20 μg/l [2].

The detection limit of 0.1 μg/ml (signal to noise ratio, 3/1) is sufficiently low to enable the determination of phenprocoumon in plasma under real life conditions. In the plasma of the patient treated with the drug, the phenprocoumon concentration was found to be 3.8 μg/ml. This results with values reported in the literature [2].

Analysis of urine samples

No sample pretreatment was necessary for the analysis of urine samples; they could be directly applied to the SPE cartridge. The electropherograms of figure 4.4.3 show that the trace-level detection of phenprocoumon is straightforward. Due to the fact that larger samples can be processed (1 ml for plasma, 8 ml for urine) the detection limit is consequently better, i.e. 0.02 µg/ml. It is also obvious from figure 4.4.3, that there is no phenprocoumon in the urine of the treated patient, even though the plasma contained ca. 4 µg/ml. This can be explained by the fact that the drug is completely metabolized. It is known to be hydroxylated, the main metabolites being 4-, 6- and 7-hydroxyphenprocoumon [4]. Unfortunately, as far as we know these compounds are not commercially available. Consequently, no attempt could be made to establish their possible presence.

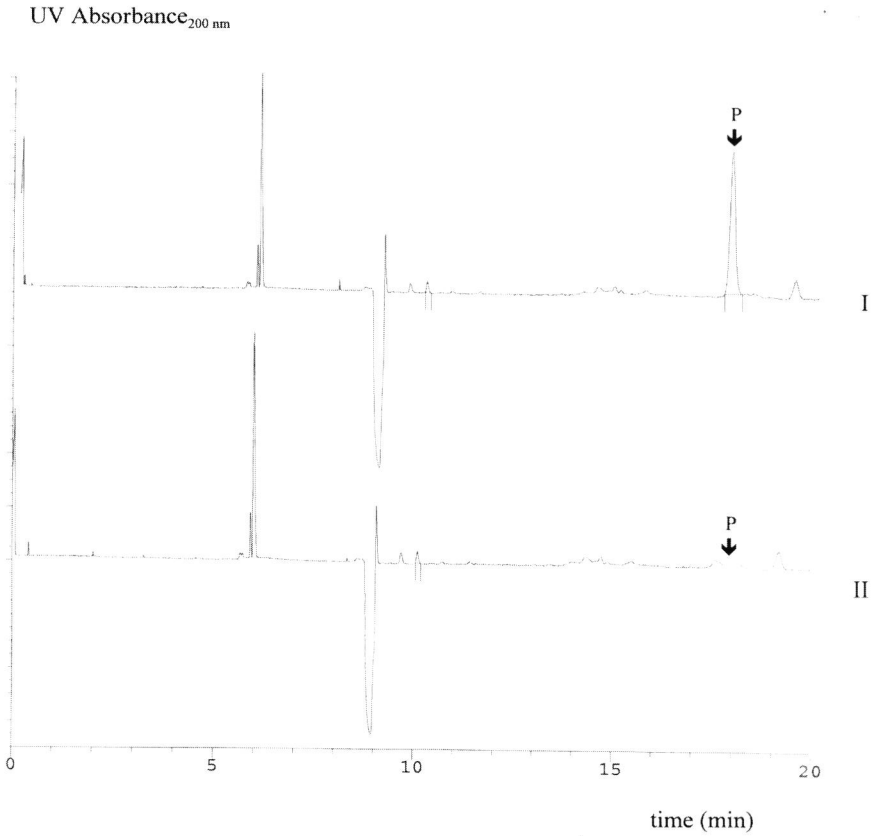


Figure 4.4.3 At-line SPE–CE electropherograms of urine samples from a patient treated with 5.0 mg phenprocoumon a day; I) spiked to a level of 3 µg/ml and II) non-spiked urine.

Conclusions

It has been shown that the fully automated at-line SPE–CE procedure can be applied for the quantitative determination of the anticoagulant phenprocoumon in plasma and urine. After the samples were placed in the ASPEC, the SPE procedure, transfer to the CE system and the CE analysis were performed automatically. This in contrast with procedures presented in the literature which includes several manual sample preparation steps. The sample throughput is 2 samples per hour. A capillary was used for the analyses of 250 samples before replacement. It was flushed after 50 samples sodium hydroxide solution. The SPE cartridges were used once for biological samples to avoid carry-over effects. No blocking of the loop, interface or capillary was found, and therefore, the system was successfully used for overnight experiments.

Plasma samples required the addition of formic acid, to disrupt the drug-protein binding, while urine could be analysed directly. The calibration curve and the detection limits in serum samples are fully adequate to cover the relevant toxic and therapeutic ranges. The concentration of phenprocoumon found in the plasma of a patient, and its absence in urine is in agreement with the literature data.

References

[1] J.R. Veraart, C. Gooijer, H. Lingeman, N.H. Velthorst, U.A.Th. Brinkman, J. Chromatogr. B., 719 (1998) 199.

[2] P.J. Brombacher, H.M.H.G. Cremers, M.J. Mol, P.H.J. Muijers, P.M. van der Plas and P.E. Verheessen, Clin. Chim. Acta, 75 (1997) 443.

[3] J.X. de Vries, R. Zimmermann, J. Harenberg, Eur. J. Clin. Pharmacol., 29 (1986) 591.

[4] J.X. de Vries, M. Simon, R. Zimmermann, J. Harenberg, J. Chromatogr. A, 338 (1995) 325.

[5] J.R. Veraart, C. Gooijer, H. Lingeman, N.H. Velthorst, U.A.Th. Brinkman, Chromatographia, 44 (1997) 581.

Chapter 5

Dialysis–Solid-phase Extraction Coupled on-line to Capillary Electrophoresis

5.1

On-line dialysis–solid-phase extraction coupled to capillary electrophoresis: determination of amphoteric solutes in biological samples

Summary

A fully automated dialysis–solid-phase extraction (SPE) sample preparation procedure is coupled on-line to capillary electrophoresis (CE) for the first time. The system is used to determine sulphonamides in serum and urine. The dialysis unit serves to remove proteins and particulate matter. Reconcentration of the analytes is performed with a small SPE column while simultaneously (in)organic salts and other interferences are removed. Finally, the analytes are desorbed and injected, via a home-made interface, into the CE system. Limits of detection (LOD) of 0.05–0.1 and 0.05–0.3 μg/ml are obtained in urine and serum, respectively. The within-day and between-day precisions are in the range of 2–6% and 3–8%, respectively, for a concentration of five times the LOD. The dialysis–SPE–CE system was used over a period of six months for the analysis of over 500 serum and urine samples without problems such as clogging of the CE capillary or SPE column.

Introduction

Capillary electrophoresis (CE) is a powerful separation technique which is, in particular, suitable for the analysis of biological samples. Because of the relatively high concentrations and wide variety of interfering solutes, an efficient sample treatment procedure is of major importance [1–3]. Different approaches are used to clean and/or concentrate this type of samples by means of chromatographic or electrophoretic techniques [1–3].

One possibility is to use a dialysis membrane to remove particulate matter and proteins. The cut-off membrane separates a donor from an acceptor compartment [4]. The sample is introduced into the donor channel. The non-protein-bound analytes will pass the membrane, while the higher-molecular-weight proteins will remain in the donor phase. Finally, an enrichment column serves to reconcentrate the analytes and, also, to selectively remove interferences. The main advantages of dialysis as sample pretreatment technique are the considerable clean-up which enables the determination of the free fraction of a drug and the robustness of the technique [4]. Therefore, this sample preparation technique is often coupled to LC for the analysis of biological samples [5].

In this study a dialysis unit is coupled on-line with CE, which is not presented so far, via the same home-made interface as described for the coupling of LC and CE [2], and SPE and CE [1, 3].

Experimental

Chemicals and samples

Decanoic acid and sodium dihydrogen phosphate were obtained from Merck (Darmstadt, Germany). Acetic acid, disodium hydrogen phosphate and sodium acetate were purchased from Riedel-de Haën (Seelze, Germany). Acetonitrile (ACN), phosphoric acid, tetrahydrofuran (THF) and methanol were obtained from J.T. Baker (Deventer, The Netherlands), and sulphadiazine (SDI), sulphamethizole (SMI), sulphamethoxypyridazine (SMP), sulphamerazine (SMR), sulphamethoxazole (SMX) and sulphaquinoxaline (SQX) were obtained from Sigma (St. Louis, MO, USA). All chemicals were of the highest purity available. Water was demineralized and distilled before use.

Urine was collected from five healthy volunteers on three subsequent days. The samples were pooled and divided in portions of 100 ml and frozen at -18 °C. Serum samples were obtained from the Academic Hospital of the Vrije Universiteit, pooled and divided into portions of 10 ml. Urine and serum samples were stored for a maximum of 6 months at -18 °C.

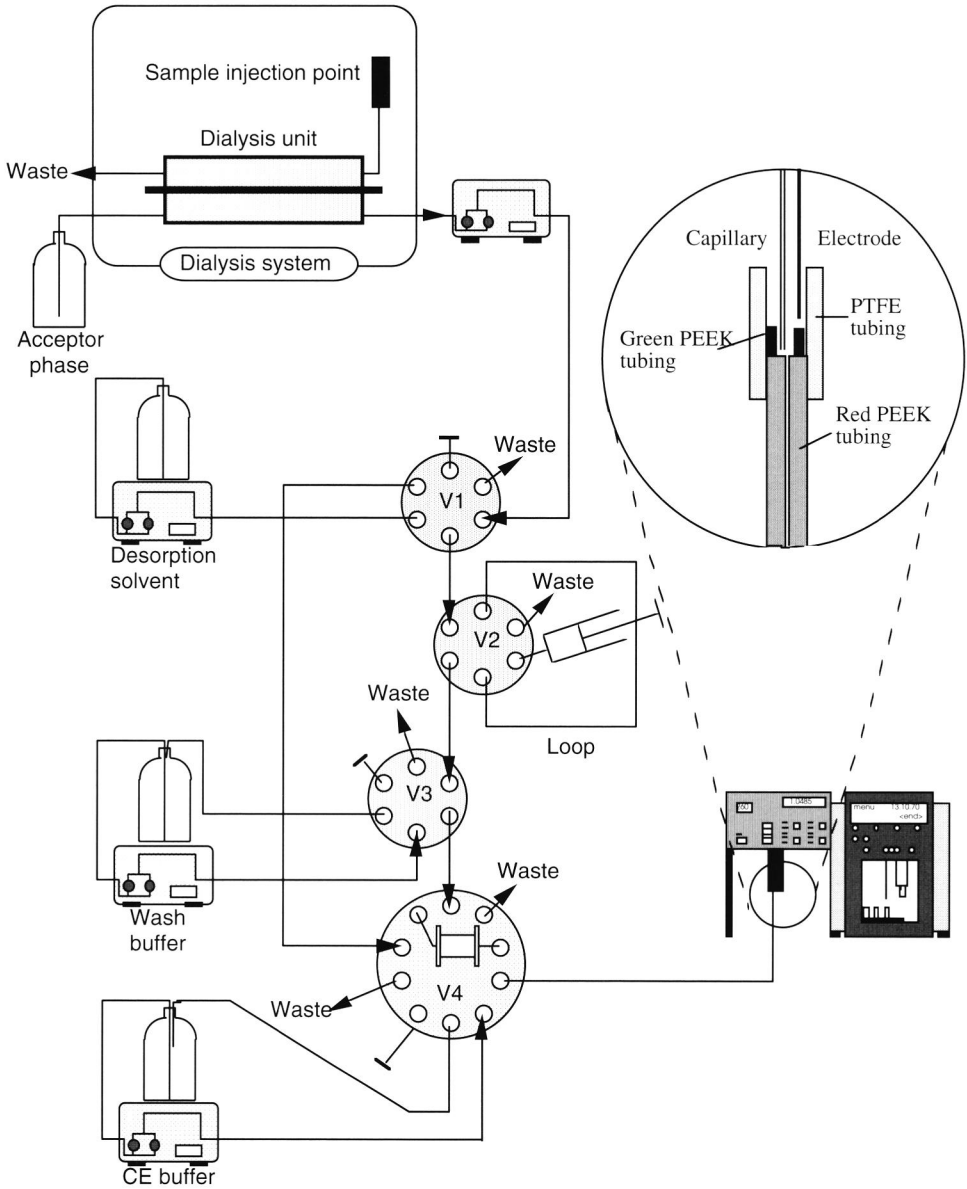


Figure 5.1.1 The dialysis–SPE–CE set-up. For details, see text.

Dialysis–SPE–CE set-up

The dialysis–SPE–CE set-up is shown in figure 5.1.1. The separate parts of the system are discussed below.

Dialysis.

The whole system was controlled by a dialysis unit (ASTED XL, Gilson, Villiers-le-Bel, France). A membrane with a cut-off value of MW 15,000, supplied by Gilson, was inserted in the dialysis block. First the donor channel (volume, 100 µl) was

washed with 3 ml of water and the acceptor channel (volume 170 µl) with acceptor solution (20 mM phosphate buffer, pH 3), both at a flow rate of 1 ml/min. Next, the donor channel was flushed with 0.4 ml of sample by means of a diluter equipped with a 1-ml syringe (0.36 ml/min). Next, the dialysis was started and acceptor phase was flushed continuously through the acceptor channel (flow rate 0.5 ml/min) to improve the dialysis efficiency. At the start of the dialysis the 0.6 ml of sample remaining in the syringe was injected into the donor channel, using a flow of 0.18 ml/min. Dialysis was performed for 10 min.

SPE.

The SPE system consisted of four switching valves, four high-pressure pumps and an SPE column as enrichment column to reconcentrate the analytes, which was a 50 x 2 mm stainless-steel column home-packed with 5 µm PLRP-S copolymer (Polymer Laboratories, Church Stretton, Shropshire, UK). After each run, the enrichment column was washed with 0.6 ml of desorption fluid (water–THF, 25/75 v/v) and 1.5 ml of acceptor solution at flow rates of 0.4 ml/min and 1.0 ml/min, respectively. As regards the switching valves, six-port valve V1 (Rheodyne, Cotati, CA, USA) was used to select the acceptor solution or desorption solvent, six-port valve V2, mounted on a MUST system (Spark Holland, Emmen, The Netherlands), was used to perform the injection of standard solutions onto the Enrichment column, six-port valve V3 was used to remove interferences from the enrichment column with the wash buffer (10 mM pH 6 phosphate buffer–acetonitrile, 90/10 v/v), and the ten-port valve V4 (VICI, Houston, TX, USA) was used to switch from the loading (loading of the column and simultaneously flushing CE buffer to interface) to the desorption (from column to interface) position. All valves were controlled by the dialysis unit. Two Model 2150 (LKB, Bromma, Sweden) pumps were used for the desorption fluid (flow, 0.4 ml/min) and CE buffer (flow, 0.2 ml/min). An Applied Biosystems (Foster City, CA, USA) pump was used for washing the enrichment column (flow, 1.0 ml/min), and a Model 305 Gilson LC pump for the acceptor solution.

Interfacing of SPE and CE.

Ten-port valve V4 was connected with the CE system via a home-made interface as which is depicted in the insert of figure 5.1.1. It was constructed from 7 mm of green PEEK tubing (0.75 mm I.D., 1/16" O.D.) positioned above a 30 cm piece of long red PEEK tubing (0.13 mm I.D., 1/16" O.D.) which were both placed inside a piece of PTFE tubing with a length of 37 mm (1/16" I.D., 1/8" O.D.). 18 mm of the red PEEK tubing were inserted in the PTFE tubing, while its other end was connected with valve V3. The CE capillary was inserted in, and the electrode on top of, the green PEEK tubing. The interface has been used before to interface LC and CE, on-line [2] and SPE and CE, at-line [1, 3]. After the dialysis–SPE procedure has been completed, the sample preparation unit sends a 'dialysis-ready' signal to the CE

system. Desorption from the dialysis–SPE system does not start until a 'CE-ready' signal is received. The valve is then switched and the analytes are desorbed from the enrichment column. After 20 s (Δt) they will reach the tip of the capillary. While the analyte-containing zone passes the tip of the CE capillary in the interface, injection is performed during 15 s by applying an under-pressure of 80 mbar at the other end of the CE capillary. Evidently, only a minor part of the SPE effluent will be introduced in the CE capillary; the remaining liquid is flushed to waste. The analytes are introduced in the outlet side of the CE capillary because that electrode was grounded and more easy assessible.

The CE set-up.

The CE experiments were performed using a PrinCE CE system (Prince Technologies, Emmen, The Netherlands). The CE capillary (50 µm I.D., 375 µm O.D.), with a total length of 105 cm and an effective length of 40 cm (LC-service, Emmen, The Netherlands), was equipped with a thermostating device (set at 20 °C), which blows air around the capillary (35 m/s) [6] to avoid Joule heating effects. The capillary was conditioned using a pressure of 2000 mbar with, subsequently, a 1 M sodium hydroxide solution (5 min), water (15 min) and 20 mM pH 7.0 phosphate buffer CE buffer (30 min). Before injection, the capillary was flushed with CE buffer. Analyses were performed using a voltage of -30 kV (the electrode in the interface was grounded) for 10 min and detection at 260 nm was performed using a Model 759A UV-VIS detector (Applied Biosystems).

Results and Discussion

To facilitate the presentation and discussions of the results, five topics are distinguished, i.e. (i) optimizing the CE buffer, (ii) optimizing the interfacing between enrichment column and CE unit, (iii) coupling of the dialysis unit to the SPE–CE unit and testing it injecting of standard solutions, (iv) analysis of biological samples and (v) validation of the total system.

CE buffer

The pH of the CE buffer is the most important parameter when optimizing a CE procedure for amphoteric compounds. The experiments were performed using 20 mM phosphate buffers with pH between 2 and 8, except for pH 3.8–5.8 where 20 mM acetate buffers were used. The plots of figure 5.1.2 show that the separation between the test compounds is strongly pH dependent. This is not unexpected in view of the fact that sulphonamides are characterized by two functional groups, one with a pK_a around 2 and the other with pK_a values ranging from 5 to 7 [7, 8]. This implies that

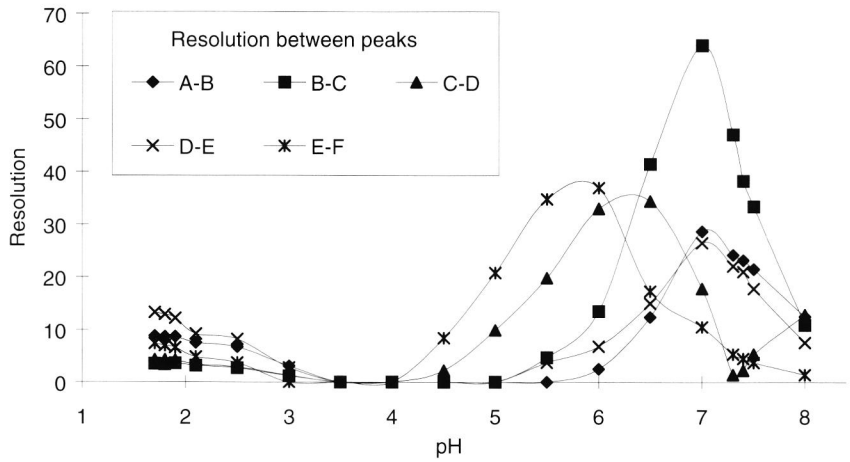


Figure 5.1.2 Resolution of the test compounds as a function of the pH of the CE buffer. The letters refer to the migration position in the electropherogram (A = first migrating peak, B = second migrating peak, *etc.*).

the sulphonamides will be essentially neutral at a pH of about 4, while they will be positively charged at lower pH values, and negatively charged at higher pH values. Since the values of the higher pK_a also depend rather strongly on the structure of the test analytes, and CE migration times depend on both charge and size, good separations can be obtained in the pH 5–7 region. This is in contrast with the situation at pH < 4 because of the closely similar values of the pK_a then involved. Sufficient resolution for all analyte pairs was observed for pH values of 6.5–7.0, and a buffer of pH 7 was used in all further experiments.

Interfacing of SPE and CE

Optimization of the interface between the enrichment column and the CE unit comprises selection of (i) an enrichment column, (ii) the desorption volume and timing and, (iii) the injection volume of the desorbed compounds into the CE capillary.

Enrichment column.

Because of the experimental conditions used, the sorbent of the enrichment column should quantitatively retain all analytes from at least 10 ml of dialysis acceptor solution. The breakthrough volumes on a 50 x 2 mm column packed with 5 μm PLRP-S copolymer were determined according to the procedure described in [9]. First, the column was washed with methanol (5 ml), water (5 ml) and 10 mM of pH 2 phosphate buffer (5 ml). Next, the column was loaded with a buffered solution containing 10 μg/ml of one of the test compounds, and the UV signal monitored at 260 nm. All analytes were studied separately. The pH dependence of the breakthrough

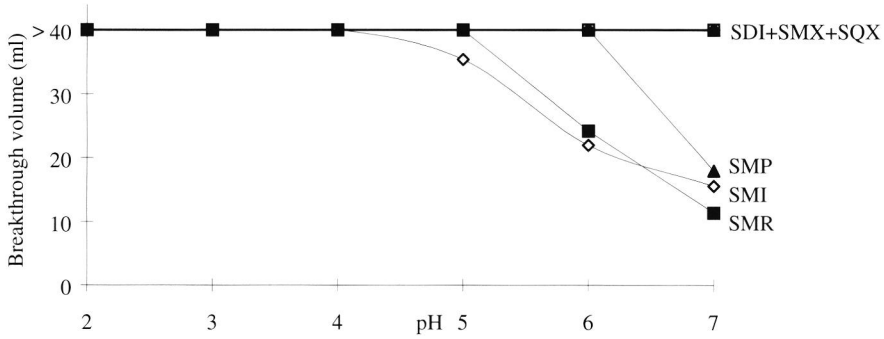


Figure 5.1.3 pH dependence of the breakthrough volumes of the sulphonamides on the PLRP-S column.

was determined using both 10 mM phosphate buffers (pH 2, 3, 6 and 7) and 10 mM acetate buffers (pH 4 and 5). Figure 5.1.3 shows that all six analytes have high breakthrough volumes at pH 4 or lower, while early breakthrough of some of them starts at pH 5–7. Obviously, the PLRP-S column can be used to reconcentrate the analytes.

Desorption volume and timing.

The analytes should be desorbed from the enrichment column in a volume which is as small as possible in order to maintain a high overall preconcentration factor. Furthermore, the electrical conductivity of the desorption solvent should be lower than that of the CE buffer, to allow injection of a large fraction of the desorption solvent into the CE system and to decrease the plug length when using a stacking procedure. A desorption solvent composed of THF–water (75/25 v/v) was selected. Although high percentages of other modifiers such as methanol and acetonitrile can also be used to desorb the analytes, THF was chosen to avoid bubble formation during the subsequent CE analysis.

As regards the time constraints in the final on-line system, three parameters have to be optimized: (i) the time needed to desorb all compounds quantitatively at an arbitrarily selected flow rate of 0.4 ml/min, (ii) the delay time, Δt, between the start of the desorption and the arrival of the desorbed solutes at the interface and (iii) the time of injection, t_{inj}, during which the analytes pass the tip of the capillary. To determine the desorption time, the enrichment column was conditioned with desorption solvent (1.2 ml) and, next, the acceptor buffer (3 ml) at 0.4 ml/min and 1 ml/min, respectively. Next, 100 µl of the test compounds in acceptor buffer (10 µg/ml each) were loaded on the enrichment column. When studying the desorption time, the other parameters, which were optimized later, were set at values which ensured the injection of all analytes in the CE system (Δt = 0 s, t_{inj} = 120 s and an injection underpressure of 10 mbar). Plots of the amounts of analyte eluted, expressed as peak

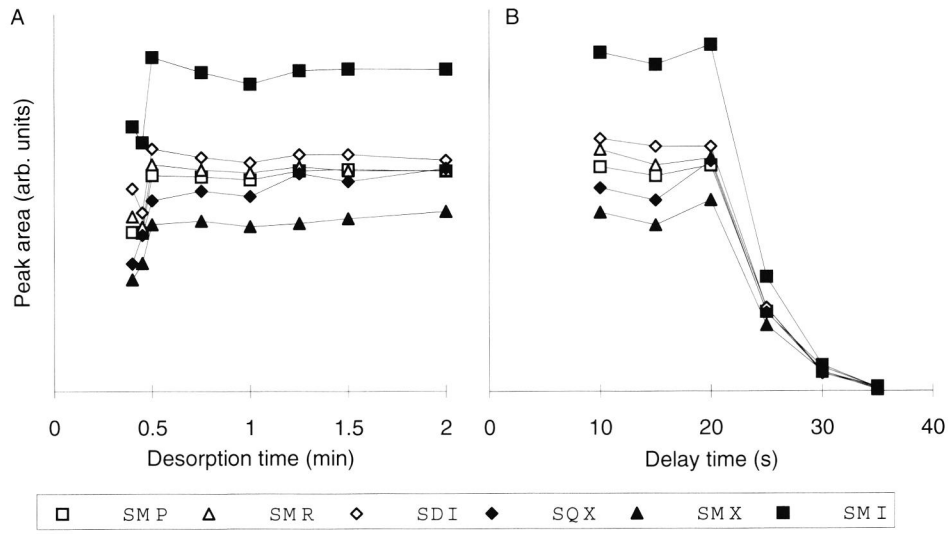


Figure 5.1.4 Influence of (A) desorption time and (B) delay time of injection on the peak area of the test compounds. For details, see text.

areas, versus the desorption time are shown in figure 5.1.4A. It is clear that desorption is complete after 30 s. Shorter desorption times give sharply decreased peak areas, which indicate non-quantitative desorption. A desorption time of 30 s with a flow rate of 0.4 ml/min was used in all further experiments.

Next, the values of Δt and t_{inj} had to be adjusted in such a way that only analyte-containing desorption solvent will be injected. The column was loaded with testcompounds, which were then desorbed and injected in the CE capillary using delay times increasing from 0 to 35 s. The injection time and underpressure were set at 120 s and 10 mbar, respectively. The peak area plots of figure 5.1.4B indicate that a plateau condition exists between 10 and 20 s. For Δt values of over 20 s, the peak areas of all test compounds decrease dramatically and they become zero for $\Delta t = 35$ s. In other words, the analytes pass the tip of the CE capillary between 20 and 35 s after switching valve V4; during this period (t_{inj}) injection into the CE capillary has to be performed. Values of $\Delta t = 20$ s and $t_{inj} = 15$ s were used in all further experiments.

Injection volume.

To achieve maximum analyte detectability, the volume of desorption solvent injected into the CE capillary should be as large as possible. In the next step, the analytes in this volume are concentrated by the sample stacking effect. However, the solvent volume that can be focused by sample stacking is limited: with a too large volume, additional band broadening will occur and a non-linear relation between the response and the applied underpressure will be found [1]. Since in the present instance the injection time has been optimized already (see above), the injection volume has to be increased by increasing the injection pressure. The results of an

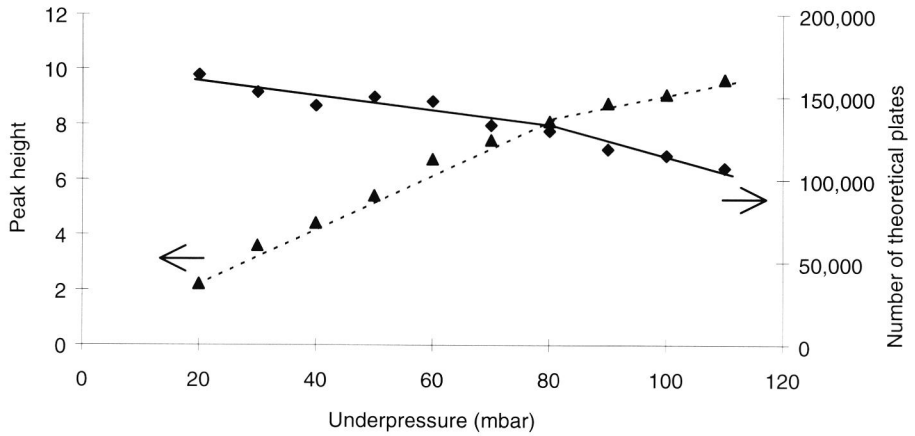


Figure 5.1.5 Peak height (dashed line) and efficiency (solid line) of sulphamethoxazole as a function of the injection underpressure.

increase from 20 to 110 mbar are shown in figure 5.1.5 for sulphamethoxazole. The peak height is seen to increase over the whole range of underpressures tested, but the increase is linear only up to about 80 mbar. Since there is simultaneously a loss of efficiency, an underpressure of 80 mbar was used in all further experiments.

Optimization of the dialysis system

The pH of the donor and acceptor phases can influence the recovery of ionisable compounds in the dialysis process, because differently charged compounds may react in different ways with a cellulose-based dialysis membranes [4]. Therefore, the pH was varied over the range of 2.0 to 7.0. Although the recovery of most test compounds was slightly lower for pH < 3 (data not shown), pH 3 was selected because of the larger breakthrough volumes of the analytes on the enrichment column (*cf.* figure 5.1.3).

To study the influence of the flow rate of the acceptor solution, the donor compartment was filled with a 10 µg/ml solution of the test compounds in pH 3 buffer. The dialysis time was varied from 1 to 15 min using acceptor flows of 0.25, 0.50 or 0.75 ml/min. The plots of figure 5.1.6 show that, whereas a flow rate of 0.25. ml/min results in the recoveries of only some 40% considerably higher recoveries are obtained at 0.50–0.75 ml/min. A flow of 0.5 ml/min was selected because a higher flow does not improve performance.

The response of the drugs can be further improved by increasing the amount of sample, e.g. by refilling the donor compartment during the dialysis procedure. First the donor compartment was filled with 0.4 ml (flow rate, 0.36 ml/min), out of the 1.0 ml of sample available. Next, dialysis was started by flushing the acceptor compartment with 20 mM phosphate buffer (pH 3) at 0.5 ml/min. At the same time the remaining 0.6 ml of sample was flushed through the donor compartment at a rate

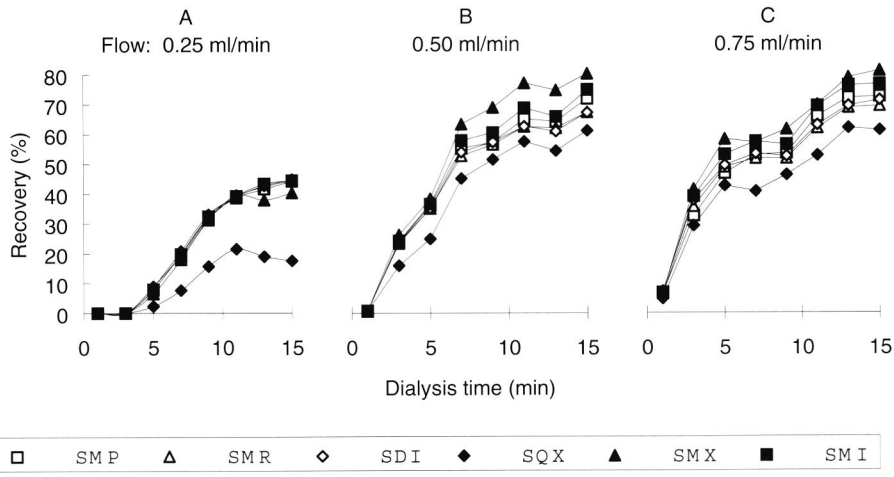


Figure 5.1.6 Analyte response versus dialysis time for different flow rates of the acceptor phase. For details, see text.

of either 0.18 or 0.36 ml/min which took either 3.4 or 1.7 min. In this way the concentration gradient over the membrane is improved. After 1.7 or 3.4 min the flow in the donor compartment was stopped, while flushing of the acceptor compartment was continued for thepredetermined time of up to 15 min. The data of figure 5.1.7B and 5.1.7C show that applying a acceptor flow of 0.18 ml/min provides the highest responses. The somewhat lower values found for 0.36 ml/min can be explained by the fact that the process is diffusion controlled: part of the analytes cannot reach the membrane before they are flushed out of the donor channel. However, at a donor flow of 0.36 ml/min the responses are higher than with a single injection (figure 5.1.7A)

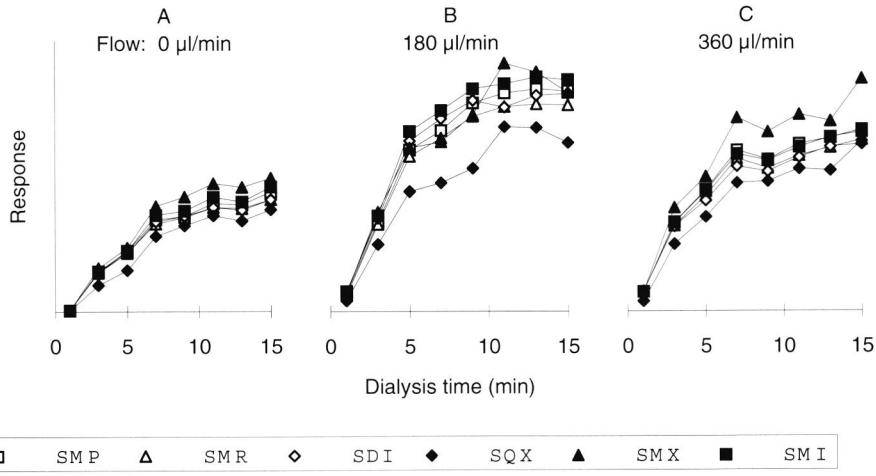


Figure 5.1.7 Analyte response versus dialysis time for different flow rates of the donor phase. For details, see text.

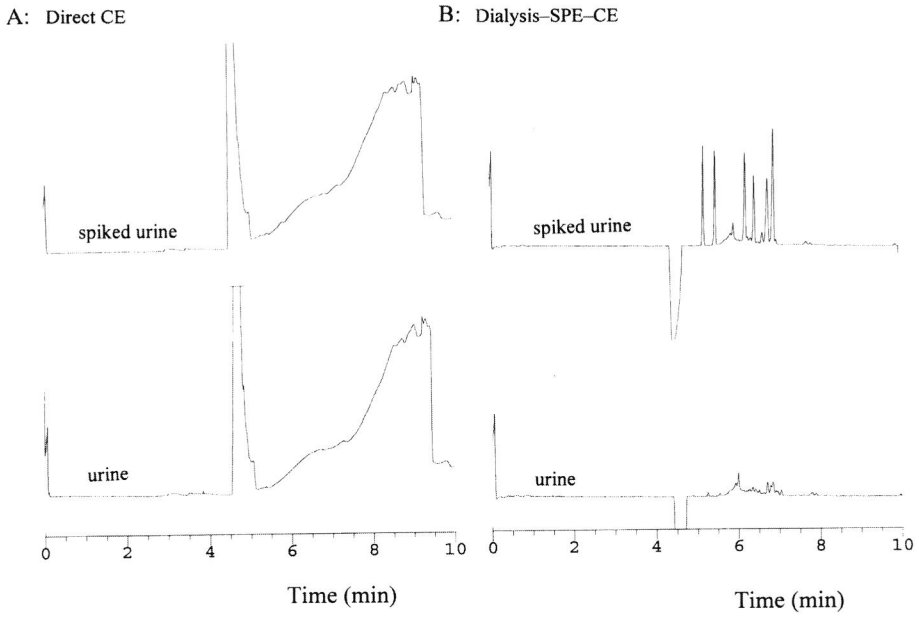


Figure 5.1.8 Electropherograms of spiked (10 µg/ml each test compound) urine and blank urine with direct CE and dialysis–SPE–CE. Migration order: SMP, SMR, SDI, SQX, SMX and SMI. UV absorbance detection at 260 nm.

because the total sample volume was 1.0 ml rather than 0.4 ml. An acceptor flow of 0.18 ml/min and a total dialysis time of 10 min were selected for further work.

Dialysis–SPE–CE of biological samples

As an introductory example, figure 5.1.8A shows that the direct CE analysis of urine is not possible because of the presence of a large number of endogenous compounds. When the analysis was performed with the complete dialysis–SPE–CE procedure outlined above, the electropherogram still contained too many interferences (data not shown). Further improvement was obtained by washing the enrichment column with water before elution of the analytes. The procedure was optimized with respect to volume (1–3 ml), amount modifier (acetonitrile 0–10%) and pH (4–7). A fully satisfactory result was obtained by using 2 ml of 10 mM acetate buffer (pH 6)–acetonitrile (90/10 v/v). At lower pH, and with a smaller volume of water or amount of acetonitrile, too many interferences were still present in the electropherograms. Higher pH values and larger volumes of water or amount of acetonitrile rapidly caused breakthrough of the analytes. Figure 5.1.8B shows the good performance now obtained for analysis of urine.

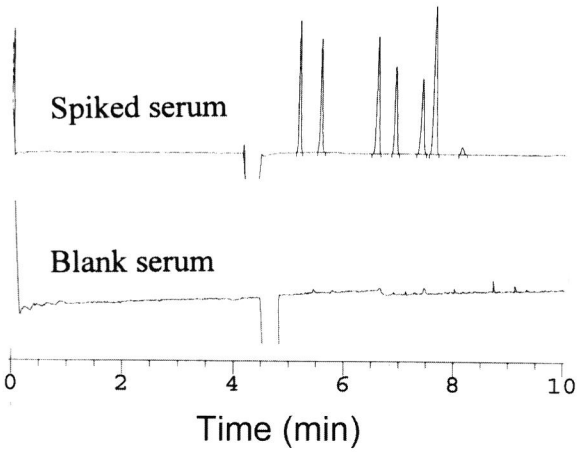


Figure 5.1.9 Electropherograms of spiked (10 μg/ml each test compound) and blank serum, both with adding an additive (8 mM decanoic acid and 20 vol.% acetonitrile). Migration order: SMP, SMR, SDI, SQX, SMX and SMI. UV absorbance detection at 260 nm.

The main problem that has to be dealt with in the analysis of serum is drug–protein binding. The percentage of drug–protein binding calculated by using the present dialysis–SPE–CE procedure for spiked samples (at a 10 μg/ml level) are shown in table 5.1.1. The results are closely similar to those reported in the literature. Even so the data reported by us should be considered as approximate values because drug–protein equilibria will shift during the dialysis process.

Several methods to release drugs from their proteins have been reported in the literature [12]. Well-known approaches are the addition of a competitor for the active

Table 5.1.1 Drug–protein binding of sulphonamides according to the literature and determined via with dialysis–SPE–CE.

Analyte	Drug–protein binding (%)[a]:		
	Lit. [10]	Lit. [11]	This study
SDI	20-50	45	40
SMI	90	85	85
SMP	85	90	75
SMR	60-80	-	65
SMX	65	60	65
SQX	-	-	99

[a] the standard deviations are typically ± 5% for all results

Table 5.1.2 Influence of modifiers on the recovery of sulphonamides from serum in dialysis–SPE–CE.

Analyte	Addition of			
	none	0.5 mg/ml sulphacetamide	20 vol% ACN and 0.5 mg/ml sulphacetamide	20 vol% ACN and 8 mM decanoic acid
SDI	60	65	60	75
SMI	15	35	35	75
SMP	25	50	50	80
SMR	35	60	55	80
SMX	35	55	55	75
SQX	<1	3	5	60

site (such as, in this case, sulphacetamide), or the addition of a compound such as decanoic acid that changes the physicochemical properties of the analyte or the bonding protein (e.g., degree of protonation, spatial structure, solvatation) [12]. As can be seen from table 5.1.2, the addition of decanoic acid in combination with acetonitrile (20 vol.%) resulted in the highest recoveries. Therefore, this alternative was used for the analysis of serum. Typical electropherograms of spiked and blank serum using dialysis–SPE–CE are shown in figure 5.1.9.

Validation of the dialysis–SPE–CE system

Limits of detection (LOD) of the test compounds in both serum and urine are shown in table 5.1.3. The LODs in urine (50–100 ng/ml) are slightly lower than those in serum (50–300 ng/ml), which is explained by the residual protein binding of the sulphonamides. Calibration curves of the test compounds were linear from the LOD up to 10 μg/ml in both sample types (table 5.1.3). The precision was studied at analyte concentrations of five times the LOD. Table 5.1.3 shows RSD values of 2–6% for the within-day precision and 3–8% for the day-to-day precision. These are fully satisfactory results.

Over a period of six months, over 500 samples were subjected to dialysis–SPE–CE without problems such as clogging of capillary or enrichment column. The procedure was successfully used for overnight runs. The dialysis membrane was replaced every 250 samples.

Table 5.1.3 Quantitative data on dialysis–SPE–CE of sulphonamides in biological fluids.

Analyte	Calibration curve[1]			LOD	RSD[2]	
	intercept (std)	slope (std)	correlation coefficient	(ng/ml)	within-day	day-to-day
Urine						
SDI	0.00 (0.02)	0.24 (0.003)	0.9991	100	2.5	3.8
SMI	-0.01 (0.01)	0.16 (0.001)	0.9997	50	2.9	4.7
SMP	-0.01 (0.02)	0.23 (0.004)	0.9988	100	2.7	4.6
SMR	0.03 (0.02)	0.29 (0.003)	0.9993	100	3.2	4.4
SMX	0.18 (0.03)	0.20 (0.006)	0.9950	50	1.8	2.5
SQX	0.01 (0.01)	0.20 (0.002)	0.9997	50	5.4	5.9
Serum						
SDI	0.00 (0.02)	0.22 (0.004)	0.9984	100	4.8	6.8
SMI	0.01 (0.03)	0.25 (0.006)	0.9970	100	4.2	5.4
SMP	0.01 (0.02)	0.20 (0.004)	0.9982	100	3.2	7.1
SMR	0.01 (0.02)	0.21 (0.004)	0.9977	100	5.1	6.5
SMX	0.01 (0.01)	0.21 (0.002)	0.9983	100	2.7	4.5
SQX	-0.01 (0.01)	0.09 (0.003)	0.9947	300	6.4	8.1

[1] Calibration curve from LOD to 10 µg/ml with 7 data points in duplicate.

[2] Within-day, n=5 at 5xLOD; day-to-day, n=5 on five successive days.

Conclusions

A fully automated dialysis–SPE–CE set-up is presented for the determination of amphoteric drugs in biological fluids. To our knowledge, it is the first time that such a sample pretreatment coupled to CE has been developed which includes removal of the (in)organic salts, particulate matter and interferences, reconcentration of the analytes, and release of the drugs from their protein bonds. For six sulphonamides as test compounds and urine and serum as biological matrices, the detection limits were around 100 ng/ml in all instances. Linearity was good over about two orders of magnitude and the day-to-day and within-day precision data were much better than 15%, which is an acceptance value for quantification in biomedical analysis [13]. Therefore, on-line dialysis–SPE–CE can be recommended for both serum and urine samples, i.e. for clinical studies and in therapeutic drug monitoring.

The same set-up can be used for the determination of other classes of compounds. The only changes needed are adaptation of the buffer composition, the injection timing parameters and the injection pressure.

References

[1] J.R. Veraart, C. Gooijer, H. Lingeman, N.H. Velthorst en U.A.Th. Brinkman, J. Chromatogr. A, 856 (1999) 483.

[2] J.R. Veraart, C. Gooijer, H. Lingeman, N.H. Velthorst and U.A.Th Brinkman, Chromatographia, 44 (1997) 581.

[3] J.R. Veraart, C. Gooijer, H. Lingeman, N.H. Velthorst en U.A.Th. Brinkman, J. Pharm. Biomed. Anal., 17 (1998) 1161.

[4] N.C. van de Merbel, J.M. Teule, H. Lingeman and U.A.Th. Brinkman, J. Pharm. Biomed. Anal., 10 (1992) 225.

[5] N.C van de Merbel, J.J. Hageman and U.A.Th. Brinkman, J. Chromatogr., 634 (1993) 1.

[6] J.R. Veraart, C. Gooijer and H. Lingeman, Chromatographia, 44 (1997) 129.

[7] M.C. Ricci and R.F. Cross, J. Microcol. Sep., 5 (1993) 207.

[8] H. Stober and W. de Witte, Analytical profiles of drug substances, Amer. Pharm. Assoc., 11 (1982) 523.

[9] C.E. Werkhoven-Goewie, U.A.Th. Brinkman and R.W. Frei, Anal. Chem., 53 (1981) 2072.

[10] Informatorium Medicatorium, Kon. Ned. Maatsch. Bevord. Pharm., 's-Gravenhage, The Netherlands, Part I, (1990) 139.

[11] W. Lammers, F.A. Nelemans, Th.J. Bouwman, J. van Noordwijk, L. Offerhaus, W.M. Rosinga and A. Vermeulen, Algemene Farmacotherapie, (1980) 672.

[12] W.E. Lindup, in J.W. Bridges, L.F. Chasseaud and G.G. Gibson (Eds.), 'Progress in Drug Metabolism', Vol. 10, Taylor and Francis, New York, (1987) 141.

[13] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman and S. Spector, J. Pharm. Sci., 309 (1992) 306.

5.2

On-line dialysis–SPE–CE of acidic drugs in biological samples

Summary

A fully automated method is presented for the determination of acidic drugs in urine and serum using on-line dialysis–solid-phase extraction (SPE)–capillary electrophoresis (CE) with UV detection. With non-steroidal anti-inflammatory drugs (NSAIDs) as test compounds, detection limits in the biological samples were 0.05–1.0 μg/ml. Calibration plots were linear over two orders of magnitude and the within-day and between-day repeatability were better than 10%. The CE capillary and SPE column were used for over 500 analyses; the dialysis membrane was replaced after 250 analyses.

A general protocol for dialysis–SPE–CE was devised which can be used for amphoteric and acidic drugs. The present experiences show that this protocol has rather general validity and can be recommended for future work on other classes of drugs.

Introduction

Capillary electrophoresis (CE) has a high separation power and can be used for the separation of charged analytes as present in biological samples. Unfortunately, such matrices contain quite a number of compounds which can interfere with the analysis, such as salts, proteins and particulate matter. These compounds must be removed prior to injection by applying a proper sample preparation procedure. Off-line methods like liquid–liquid extraction (LLE), precipitation and off-line solid-phase extraction (SPE) are most frequently used. These are, however, rather time-consuming and degradation of the analytes can occur because of (nearly) irreversible adsorption effects and if solvent evaporation steps cannot be avoided. However, in bioanalysis, large numbers of samples often have to be processed, which means that automated procedures are preferred.

Papers describing CE procedures for the determination of NSAIDs are mainly directed at the separation of chiral isomers [2, 3], aqueous standards [4], and pharmaceutical formulations [5]. The determination of NSAIDs using CE in serum was reported using off-line sample preparation methods like LLE [6, 7] and protein precipitation [8]. Detection limits varied from 1 to 10 µg/ml [7, 8] which are not sufficient for therapeutic drug monitoring (for data, see table 5.2.1).

The final result, a fully automated method for the direct determination of NSAIDs in biological samples, is presented for the first time.

In a recent study, on-line dialysis–SPE–CE was presented for the first time for the determination of sulphonamides in urine and serum [1]. Dialysis is applied to remove proteins and particulate matter. The analytes are diluted during this procedure; therefore, in a second step they are trapped on an SPE column while salts are removed simultaneously. During the following washing step, compounds that will interfere during the CE run can be removed. Finally, the analytes are desorbed and injected into the CE capillary via a home-made interface. In the present paper, it will be shown that this set-up and the optimization strategy in general can be used also to determine other compounds classes such as strongly acidic drugs, with non-steriodal anti-inflammatory drugs (NSAIDs) as test compounds.

Table 5.2.1 Therapeutical level of the NSAIDs in serum and their absorbance maximum.

Compound	Concentration (µg/ml)	Absorbance maximum (nm)
Ibuprofen	25 – 50	264
Naproxen	25 – 70	273
Fenoprofen	20 – 50	273
Ketoprofen	0.5 – 6	261
Flurbiprofen	2 – 12	247

Experimental

Chemicals and samples

Ibuprofen, naproxen, fenoprofen, ketoprofen and flurbiprofen were obtained from Sigma (St. Louis, MO, USA). Acetonitrile, boric acid, methanol and phosphoric acid were obtained from J.T. Baker (Deventer, The Netherlands). n-Decanoic acid, sodium dihydrogen phosphate monohydrate and disodium hydrogen phosphate 12-hydrate came from Merck (Darmstadt, Germany). Acetic acid and sodium acetate were from Riedel-de Haën (Seelze, Germany). Water was demineralized and distilled before use. In all cases, chemicals of the best available quality were used.

Urine was collected from five healthy volunteers during three subsequent days. The samples were pooled and divided into 100-ml portions and frozen at –18 °C. Bovine serum of untreated objects was purchased from Sigma; it was divided into 10-ml portions and frozen at -18 °C. The biological samples were stored for a maximum period of three months.

Methods

The set-up of the dialysis–SPE–CE system and the instruments used were described in detail in ref. [1]. A schematic of the set-up is presented in figure 5.1.1. The experimental parameters are discussed in the next section and are summarized in table 5.2.2.

Results and discussion

The main aim of the present project was to devise a generally valid optimization strategy for dialysis–SPE–CE. An overview of all relevant parameters is given in table 5.2.2; data related to an earlier first attempt (i) are included for the sake of convenience. Because of practical aspects, several parameters were kept the same. These included the sample injection into the dialysis block (which can certainly be improved if there would be insufficient analyte detectability), the dimensions of the CE capillary, and the CE capillary rinsing procedure. In the next paragraphs, the method development steps are briefly introduced and the results found for the NSAIDs are presented and, if relevant, compared with that of the sulphonamides.

i. The CE analysis is optimized. The first choice of buffer composition is a relatively high pH to ensure complete ionization of the analytes. If co-migration is observed, optimization is carried out using pH values closer to the pKa values of the analytes dealt with.

Table 5.2.2 Experimental conditions used for NSAIDs (this study) and sulphonamides [1].

Determination of:	NSAIDs	Sulphonamides
Sample treatment		
Urine	None	
Serum, free concentration	None	
Serum, total concentration	Addition of 20% ACN + 8 mM decanoic acid	
Dialysis		
Dialysis block	Donor phase 100 µl, acceptor phase 170 µl	
Membrane	Cellulose acetate, MWCO[1] 15 kDa	
Sample injection	0.4 ml prior to starting dialysis and 0.6 ml at 0.18 ml/min after starting dialysis	
Acceptor solution	10 mM phosphate buffer, pH 2	20 mM phosphate buffer pH 3
Acceptor solution flow rate	1.0 ml/min	0.5 ml/min
Dialysis time urine	10 min	9 min
serum	19 min	9 min
SPE		
Column	20x2.1 mm, 5 µm C18	50x2 mm, 5 µm PLRP-S
Desorption buffer	27 mM phosphate buffer pH 7 – ACN (30/70, v/v)	water – THF (25/75, v/v)
Desorption buffer flow rate	0.2 ml/min	0.4 ml/min
Wash buffer	12.5 mM phosphate buffer pH 4.5 – ACN (80/20, v/v)	10 mM phosphate buffer pH 6 – ACN (90/10, v/v)
Wash buffer flow rate	1.0 ml/min	
Wash time	1.5 min	2.0 min
SPE–CE interfacing		
Interface	home-made interface	
CE buffer flow rate	0.2 ml/min	
Δt[2]	18 s	20 s
t_{inj}[3]	18 s	15 s
CE		
Capillary	Bare silica, 50 µm i.d., 375 µm o.d. total length: 106 cm, effective length: 40 cm	
Capillary rinsing	2000 mbar for 2 min	
Injection pressure	- 80 mbar during t_{inj}	
CE buffer	50 mM acetate buffer pH 4.6 – MeOH (90/10, v/v)	20 mM phosphate buffer pH 7.0
UV detection at	200 nm	260 nm
Analysis time	15 min	10 min

[1] MWCO, molecular weight cut-off.

[2] Δt, the time between start of elution and analytes passing the tip of the CE capillary.

[3] t_{inj}, total length of time in which the analytes pass the tip of the CE capillary.

With the NSAIDs, complete resolution required pH adjustment to 4.6, a value very close to the pKa of all test analytes; furthermore, 10 vol.% methanol had to be added to reduce the electroosmotic flow and to improve the separation.

NSAIDs have absorption maxima in the 247–273 nm range (*c.f.* table 5.2.1), but the absorption wavelength of the maxima differ considerably. Therefore, to detect all analytes, 'end absorption', i.e. detection at 200 nm had to be used, a wavelength choice which of course, adversely effects the selectivity of the detection.

ii. The composition of the dialysis acceptor solution and the type of SPE cartridge largely determine the selection range of most of the other parameters. For example, the acceptor solution should not contain compounds which can interfere in the CE analysis (such as e.g. cationic ion-pairing reagents). To facilitate dialysis and increase analyte retention on hydrophobic SPE sorbents, it should also have a pH at which the analytes are neutral. As regards analyte trapping, in many instances the use of a small SPE cartridge with 20 x 2.1 mm I.D. dimensions packed with a 5 μm sorbent is a good choice. To be on the safe side, the SPE cartridge should have a breakthrough volume for the analytes, that is sufficient to retain them out of twice the volume of acceptor phase generated during the dialysis step (typically 5 – 20 ml).

For obvious reasons, an acceptor solution with low pH was selected for the NSAIDs. No breakthrough then occurred for any analyte after loading up to 50 ml of a pH 2 phosphate buffer, when using C18-bonded silica as SPE sorbent. This is in marked contrast with the sulphonamides which required a hydrophobic styrene–divinylbenzene copolymer, PLRP-S, to achieve sufficient retention.

iii. Interfacing of the SPE and CE involves three steps, desorption of the analytes from the SPE column, their transport to the interface, and their injection into the CE capillary. A critical aspect in SPE–CE interfacing is the selection of an organic desorption buffer which, preferably, should have a low ionic strength to ensure a good stacking effect in the CE system.

In order to desorb the analytes in a relatively small volume, at least 70 vol.% of acetonitrile should be added to the desorption buffer. In addition the pH should be increased to 7 to ensure that all analytes are fully deprotonated during the desorption step. Because of the relatively small volume of the SPE cartridge, the time between starting the elution from the SPE cartridge and the analytes passing the tip of the CE capillary, Δt, and the total time in which the analytes pass of the tip of the CE capillary, t_{inj}, are rather critical. Since a decrease of t_{inj} requires a significantly higher pressure during a short time during injection, the repeatability of the procedure will be adversely affected. As a result the maximum desorption flow rate is 0.2 ml/min.

iv. To improve sensitivity, more sample can be injected. Because the time during which injection is performed, has already been set (t_{inj}; *c.f.* above), the only way to inject more analyte is to increase the pressure during injection. Optimization can

be performed by injecting increasing amounts of sample and plotting the peak height versus the injection pressure.

With our set-up, analyte detectability can be improved by injecting at pressures of up to 80 mbar without a dramatic loss of efficiency due to band broadening. Increasing the pressure 8-fold resulted in an 8-fold increase of the peak heights, with a concomitant plate number loss of only 20%.

v. Optimization of the dialysis step usually involves the selection of the proper (often pulsed-type) dialysis mode and the sample volume subjected to analysis. As regard the mode pulsed dialysis is often preferred since it effects in an improvement of concentration detection limits. To facilitate the comparison of the protocol for NSAIDs and sulphonamides, these above parameters were kept the same as in ref. [1]. However, because of the good retention of the NSAIDs on the SPE cartridge, a somewhat higher acceptor solution flow rate was used to decrease the dialysis time, the main danger of early analyte breakthrough being negligible (*c.f.* above).

vi. The dialysis–SPE–CE–UV system is used for the analysis of urine. If too many interferences show up in the electropherogram, the SPE cartridge has to be subjected to a wash step prior to desorption of the analytes. The volume of the wash solution, its pH (often around the pKa values of the analytes) and the fraction of organic modifier (more rapid removal of interferences vs. increasing loss of analytes) are the main variables.

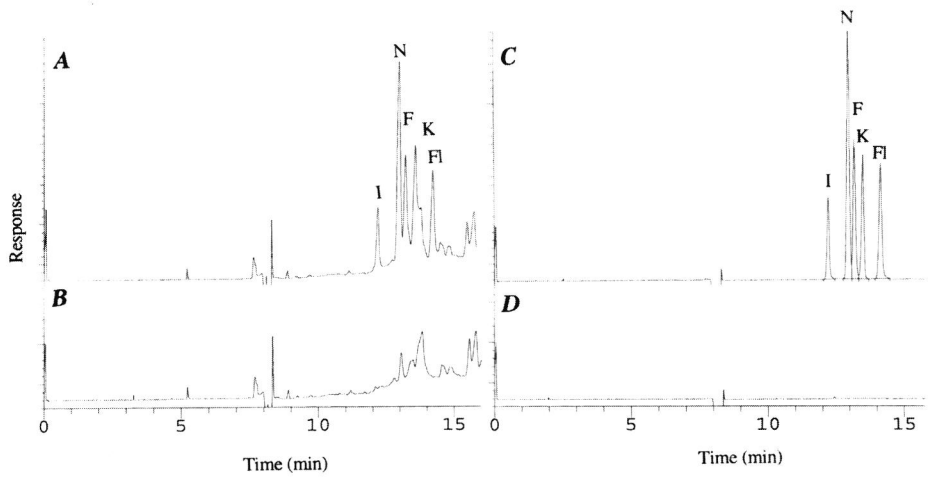


Figure 5.2.1 On-line dialysis–SPE–CE of blank (B and D) and spiked (10 µg/ml level of each NSAID) (A and C) urine. No washing of the SPE cartridge after loading (A and B) or washing with 1.5 ml of 12.5 mM phosphate buffer, pH 4.5 – acetonitrile (80/20 v/v) (C and D).

Figure 5.2.1A and B show that quite a number of interferences show up in the analyte window if no a wash step is included. The beneficial effect of a 1.5 min clean-up procedure (in presence of 10 vol.% of organic modifier and with a phosphate buffer of the expected pH value becomes obvious from figures 5.2.1C and D.

vii. The analysis of serum generally requires a somewhat modified strategy because of the more complex sample configuration and its higher viscosity (which tend to affect analyte detectability) and drug–protein bonding. If total drug concentrations in serum have to be determined, a significant amount of an organic solvent and/or a properly selected displacer have to be added to the sample to disrupt the drug–protein bonding.

For the acidic drugs concerned, the addition of acetonitrile and decanoic acid (final concentrations of 20 vol.% and 8 mM, respectively) was found to give the best results. If such a step has to be included in the final protocol, it is recommended to reoptimize the duration of the dialysis procedure. For the NSAIDs, the optimum was found to be 19 min. The increase of the dialysis time can probably be attributed to a rather unfavourable drug–protein equilibrium which is shifted during the dialysis procedure. With serum, a wash step was also necessary, even though the situation is less critical than with urine (figures 5.2.2A and B). When the same washing procedure was applied as for urine, fully satisfactory results were obtained (figures 5.2.2C and D).

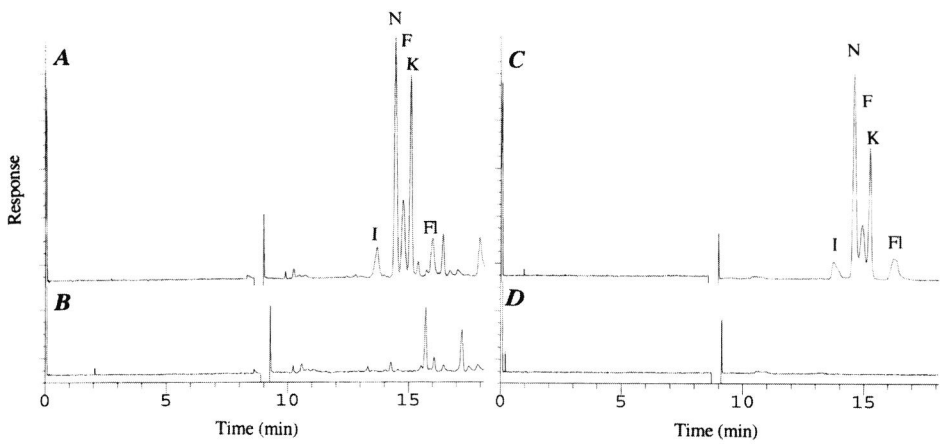


Figure 5.2.2 On-line dialysis –SPE–CE of blank (B and D) and spiked (10 µg/ml level of each NSAID) (A and C) serum. No washing of the SPE cartridge after loading (A and B) or washing with 1.5 ml of 12.5 mM phosphate buffer, pH 4.5 – acetonitrile (80/20 v/v) (C and D).

Table 5.2.3 Calibration data for the determination of NSAIDs using dialysis–SPE–CE. Concentration range: detection limit–10 µg/ml for urine and detection limit–100 µg/ml for serum.

Analyte	Detection limit (µg/ml)	Within-day precision (%)[1]	Between-day precision (%)[1]	Intercept (STD)	Slope (STD)	Correlation coefficient (r^2)
Urine (n=7–8)						
Ibuprofen	0.10	5.9	6.5	-0.03 (0.01)	0.46 (0.002)	0.9997
Naproxen	0.05	1.5	4.0	0.06 (0.05)	1.47 (0.01)	0.9992
Fenoprofen	0.05	2.5	3.6	0.01 (0.02)	0.84 (0.004)	0.9997
Ketoprofen	0.10	2.2	5.1	0.00 (0.05)	0.69 (0.01)	0.9973
Flurbiprofen	0.10	8.0	4.7	-0.01 (0.02)	0.73 (0.004)	0.9997
Serum(n=7–10)						
Ibuprofen	1.00	3.5	6.3	-0.08 (0.04)	0.10 (0.001)	0.9997
Naproxen	0.10	3.0	5.6	-0.04 (0.11)	0.52 (0.003)	0.9997
Fenoprofen	0.50	2.8	8.5	-0.13 (0.10)	0.20 (0.002)	0.9991
Ketoprofen	0.20	4.6	7.7	0.03 (0.11)	0.29 (0.003)	0.9992
Flurbiprofen	1.00	6.1	8.8	-0.10 (0.08)	0.12 (0.002)	0.9988

[1] determined using a concentration of 5 times LOD.

Finally, the optimized procedure for the determination of the NSAIDs was validated with serum and urine. The detection limits were 0.05–0.1 µg/ml for urine and 0.1–1.0 µg/ml for serum (table 5.2.3). As indicated above the higher detection limits in serum can be attributed to small losses caused by drug–protein binding and to a somewhat higher background. Calibration curves were taken from the detection limits up to 10 mg/ml(urine) or 100 mg/ml (serum). They were linear (table 5.2.3) and cover the therapeutical levels in serum (0.5 – 70 mg/ml) listed in table 5.2.1. The RSD values of the within-day and between-day precision for urine were < 8% and for serum, < 9% (table 5.2.3). This is significantly better than the value of 15% which is an often used acceptance level for quantification results of biological samples [9].

Obviously, the present protocol can be used to optimize dialysis–SPE–CE procedures for the low-mg/ml determination of different classes of drugs. Since the set-up is fully automated, analyses can be and were indeed, run unattendedly. The CE capillary and SPE cartridge were used without any problems for over 500 analyses. The dialysis membrane was replaced every 250 analyses.

Conclusions

The present study shows that the optimization procedure earlier used for sulphonamides can be applied also for other classes of drugs: there are, of course, differences in the numerical values found for several parameters, but no major changes occur. However, as we will show in the next paper, complicating effects do occur if unusual CE conditions are encountered. This was observed when cationic drugs were studied and a non-standard CE buffer had to be used as is discussed in 5.3 of this thesis [10].

In this paper on-line dialysis–SPE–CE of NSAIDs with UV detection was performed in a fully automated way, being controlled by the SPE unit and dialysis module. The procedure is robust, and 250–500 analyses can be performed without any need for maintenance or exchange of parts. The analytical characteristics are fully satisfactory for both urine and serum, and allow the use of dialysis–SPE–CE–UV for metabolic studies and clinical analyses [9].

References

[1] J.R. Veraart, J. van Hekezen, M.C.E. Groot, C. Gooijer, H. Lingeman, N.H. Velthorst, U.A.Th. Brinkman, Electrophoresis, 19 (1998) 2944.

[2] A. Guttman, N. Cooke, J. Chromatogr. A, 685 (1994) 155.

[3] S. Fanali, Z. Aturki, J. Chromatogr. A, 694 (1995) 297.

[4] M.G. Danato, E. van den Eeckhout, W. van den Bossche, P. Sandra, J. Pharm. Biomed. Anal., 11 (1993) 197.

[5] M.G. Danato, W. Baeyens, W. van den Bossche, P. Sandra, J. Pharm. Biomed. Anal., 12 (1994) 21.

[6] H. Soini, M.V. Novotny, M.-L. Riekkola, J. Microcol. Sep., 4 (1992) 313.

[7] C.W. Maboundou, G. Paintaud, M. Berard, P.R. Bechtel, J. Chromatogr. B, 657 (1994) 173.

[8] Z.K. Shibabi, M.E. Hinsdale, J. Chromatogr. B, 683 (1996) 115.

[9] F. Lapique, P. Netter, B. Bannwarth, P. Trechot, P. Gillet, H. Lambert, R.J. Royer, J. Chromatogr., 496 (1989) 301.

[10] J.R. Veraart and U.A.Th Brinkman, J. Chromatogr. A, (submitted).

5.3

Dialysis–SPE combined on-line with non-aqueous capillary electrophoresis for improved detectability of tricyclic antidepressants in biological samples

Summary

Dialysis–solid-phase extraction (SPE) sample pretreatment is combined on-line with non-aqueous capillary electrophoresis for the determination of tricyclic antidepressants in urine and serum. After clean-up and enrichment, the water is removed from the sample matrix and the analytes are eluted from the cartridge by means of an organic solvent. Next, the eluate is transported to the capillary and the injection is performed electrokinetically. This injection, which does not suffer from an adverse sample matrix effect because of the SPE step, results in further analyte concentration. The detection limits are in the 0.02 – 0.1 μg/ml range and the day-to-day repeatabilities are between 2.5 and 9.5%, which is quite satisfactory.

Introduction

Capillary electrophoresis (CE) is a technique that is highly suitable for the determination of charged compounds, with the analysis of compounds in biological fluids being a typical field of application. However, when biological fluids are injected directly into a CE capillary problems such as clogging of the capillary by particulate matter, irreversible binding of proteins and peak distortion can occur because of the high salt concentration. Proper sample preparation prior to a CE analysis is the preferred way to solve these problems.

In previous papers, solid-phase extraction (SPE) [1–4] or dialysis–SPE [5, 6] sample preparation was coupled at-line or on-line with CE, via a home-made interface. The at-line SPE–CE system was successfully used to determine positively [1] and negatively [2, 3] charged and amphoteric [4] drugs in urine and serum. The methods featuring dialysis–SPE–CE which is a truly on-line set-up, have the advantage of using less sample, and were used for negatively charged [5] and amphoteric [6] drugs in the same matrices.

One main difference between the SPE–CE studies on negative and amphoteric as against positive analytes (tricyclic antidepressants; TCADs) was that, in the latter case, a non-aqueous buffer had to be used. In the present paper, the practicality of dialysis–SPE combined on-line with non-aqueous CE will be studied for the first time. Although the same approach will be used as in-line SPE–non-aqueous CE, the present procedure is more complicated because of the on-line character of dialysis–SPE–CE. One critical step is the removal of the bulk of the water from the SPE cartridge without losing the analytes. Another important difference is that with the SPE–CE system desorption from the SPE cartridge was performed using methanol, which was subsequently mixed with acetonitrile–acetic acid and injected hydrodynamically into the CE capillary. This cannot easily be mimicked in the on-line set-up: if pure methanol is injected into the CE, no separation can be performed because methanol is a non-conducting solvent. Therefore, electrokinetic injection has to be used as an alternative. The system modifications required to ensure that no methanol will enter the capillary will be discussed below.

Electrokinetic injection usually is not one's first choice as an injection method, because a change of sample matrix, especially a change of conductivity, results in changing amounts of analyte being introduced into the CE capillary. This especially creates problems when biological samples are injected. However, in this study, an automated on-line sample preparation procedure effects the removal of the proteins, salts and water before injection of the analytes into the CE. As a result, the analyte-containing matrix that is introduced has a constant composition, viz. that of the eluent buffer. The overall result is the injection of a relatively large amount of analytes, which are contained in a very narrow injection plug. Consequently, the initial peak width is very small.

Table 5.3.1 Experimental conditions used for TCADs.

Parameter	Comment
Samples	
Urine	None
Serum, free analyte concentration	None
Serum, total analyte concentration	Add of 1 part of PRR[a] to 9 parts of serum[d]
Dialysis	
Dialysis block	Donor phase, 100 µl; acceptor phase, 170 µl
Membrane	Celluose acetate, molecular weight cut-off, 15 kDa
Sample injection	0.5 ml prior to start
Acceptor solution	10 mM phosphate buffer, pH 7.0
Acceptor solution flow rate	0.5 ml/min
Dialysis time urine	12 min[d]
serum	12 min[d]
SPE	
Cartridge	20 x 2 I.D. mm cartridge packed with 5 µm C2-bonded silica (Hewlett-Packard)
Desorption solvent	Methanol
Desorption buffer flow rate	0.1 ml/min[d]
Wash soltution	acetonitrile – water (20/80 v/v)
Wash solution flow rate	1 ml/min
Wash time	1.0 min
SPE–CE interfacing	
Interface	Laboratory-made interface
CE buffer flow rate	0.2 ml/min
Δt[b]	0.02 min[d]
t_{inj}[c]	7 min[d]
CE	
Capillary	Bare silica, 180 µm I.D. 350 µm O.D.; total length, 106 cm; effective length, 40 cm.
Capillary rinsing	1000 mbar for 0.5 min
Injection voltage	-4 kV [d]
CE buffer	1.0 M acetic acid, 25 mM ammonium acetate in acetonitrile
UV detection	214 nm
Analysis time	15 min

[a] PRR, protein releasing reagent (1 M hydrochloric acid and 25% glycerol in water).

[b] Time between start of elution and analytes passing tip of CE capillary.

[c] Time unit interval in which nalytes pass tip of CE capillary.

[d] Discussed in text.

Experimental

Chemicals and samples

Acetonitrile, boric acid, methanol and phosphoric acid were obtained from J.T. Baker (Deventer, The Netherlands). n-Decanoic acid, sodium dihydrogen phosphate monohydrate and disodium hydrogen phosphate 12-hydrate were from Merck (Darmstadt, Germany) and acetic acid and sodium acetate from Riedel–de Haën (Seelze, Germany). Water was demineralized and distilled before use. In all cases, chemicals of the best available quality were used.

Urine was collected from five healthy volunteers on three subsequent days. The samples were pooled and divided into 100-ml portions and frozen at -18°C. Bovine serum of untreated animals was purchased from Sigma; it was divided into 10-ml portions and frozen at -18 °C. The biological samples were stored for a maximum period of three months.

Methods

The set-up of the dialysis–SPE–CE system and the instruments used were described in detail in ref. [5, 6]. The experimental parameters are summarized in table 5.3.1. During optimization, the protocol elaborated in one of our earlier papers [6] was followed as far as was possible. Items that received special attention in the present study, are marked by an asterisk in table 5.3.1.

Results and discussion

In the present paper, parameters such as CE buffer selectivity, back-pressure optimization and SPE–CE interfacing were studied first. Next, the dialysis procedure was optimized, and, finally, the analysis of biological samples was studied.

CE buffer and SPE cartridge

A non-aqueous buffer containing 1 M acetic acid and 25 mM ammonium acetate in acetonitrile was selected for the CE separation. This buffer was successfully used in the literature for the CE separation of standard solutions of TCADs [7]. In a later study [1], it was shown that hydrodynamic injection of standard solutions in acetonitrile containing 1 M acetic acid yields nicely resolved analyte peaks, but that injection of aqueous standard solutions gives broad peaks. In the quoted paper it was also demonstated that methanol has to be used to elute the analytes from the SPE cartridge. Unfortunately, the presence of a small plug of methanol in the CE capillary will cause the current to drop to zero, because methanol is a non-conducting solvent. As an alternative approach, we therefore used electrokinetic injections, in combination with a buffer flow from the detection towards the injection side of the

capillary, with the conditions being selected in such a way that the analytes can, but methanol cannot, enter the capillary.

As regards the 20 x 2 mm I.D. SPE cartridge (table 5.3.1), C2-bonded silica was used as sorbent, because longer alkyl chains will result in too strong interaction with the analytes.

Back-pressure optimization

During injection, there will be a small electro-osmotic flow towards the outlet of the capillary. This will cause the presence of a small plug of methanol in the capillary, which has a low resistance and causes the current to decrease. In order to prevent this, pressure should be applied at the far end of the capillary, preferably by using a somewhat higher buffer level there.

Sample introduction was performed by adding 1 ml of aqueous sample containing 10 μg/ml of each TCAD, to the SPE cartridge. Next, elution was effected with 1 ml of methanol at a flow of 0.2 ml/min. Injection into the CE capillary was performed by using a voltage of -3 kV for 5 min, which was applied immediately upon starting the elution. Finally, the 15 min separation was performed using –30 kV, and with 1.0 M acetic acid, 25 mM ammonium acetate in acetonitrile as CE buffer. The dependence of the amount of analyte detected, expressed as the peak area, on the height difference of the buffer levels, is shown in figure 5.3.1. With differences of 10 mm or less, no peaks were observed because methanol then entered the CE capillary, making separation impossible. On the other hand, when using a height difference of 14 mm or more, no peaks showed up either, because the flow of the CE buffer from the detection to the injection side was higher than the speed of the analytes towards the CE capillary outlet; in other words, the analytes could not enter the capillary. Good, and mutually rather similar results were obtained for height differences of 11 – 13 mm, and an intermediate value of 12 mm was selected for all further work.

SPE–CE interfacing

With regard to interfacing, there are two main parameters that have to be optimized: (i) the injection window, i.e. Δt and t_{inj} in the protocol of table 5.3.1 and (ii) the flow rates used during desorption.

In earlier studies [5, 6], a long injection time of the desorption solvent resulted in band broadening. Under the present conditions this effect is absent, because the desorption solvent, methanol, does not enter the capillary anyway. Consequently, Δt was set at 0.02 min or, in other words, the voltage was applied almost immediately after starting the desorption of the TCADs from the SPE cartridge, to avoid loss of analytes.

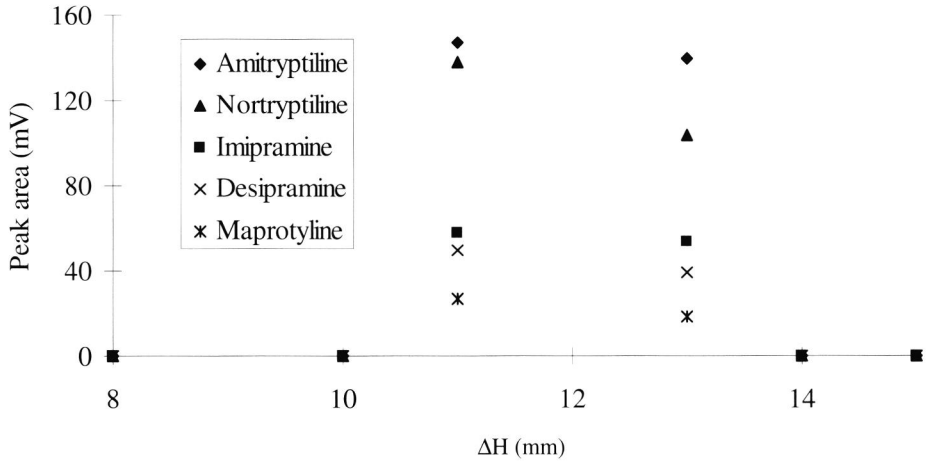


Figure 5.3.1 Dependance of analyte response (peak areas) on height difference of the two buffer vials.

As regards the effect of the flow rate of methanol on the desorption of the TCADs, the cartridges were loaded with 1 ml of a solution containing 10 μg/ml of each TCAD and were, next desorbed with 2 ml of methanol using flow rates varying from 0.1 to 2.0 ml/min. Over the range tested, increasing the flow resulted in a steadily decreasing amount of analyte desorbed per unit of volume. In addition, the amount of TCADs desorbed from the cartridge was more reproducible when using low flow rates. Because of these two observations, a low flow of 0.1 ml/min was selected for desorption. Since, on the other hand, the amount of analyte desorbed per time unit was higher at a high flow, a flow rate of 2.0 ml/min was selected for cartridge regeneration.

To study the minimum volume of methanol necessary to remove all TCADs from the cartridge, a test sample containing 10 μg/ml of each TCAD was injected onto the SPE cartridge and, next, eluted using varying amounts of methanol (0.1–1.0 ml), at a flow of 0.1 ml/min. The injection time into the CE was 10 min in all cases. Complete desorption was found when using 0.7 ml of methanol, and an injection time, t_{inj}, of 7 min and the low flow rate were used for desorption in all further experiments.

In conclusion, even when using a low flow rate during desorption, 0.7 ml of methanol is still required to desorb all TCADs; this is quite much more than the 0.2 ml typical of earlier studies. The slow desorption is probably caused by the strong interaction of the TCADs with free silanol groups with the surface of the C2-bonded silica. In principle, the interaction can be reduced by adding an excess of amines to the desorption solvent. Unfortunately, this approach cannot be used here, because the amines will also interact with the free silanol groups on the CE capillary wall. This will cause the electro-osmotic flow to fluctuate, and, the migration times to become

non-reproducible. Because of this, pure methanol was used for desorption, and a 7-min desorption time was accepted. As will be shown below, the adverse effect of analyte dilution was made up for by urine, an injection technique, which results in analyte (re)concentration.

Injection voltage

The injection voltage applied for 7 min after the desorption has been started, should be optimized to increase the sensitivity of the procedure, without causing additional band broadening as is described in the protocol as is shown in section 5.2 [6].

To study this aspect, 1 ml of standard solution (1 μg/ml of each TCAD) was applied to the SPE cartridge. Upon desoption with methanol and flushing of the analytes to the CE system, electrokinetic injection was performed for 7 min using voltages ranging from 1 to 14 kV. Unfortunately, no clear-cut picture emerged. For the rapid migrating TCADs, amitryptiline and imipramine, the peak width was found to decrease about 2-fold when the voltage was doubled; or in other words, increasing the amount injected resulted in broader peaks with the same peak height. Essentially, no effect on peak width was observed for the slow migrating nortryptiline and desipramine, while peak width was increased with an increasing voltage for the slowest migrating TCAD, maprotyline. Most probably, the explanation is that the stacking effect is more efficient for slower, as compounds compared with faster migration compounds. In all further experiments, a compromise voltage of -4 kV was used.

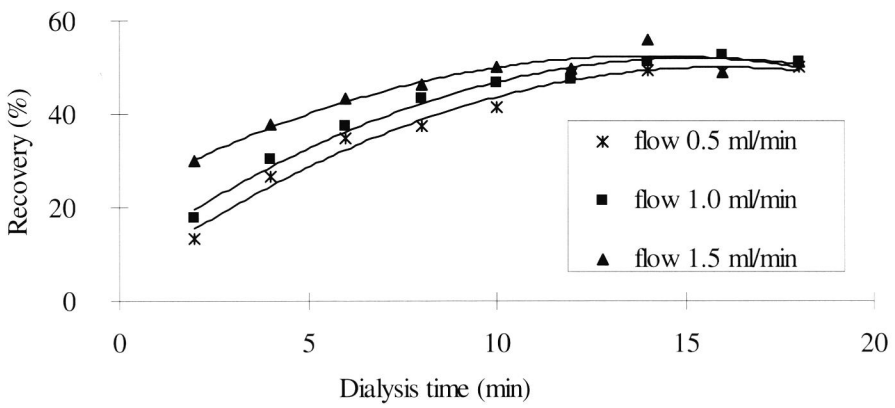


Figure 5.3.2 Effect of dialysis time on imipramine recovery for different acceptor phase flow rates.

Dialysis

As the next step, the dialysis module was connected on-line to the SPE cartridge that now essentially is a trapping column. The composition of the acceptor phase was the same as was used in earlier studies on dialysis–SPE: an aqueous 10 mM phosphate buffer (pH 7) [6].

The donor compartment was filled with a standard TCAD solution (concentration,1 μg/ml each). Dialysis times were varied over the range of 2.5 – 17.5 min and acceptor flow rates of 0.5 – 1.5 ml/min were used. At this stage of the study, the CE–UV_{214} analysis was combined on-line with the sample pretreatment. Figure 5.3.2 shows the result obtained for imipramine (similar results were obtained for other TCADs). It is obvious that, for short dialysis times of less then about 7.5 min, a high acceptor flow has to be recommended. On the other hand, if some of the speed of analysis that can be obtained is sacrificed, analyte recoveries are increased and the flow dependency is essentially lost. A low acceptor flow of 0.5 ml/min (dialysis time, 13 min) was used, to reduce the possibility of breakthrough of the analytes.

Analysis of biological samples

Two types of biological samples were used to test the optimized on-line set-up: urine and serum. As is well known, with both sample types adequate removal of interferences present on the SPE cartridge after trace enrichment of the analytes, is the main problem. In the present, non-aqueous CE, study, the earlier solution of washing with a suitable pH-stabilized buffer/organic solvent mixture cannot be used: there is a

Table 5.3.2 Calibration data for TCADs in spiked urine and serum[a].

Compound	Linearity[b]				LOD
	Slope (RSD)		Intercept (RSD)		(ng/ml)
Urine					
Ami	0.195	(0.004)	-0.16	(0.09)	40
Imi	0.38	(0.01)	-0.1	(0.2)	80
Nor	0.181	(0.005)	-0.1	(0.1)	60
Des	0.54	(0.01)	-1.0	(0.1)	60
Map	1.07	(0.03)	0.0	(0.1)	60
Serum					
Ami	0.119	(0.002)	0.04	(0.06)	60
Imi	0.364	(0.009)	0.01	(0.12)	100
Nor	0.141	(0.003)	0.13	(0.10)	90
Des	0.358	(0.007)	0.16	(0.1)	80
Map	0.76	(0.016)	0.15	(0.1)	90

[a]Sample size: urine, 0.5 ml; serum, 0.5 ml

[b]TCAD concentration range: LOD – 10 μg/ml, 5 datapoints

real risk that (part of) the solutes in the buffer will precipitate when coming into contract with the desorption solvent. Fortunately, even if no pH effect was used, and a simple acetonitrile–water (20/80 v/v) mixture, a 1-ml wash step was sufficient to remove most of the interferences.

With serum samples, adding a releasing agent solved the additional problem of the protein–drug binding. To this end, the use of a 1/9 (v/v) mixture of protein-releasing reagent (PRR; 1 M hydrochloric acid and 25% glycerol in water) and serum sample was used (c.f. ref. [8]). It should be noted that, after having mixed the solutions by shaking, analysis should proceed forthwith, because after about one-hour the viscosity starts to increase. The analyte recoveries obtained when using PRR, were 60-70% for amitryptiline, desipramine and maprotyline, 80% for nortryptiline and 95% for imipramine. This contrasted sharply with recoveries of less than 10% obtained when no PRR was used. Figure 5.3.3 shows that the proposed procedure (with the same washing step as for urine), resulted in excellent electropherograms for serum samples.

Some relevant analytical performance data are summarized in table 5.3.2. Linear calibration plots were obtained for all five TCADs in both types of biological sample. The within-day precision was 2.5–4.5% (n=5) for standard solutions, 2.5–5.5% (n=5) for urine and 4.5–9.5% (n=5) for serum. The values were all below the threshold of 15%, a value often used as acceptance level of an analytical method for to be used for quantification of analytes in biological samples [9]. In addition, table 5.3.2 shows that the LODs are in the 40–80 ng/ml range for urine, and the 60–100 ng/ml range for serum. This is a distinct improvement over earlier results obtained with at-line SPE–CE with hydrodynamic injection, where LOD, were 30–300 ng/ml for urine, and 300–1000 ng/ml for serum. The improved detectability is largely due to the use of electrokinetic instead of hydrodynamic injection. With electrokinetic injection more analyte can be injected without creating extra band broadening; with hydrodynamic injection, restrictions are more severe because next to the analytes, solvent is also injected. The improved analyte detectability can be called very gratifying, especially when one considers that the sample volumes per analysis were only 0.5 ml for both urine and serum, as against 8 ml (urine) or 1 ml (serum) in the SPE–CE study. This is an important aspect when biological fluids have to be analysed that are available in only limited amounts.

Finally, it should be added that maintenance of the automated dialysis–SPE–non-aqueous CE system was negligible during the four months of the present study, and that no technical or operational problems were encountered.

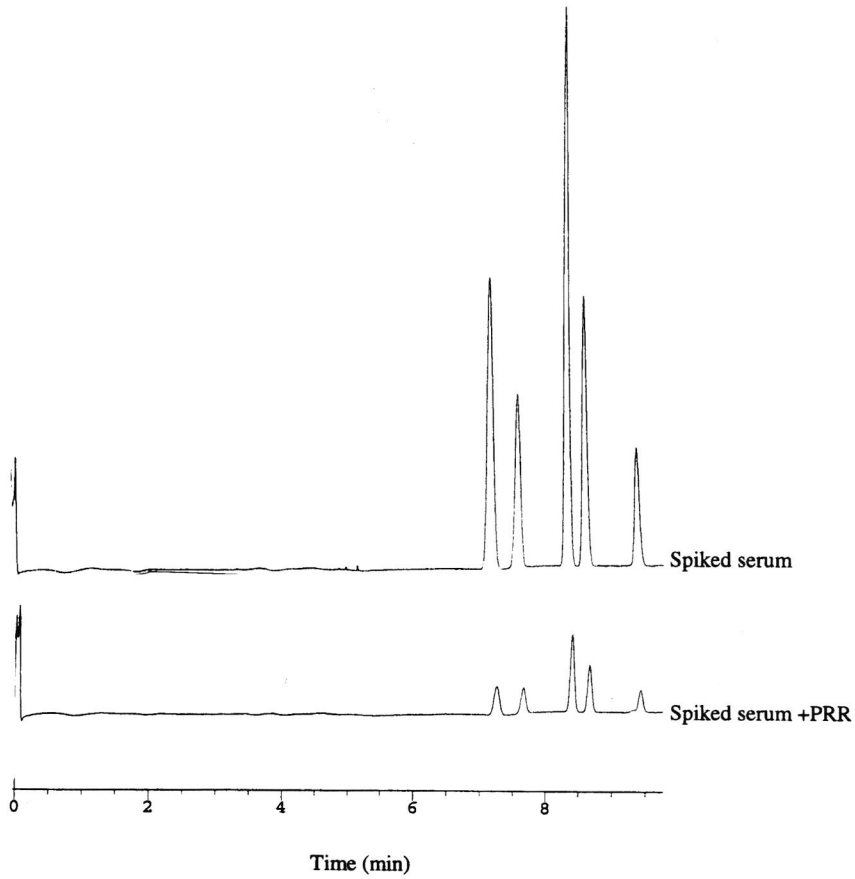


Figure 5.3.3 Electropherogram of TCADs (spiked at 1 μg/ml each) in serum with and without the addition of PRR obtained by dialysis–SPE–CE–UV_{214}. Elution order: Amitriptyline, Imipramine, Nortriptyline, Desipramine and Maprotyline. For details, see text.

Conclusions

Dialysis–SPE has successfully been combined on-line with non-aqueous CE with UV detection. To illustrate the practicality of the procedure, five tricyclic antidepressants were determined in serum and urine. The analytical performance data of the automated procedure were fully satisfactory, one main advantage being an over 10-fold improved analyte detectability compared with an earlier at-line SPE–CE approach. With 0.5-ml urine and serum samples, the urine detection limits were in the 40–100 ng/ml range. The successful outcome is primarily caused by the use of electrokinetic injection which is shown to provide excellent results if combined with a sample pretreatment which ensures that an extract is injected which has a constant composition, independent of the nature of the biological sample.

The present paper completes a series studies which demonstrate that at-line SPE–CE but, even more so, on-line dialysis–SPE–CE is a powerful method for the trace-level determination of cationic, amphoteric and anionic analytes (drugs) in serum and urine, and can be combined with both an aqueous or non-aqueous CE buffer.

References

[1] J.R. Veraart, H. Lingeman and U.A.Th. Brinkman, Chromatographia, accepted.

[2] J.R. Veraart, C. Gooijer, H. Lingeman, N.H. Velthorst and U.A.Th. Brinkman, J. Chromatogr. B, 719 (1998) 199.

[3] J.R. Veraart, C. Gooijer, H. Lingeman, N.H. Velthorst and U.A.Th. Brinkman, J. Pharm. Biomed. Anal., 17 (1998) 1161.

[4] J. R. Veraart, C. Gooijer, H. Lingeman, N.H. Velthorst, U.A.Th. Brinkman, J. High Resol. Chromatogr., 22 (1999) 183.

[5] J.R. Veraart, J. van Hekezen, M.C.E. Groot, C. Gooijer, H. Lingeman, N.H. Velthorst and U.A.Th. Brinkman, Electrophoresis, 19 (1998) 2944.

[6] J.R. Veraart, M.C.E. Groot, C. Gooijer, H. Lingeman, N.H. Velthorst and U.A.Th. Brinkman, Analyst, 124 (1999) 115.

[7] L. Bjønsdittir and S.H. Hanssen, J. Chromatogr. A, 711 (1995) 313.

[8] K. Johansen and K.E. Rasmussen, J. Pharm. Biomed. Anal., 16 (1998) 1159.

[9] V.P. Shah, K.K. Midha, S. Dighe, I.J. McGilveray, J.P. Skelly, A. Yacobi, T. Layloff, C.T. Viswanathan, C.E. Cook, R.D. McDowall, K.A. Pittman and S. Spector, J. Pharm. Sci., 309 (1992) 306.

Samenvatting

Samenvatting

Geautomatiseerde behandeling van biologische monsters voor capillaire elektroforese

Capillaire elektroforese (CE) is een analysetechniek die sinds zijn introductie in de beginjaren '70 is uitgegroeid tot een volwassen techniek. Er is een groot aantal publicaties verschenen met een breed toepassingsgebied. De techniek heeft verscheidene bijzonder gunstige eigenschappen, zoals bijvoorbeeld de enorme scheidingskracht. Aan de andere kant heeft CE ook zijn beperkingen. Een van de grootste beperkingen is de kwetsbaarheid van de techniek bij de aanwezigheid van zouten, eiwitten of vaste deeltjes in een monster. Bij de analyse van biologische monsters vormt CE een goede aanvulling op de bestaande technieken, maar juist in deze monsters komen deze storende stoffen dikwijls en in grote en wisselende concentraties voor. Op grond hiervan zou men mogen verwachten dat er veelvuldig onderzoek gedaan is naar de ontwikkeling en het strategisch gebruik van goede monstervoorbereidingstechnieken ten behoeve van CE. Verder is het wenselijk, gezien de grote hoeveelheid monsters die in de praktijk geanalyseerd moeten worden, dat de monstervoorbereiding geautomatiseerd kan worden uitgevoerd en, indien mogelijk, met een goede en directe koppeling aan CE.

Dit proefschrift beschrijft de koppeling van twee monstervoorbereidingstechnieken aan CE. Deze beide koppelingen zijn getest met basische, zure en amfotere verbindingen en met urine en serum als matrices. Er is naar gestreefd om zo veel mogelijk gelijkaardige strategieën te ontwerpen voor de verschillende analieten en matrices, zodat een snelle aanpassing van de procedures ten behoeve van nieuwe problemen gewaarborgd is.

In **Hoofdstuk 1** wordt het uitgangspunt en de strekking van het uitgevoerde onderzoek beschreven. Kenmerkend zijn de problematiek en mogelijkheden voor het gebruik van CE voor de bepaling van analieten in biologische monsters.

Hoofdstuk 2 geeft een uitvoerige beschrijving van de literatuur op het gebied van de CE analyse van biologische monsters. De aandacht is vooral gericht op het type monstervoorbereiding en de toepassingen. Om een goede indeling te krijgen met betrekking tot de koppeling van de monstervoorbereidingstechniek aan CE, is de mate van integratie van monstervoorbereidingstechniek en analysetechniek als uitgangspunt genomen. De daaruit voortvloeidende terminologie van *off-line*, *at-line*, *on-line* en *in-line* technieken wordt verder in dit proefschrift gehanteerd. De

gebruikte analysetechnieken bleken op drie principes gebaseerd te zijn: chromatografische, elektroforetische en het gebruik van membranen.

De bestudering van de literatuur leidde tot de conclusie dat er, ondanks de duidelijke noodzaak, weinig aandacht wordt besteed aan de monstervoorbereiding. Verder wordt er vaak gekozen voor het gebruik van tijdrovende, handmatige methodes. Dit heeft tot gevolg dat veel van de beschreven analysemethodes in principe van goede kwaliteit zijn, maar dat ze alleen toepasbaar zijn voor 'schone monsters', en dat hun bruikbaarheid voor de analyse van biologische monsters niet is bewezen. Twee technieken die dit manco niet vertonen zijn de in de Hoofdstukken 4 en 5 beschreven methodes.

Hoofdstuk 3 beschrijft (i) de invloed van de temperatuur en het hanteren van een juiste thermosteringsmethode van het capillair in CE en (ii) de ontwikkeling van een interface tussen het monstervoorbereidings- en het CE deel van de totale opstelling. Deze twee ontwikkelingen vormen de basis van de in de Hoofdstukken 4 en 5 beschreven studies.

Er zijn verschillende thermosteringsmethoden bekend in CE. Van een aantal hiervan is bekeken wat het gegenereerde vermogen tijdens de analyse is en hoe dit het verschil tussen de ingestelde en de werkelijke temperatuur beïnvloedt. Het blijkt dat er twee temperatuurverschillen zijn te onderscheiden, een vermogen-onafhankelijke en een vermogen-afhankelijke. Voor lage gegenereerde vermogens (< 1 watt per meter capillair), zoals gebruikelijk bij de meeste CE analyses, worden vergelijkbare resultaten behaald met de twee meest gebruikte methoden, geforceerde luchtkoeling en geforceerde vloeistofkoeling. Het gebruik van natuurlijke convectie en koeling met behulp van een ventilator (zoals in de CE apparatuur gebruikt in de volgende hoofdstukken) is niet voldoende. Daarom is de gebruikte CE apparatuur zodanig aangepast dat deze wel voldeed aan de gestelde eisen.

In het tweede deel van dit hoofdstuk is een interface ontwikkeld dat gebruikt kan worden om uit vloeistofstromen injecties uit te voeren. In het huidige voorbeeld is een vloeistofchromatografie (LC) systeem gekoppeld aan de CE. Met behulp van standaardoplossingen van verschillende analieten die hoge concentraties zout bevatten, is aangetoond dat directe injectie van dergelijke oplossingen niet mogelijk is. Als de monsters evenwel via het LC systeem worden geïnjecteerd, worden de zouten verwijderd. Door een onderdruk aan de andere kant van het capillair aan te leggen worden de analieten in het capillair geïntroduceerd waarna de analyse kan worden uitgevoerd.

In **Hoofdstuk 4** wordt de eerste koppeling beschreven, *at-line SPE–CE*. De monsters worden in reactievaatjes in een geautomatiseerd monstervoorbewerkingssysteem geplaatst, waar ze met behulp van een robotarm worden onderworpen aan vaste-fase extractie (SPE). Na deze monsterbehandeling wordt het eluaat, dat wèl de analieten, maar niet meer de zouten, eiwitten en vaste deeltjes bevat, in een monsterlus gebracht, waarna de inhoud hiervan met de

vloeistofstroom naar het interface wordt gespoeld (zoals in Hoofdstuk 3 beschreven) en wordt geïnjecteerd in het CE capillair.

De bruikbaarheid van de at-line SPE–CE benadering is aangetoond voor de bepaling van zure, basische en amfotere modelverbindingen. De gekozen testverbindingen waren respectievelijk tricyclische anti-depressiva (TCADs), niet-steroïde anti-koortsmiddelen (NSAIDs) en sulfonamiden. De bepalingen werden uitgevoerd in serum en in urine. Voor beide matrices werden goede analytische karakteristieken verkregen. De herhaalbaarheden (binnen een dag en over een periode van verscheidene dagen) waren goed: 2–10% voor serum en 4–11% voor urine. De verkregen detectielimieten (UV detectie) lagen in de 10–80 ng/ml range voor urine en in de 200–1000 ng/ml range voor serum. Het grote verschil in detecteerbaarheid werd voornamelijk veroorzaakt door het verschil in de gebruikte hoeveelheden monster: 1 ml voor serum en 8 ml voor urine. Verder is, als 'echte' bepaling, phenprocoumon in urine en serum bepaald. Bovendien is in dit hoofdstuk aangetoond dat er twee injectietechnieken kunnen worden gebruikt, hydrodynamische en elektroforetische injectie. Tenslotte zijn de analyses van biologische matrices gecombineerd met zowel waterige als niet-waterige buffers.

In **Hoofdstuk 5** wordt de tweede methode beschreven, *on-line dialyse–SPE–CE*. Bij deze techniek wordt het monster met behulp van een automatisch werkende injector aan de donorzijde van een membraan gebracht. Kleine deeltjes zoals analieten, maar ook zouten, kunnen de poriën van het membraan passeren - grote deeltjes en eiwitten echter niet. De passage wordt versneld door aan de acceptorzijde van het membraan gebruik te maken van een stromende vloeistof. Hierna wordt een SPE stap uitgevoerd om het dialysaat te concentreren (het volume is vergroot van 0.1 ml naar ongeveer 10 ml) en om de zouten te verwijderen. Tevens kunnen tijdens deze stap, indien noodzakelijk, andere storende bestanddelen verwijderd worden.

Net zoals in Hoofdstuk 4 is ook hier de koppeling van de monstervoorbewerking aan CE uitvoerig getest met modelverbindingen. Om een goede vergelijking mogelijk te maken, zijn dezelfde klassen testverbindingen en dezelfde matrices gebruikt als in het vorige hoofdstuk. De analytische karakteristieken zoals lineariteit en herhaalbaarheid waren even goed als bij de *at-line* procedures. De detectiegrenzen waren evenwel duidelijk beter, namelijk 40–100 ng/ml voor urine en 50–300 ng/ml voor serum bij gebruik van slechts 0,5 ml monster! Speciale aandacht werd besteed aan het efficiënt verbreken van analiet–eiwit bindingen.

Afsluitend kan men concluderen dat er nu voor alle typen analiet in urine en serum een robuuste, geautomatiseerde en geïntegreerde CE–UV procedure beschikbaar is voor kwantitatieve analyse op in de praktijk gewenste concentratieniveaus.

List of Publications

List of publications

This thesis is based on the following papers which have been or will be published as regular contributions to scientific journals:

- J.R. Veraart, C. Gooijer, H. Lingeman, N.H. Velthorst and U.A.Th. Brinkman, Coupling of biological handling and capillary electrophoresis (Review), J. Chromatogr. A, 856 (1999) 483. (**Chapter 2**)
- J.R. Veraart, C. Gooijer, H. Lingeman, N.H. Velthorst and U.A.Th Brinkman, Thermostating in capillary electrophoresis, Chromatographia, 44 (1997) 581. (**Chapter 3.1**)
- J.R. Veraart, H. Lingeman and C. Gooijer, Temperature control in capillary electrophoresis, Biomed. Chromatogr., 9 (1995) 271. (**Chapter 3.1**)
- J.R. Veraart, C. Gooijer and H. Lingeman, On-line chromatographic pretreatment of samples with varying salt concentrations for capillary electrophoresis, Chromatographia, 44 (1997) 129. (**Chapter 3.2**)
- J.R. Veraart, C. Gooijer, H. Lingeman, N.H. Velthorst and U.A.Th. Brinkman, At-line solid-phase extraction for capillary electrophoresis: application to negatively charged solutes, J. Chromatogr. B, 719 (1998) 199. (**Chapter 4.1**)
- J.R. Veraart, C. Gooijer, H. Lingeman, N.H. Velthorst and U.A.Th. Brinkman, At-line solid-phase extraction coupled to capillary electrophoresis: Determination of amphoteric compounds in biological samples, J. High Resol. Chromatogr., 22 (1999) 183 (**Chapter 4.2**).
- J.R. Veraart, M.C.E. Reinders, H. Lingeman and U.A.Th. Brinkman, Non-aqueous capillary electrophoresis of biological samples after at-line solid-phase extraction, Chromatographia, in press (**Chapter 4.3**).
- J.R. Veraart, C. Gooijer, H. Lingeman, N.H. Velthorst and U.A.Th. Brinkman, Determination of phenprocoumon in plasma and urine using at-line solid phase extraction–capillary electrophoresis, J. Pharm. Biomed. Anal., 17 (1998) 1161. (**Chapter 4.4**)

- J.R. Veraart, J. van Hekezen, M.C.E. Groot, C. Gooijer, H. Lingeman, N.H. Velthorst and U.A.Th. Brinkman, On-line dialysis–solid phase extraction coupled to capillary electrophoresis: determination of amphoteric solutes in biological samples, Electrophoresis, 19 (1998) 2944. (**Chapter 5.1**)
- J.R. Veraart, M.C.E. Groot, C. Gooijer, H. Lingeman, N.H. Velthorst and U.A.Th. Brinkman, On-line dialysis–SPE–CE of acidic drugs in biological samples, Analyst, 124 (1999) 115 (**Chapter 5.2**)
- J.R. Veraart and U.A.Th. Brinkman, Dialysis–SPE combined on-line with non-aqueous capillary electrophoresis for improved detectability of tricyclic antidepressants in biological samples, J. Chromatogr. A, submitted (**Chapter 5.3**).

Additional papers on related subjects:

- Y. Schouten, J.R. Veraart, C. Gooijer and H. Lingeman, Improved protein separation using capillary electrophoresis and phytic acid, Biomed. Chromatogr., 9 (1995) 269.
- J.R. Veraart, Y. Schouten, C. Gooijer and H. Lingeman, Evaluation of phytic acid as a buffer additive for the separation of proteins in capillary electrophoresis, J. Chromatogr. A, 768 (1997) 307.
- G.A. Kleter, J.J.M. Damen, J.J. Kettenes-van den Bosch, R.A. Bank, J.M. te Koppele, J.R. Veraart and J.M. ten Cate, A novel pyrroleninone cross-link from bovine dentine, Biochim. Biophys. Acta, 1381 (1998) 179.
- J.R. Veraart, S.J. Kok, J.M. te Koppele, C. Gooijer, H. Lingeman, N.H. Velthorst and U.A.Th. Brinkman, Capillary electrophoresis of the collagen crosslinks HP and LP utilizing absorbance, wavelength-resolved laser-induced fluorescence and conventional fluorescence detection, Biomed. Chromatogr., 12 (1998) 226.
- J. Dallüge, R. Ou-Aissa, J.J. Vreuls, U.A.Th. Brinkman and J.R. Veraart, Fast temperature programming in gas chromatography using resistive heating, J. High Resol. Chromatogr., 22 (1999) 459.

Dankwoord

Dankwoord

In mei 1994 maakte ik een volgens sommigen een ondenkbare stap: ik ging een promotieonderzoek beginnen aan die andere universiteit in Amsterdam, waar ik in voorgaande jaren altijd met de tram voorbij reed. In het begin heb ik het ervaren als een cultuurschok, maar ik ben me steeds meer op mijn gemak gaan voelen bij de vakgroep AAC. Deze vakgroep bestaat uit een grote verzameling van de meest uiteenlopende mensen, met ieder een totaal andere invalshoek en persoonlijkheid. Toch ben ik van mening dat deze grote variëteit leermeesters (m/v) de reden is dat ik snel een plaats heb gevonden binnen de VU en binnen AAC.

Een promotie is iets wat je aan de ene kant helemaal alleen moet doen, maar aan de andere kant onmogelijk is om alleen te doen. Bij deze wil ik dan ook diverse mensen bedanken die een grote of kleinere bijdrage hebben geleverd aan het proefschrift, mijn verblijf bij AAC, bij de VU en in Amsterdam.

Tijdens mijn AIO-tijd en daarna werd ik begeleid door een kwartet met ieder een ander aandachtsgebied. Soms ging ieder een andere windrichting uit, zodat ik vertwijfeld midden in de roos bleef staan. Een andere keer rende ieder dezelfde kant op zodat ik jullie amper kon bij houden. Udo, je was een ware motor van mijn proefschrift. Je was niet altijd fysiek op de VU aanwezig, maar mbv. fax en telefoon en (on)verwachte live gesprekken wist je mij altijd te bereiken. Henk, op de juiste momenten wist je met goede ideeën, aanvullingen en biomedisch realisme te komen. Verder staan de congresbezoeken me nog vers in het geheugen. Cees, groot gebak liefhebber en daarnaast ook oog voor CE details. Nel, berucht om de oogverblindende fotoacties tijdens borrels, wist met op het eerste gezicht eenvoudige vragen over mijn artikelen, een fundamenteel punt aan te boren dat door anderen over het hoofd was gezien.

Veel van het werk kon alleen gedaan worden omdat ik versterking had van studenten. Yvonne, je was de eerste echte stagiaire die ik mocht begeleiden en ik heb samen met jou de eerste CE aan de praat gekregen. Patrick, Abba fan, al je was geen prater, je was toch altijd aanwezig. Judith, de dialyse–SPE-pionier, bleef onder alle omstandigheden de rust zelve. Peter, je was de enige student die mijn muziek enigszins wist te waarderen. Gloria, a very relaxed Spanish girl, always ending a sentence with "no?" instead of a full stop. Marjolein, je bouwde indrukwekkende piramides van spaflesjes en -blikjes, labjournaals, literatuur en boeken en vervolgens wist je hier ook nog genoeg resultaten uit te toveren voor twee artikelen. Jelmer, cafeïne verslaafde, je had koffie gedronken, je dronk koffie of ging koffie drinken.

Maaike, iedereen kent je als fervente roeifanaat. Pim Lagas, je was de enige die op maandag nog moeier uitzag dan ik me voelde. Denise, de vrolijke farmaceut, zelfs als alles mislukte, kon dat je lach nog niet laten verdwijnen.

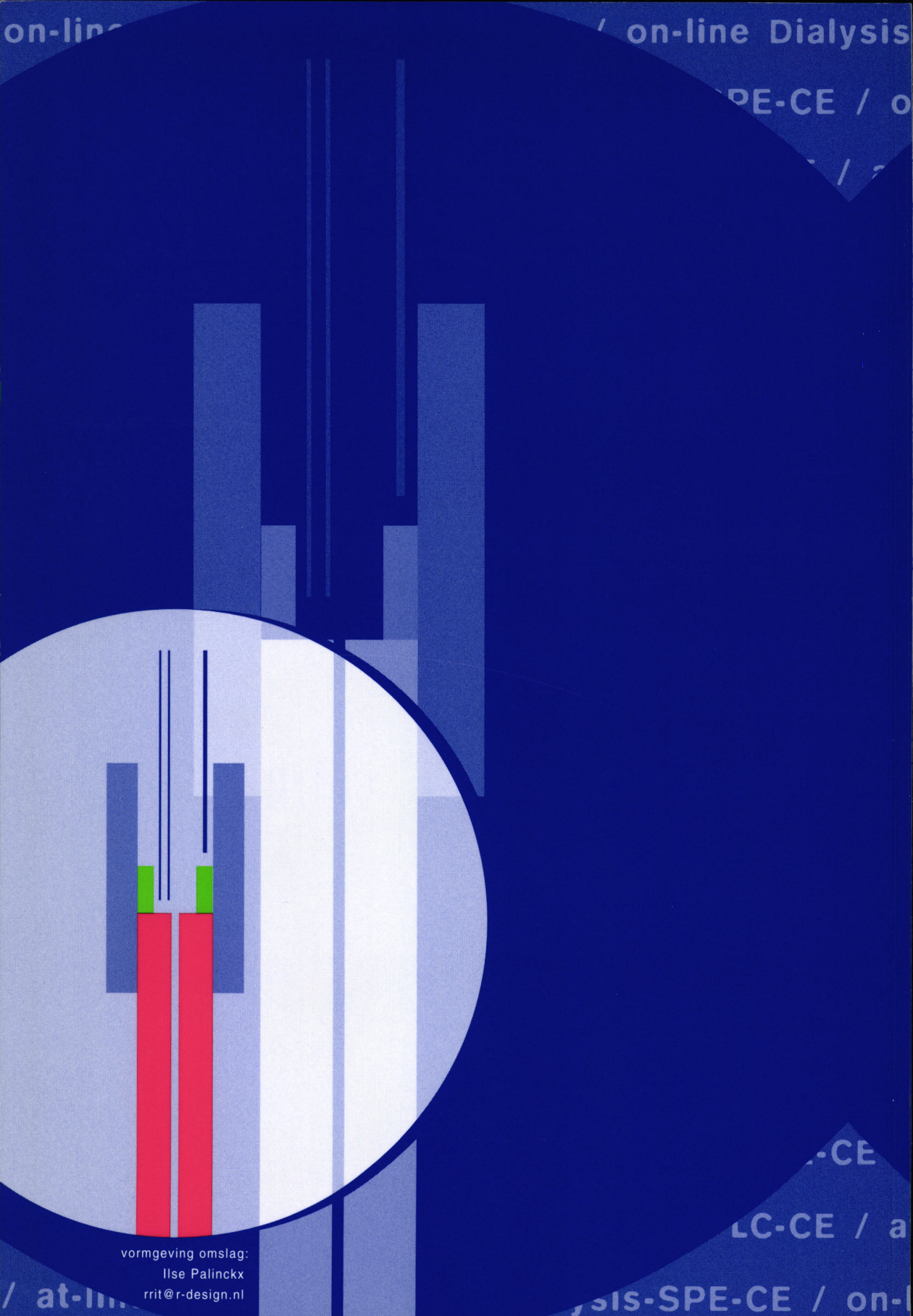
Ondersteuning heb ik van velen mogen ontvangen. Ten eerste van mijn kamergenoot Pim Voogt, de dipswitchjesexpert, jarenlang zaten we in ons overvolle hokje als haringen in een ton, maar sinds ik weg ben, heb je door annexatie van het voorraadhok een van de meest royale zitkamers van de VU. De mannen van de elektronische en mechanische werkplaats, die iedere hersenschim van mij wisten te vertalen in een werkend product. Verder wisten jullie vakkundig gesloopte onderdelen in een recordtijd te maken. Gerard, je wist alles wat met licht te maken had, ook al zat je meestal in het donker. Dik, je was de meest gezochte man van de hele VU. Gisèle, de computerfanaat, waarvan je altijd wist waar ze was, vooral als Pim Voogt je op de kast had gejaagd.

Verder wil ik de vele lotgenoten nog bedanken die ik ben tegengekomen tijdens mijn promotietijd. De borrels, barbecues, dakterrassessies, de vele mensa maaltijden en andere sociale activiteiten waren een welkome afwisseling op het geploeter van alle dag. Steven, de laserboy die het ook op O3 naar zijn zin leek te hebben, nog bedankt voor het lenen van je bril als dat nodig was. Jens, hoffotograaf op de latere uren, geen ruit was voor je veilig. Leo de meest hygroscopische AIO van allen, zowel 's avonds tijdens, als 's morgens na de borrel. Sjaak, de destillatiekampioen die de Buitenveldertse brandweermannen na kantooruren naar de enige ingang liet zoeken die niet afgesloten was. Ruud, medeoverloper van de UvA, die ook de VU verkoos om te promoveren. Koos en Mohammed, jullie wisten van AAC een ware geurensensatie te maken; heel O3 rook naar maggi en M3 naar boter. Marleen, de lieftallige assistente, je parkeerstand was onverslaanbaar. Pim Leonards, je gebruikte je bureau voor alles, behalve om er aan te zitten. Nico en Bart, de symbiose van jullie twee opstellingen is tot bloei gekomen als de dialyse–SPE–CE en SPE–CE, nogmaals bedankt voor de "vrijwillige" donatie van jullie apparatuur. Thomas, vele spullen zijn verdwenen in jouw Bermuda driehoek, je kasten met reserve onderdelen waren een ruime voorraad toen je weg was. Later heb ik je leren kennen als een kamergenoot met dezelfde temperatuurvoorkeur. Marco, je was een goeie barbecue, borrels, en lasergame organisator, maar geen CE was voor je veilig. Johan, je hebt me leren overleven in de jungle van het eerstejaarspracticum. Govert, je was een collega die altijd zijn woord klaar had, daarom ben je nu ook mijn opponent. René, ik bewonder je dat je zo'n korte nachtrust nodig had, na een zware avond. Hans, na een korte kennismaking op de VU verhuisde je naar TNO, waar we jaren later collega's geworden zijn. Reyer, de Spanje liefhebber, na jaren aan diverse MS-sen gesleuteld te hebben, heb je toch nog het licht gezien. Isabel, het zal een stuk stiller geworden zijn op O3 nu je een AIO carrière begonnen bent in Utrecht. Maria, je GC detector maakte meer geluid dan jij tijdens het werk. Tjipke, de Mac fanaat, het is nog steeds een raadsel waar je gebleven was tijdens de Kerstborrel. Jaroslav, je was altijd goedlachs.

Arjan L. en Arjan M. jullie voornaam was jullie enige overeenkomst. Sander, die na een verblijf in de donkere kamer van de VU ook koos voor TNO. Ook anderen waar ik me geen smeuïge details meer van weet te herinneren zoals Caroline, Olaf en Herman bedank ik voor hun bijdrage aan het plezier tijdens mijn verblijf bij de VU. Tevens wil ik ook de vele andere mensen die voor kortere of langere tijd bij de VU bivakkeerden hierbij bedanken.

Met plezier kijk ik terug naar Uilenstede dat een surrogaat thuis vormde: met name Eala en Clarissa, en beide heren van het Polderblok: Remco en Johan. We hebben ons vermaakt met watergevechten met tegenoverliggende keukens, de koffie-, thee-, bier- en berenburg avonden en happy-hours waren een welkom rustpunt.

Als laatste, maar niet minst belangrijkste wil ik het Brabantse thuisfront (schoon)ouders, Eric en Joyce bedanken voor alle steun en geduld tijdens mijn studie, AIO-tijd en de tijd daarna. Vooral wil ik mijn ouders bedanken voor de onvoorwaardelijke steun die jullie mij op allerlei manieren hebben gegeven. And last but not least: Danny, lang heb je liefdevol geduld moeten hebben voordat je mijn door-de-weekse-afwezigheid kon omruilen voor het fulltime samenwonen. Ook al wilden we niets liever, nooit heb je je hierover beklaagd. Nu het proefschrift echt helemaal af is, ben ik minder vaak in de avonden en weekends achter de computer te vinden.



vormgeving omslag:
Ilse Palinckx
rrit@r-design.nl