Chapter 4

On-line large-volume capillary electroextraction coupled to LC-MS to improve detection limits of peptides

Based on
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Abstract

In this research-paper we demonstrate a new electroextraction (EE) set-up and its on-line coupling with LC-MS. EE takes place in a two-phase liquid-liquid system, consisting of an aqueous and an organic phase, where an applied electric field causes ions to be extracted from one phase into the other, to be concentrated close after the liquid-liquid interface.

The extraction takes place in a wide-bore capillary that is connected to a 2-way 10-port switching valve, which serves to couple capillary EE (cEE) with LC-MS. In this set-up, volumes as high as 100 µL can be extracted, which is a ten times larger volume than has been reported, earlier, when cEE took place from a vial into a capillary. Moreover, it is faster. First, the feasibility of this set-up was studied using the cationic purple dye crystal violet. Then the method was coupled to LC-MS and large volume cEE of several model peptides was optimised. Both liquid phases, the extraction voltage and the extraction time were optimised and it was found that the addition of trifluoroacetic acid (TFA) improved extraction dramatically. Moreover, the presence of some ACN at the liquid-liquid interfaces improved cEE. The whole procedure was automated and could be used routinely. Calibration curves of 5 test peptides in water have been constructed, resulting in good repeatability, good linearity and LOD values between 0.5 and 10 nM.

Finally, the method was applied to plasma analysis and calibration curves of the relevant plasma peptides angiotensin 1 and 2 as well as the 3-8 fragment of angiotensin 2 (angiotensin 2 (3-8)) were constructed which had good linearity and repeatability; LOD values were 10-50 nM. Analysis of unspiked plasma allowed the detection of about 60 putative endogenous peptides, underlining the great potential of EE as on-line sample concentrating technique. On-line large volume cEE-LC-MS allows for enrichment, separation and detection of plasma peptides from large sample volumes, minimises sample handling and can be an important step in full automation of analytical procedures.

1. Introduction

The quickly erupting field of systems biology and metabolomics research is putting analytical methods for major challenges. In order to be able to characterise a biological system and come to a greater understanding of the system, data should be collected on as many relevant biological components as possible, preferably with one analytical method [1]. A complicating factor is the fact that many biologically relevant compounds occur at trace levels, as is for example the case for many peptides [2]. Therefore, to be able to acquire relevant metabolomics data, limits of detection (LOD) should be improved.

An option to improve the LOD is to use electroextraction (EE) in the sample pretreatment procedure. EE takes place in a two-phase liquid-liquid system, consisting of an aqueous and an organic phase, where, under the proper conditions, an applied electric field causes ions to be extracted from one phase into the other, to be concentrated just after the liquid-liquid interface [3]. At the start, the analyte ions are in the organic phase and since the conductivity of this phase is much lower than in the aqueous phase, a very high electric field strength exists there that causes these analytes to migrate very fast into the aqueous phase. Since the electric field strength that is present in the aqueous phase is very low, the analytes migrate with a very low speed. As a consequence, they are concentrated as they reach the aqueous phase. A crucial requirement of the organic phase is that it contains some water, in order to hydrate ions, enabling the presence of ions; for example ethyl acetate (EtOAc) can contain approximately 2.5% water and is therefore suitable for EE experiments. Initially, EE was developed to enhance product yields in industrial-scale solvent-solvent extractions [4-10]. In the 1990s, analytical EE was developed, coupled to CZE and LC and demonstrated with simple test solutions [11-13]. Recently, we reported capillary electroextraction (cEE) coupled to LC-MS to improve the LOD of peptides in complex samples [14]. Within 10 min, peptide peaks in the resulting chromatograms could be increased ~100 times by performing on-line cEE as sample preconcentration compared to a conventional sample injection of 0.1 μL. However, we believed that the enrichment factor could be higher, without increasing the extraction time but rather decreasing it.

The enrichment factor is mainly dependent on the volume of organic phase where an electric field is present. In the earlier cEE set-up, this volume was between the capillary inlet and the tip of the electrode, and was around 10 μL, the capillary tip being close to the bottom of the sample vial and the electrode tip being close the liquid surface [14]. This extraction volume was not fixed, but just a part of the total sample volume. On top of this,

the aqueous phase was in the capillary, above the organic phase, while the organic phase had a lower density. Dipping the capillary, with the plug close to its end, in and out vials during the measurement frequently resulted in sample plug loss, introducing unreliability in the cEE set-up. With an increased extraction volume, higher enrichment factors and therefore better LOD values should be achievable. In literature, modified electrode configurations to improve injection amounts in electrokinetic injection in CZE have been described [15, 16], but these solutions require complicated alterations of the CE apparatus and/or sample vials and increase the risk of sample carry-over.

In the approach presented in this work, large volume cEE takes place in a wide-bore capillary (internal diameter of 1 mm). The liquid-liquid interface is located just in front of a valve with a sample loop. After cEE, the concentrated sample is pushed into a sample loop by shortly applying hydrodynamic pressure. The use of wide-bore capillaries in CE is not favourable due to extensive Joule heating, but in this case the large zone of low-conductive organic phase limits the current and as a consequence heat generation. Therefore, a high electric field strength could be applied.

The large volume EE set-up was evaluated by performing experiments on solutions comprising several test peptides. The main system components were optimised, namely the composition of the organic and aqueous phases, the extraction voltage and the extraction time. It was found that adding a counter ion to the organic phase dramatically improved the EE of peptides. Acetic acid, formic acid (FA) and TFA were studied and it was found that TFA improved peptide extraction best. First, the composition of the aqueous and organic phase, the extraction time and the extraction voltage was optimised for several test peptides in water. Then the method was transferred to plasma analysis and successfully validated.

2. Experimental

2.1 Chemicals

All reagents were of analytical grade or higher. A Millipore Q-guard water purifying system (Billerica, MA, USA) was used to obtain pure water. ACN, EtOAc, methanol, iso-propanol, dimethyl sulphoxide (DMSO), TFA and FA were obtained from Biosolve (Valkenswaard, The Netherlands), acetic acid from JT Baker (Philipsburg, NJ, USA) and the test peptides (bradykinin (1-5), mass 572; bradykinin (1-6), mass 660; ACIE-bradykinin, mass 964; bradykinin, mass 1060; angiotensin 2, mass 1296, angiotensin 1, mass 1046; and substance P, mass 1347) from Genscript Corporation (Piscataway, NJ, USA). A protease

inhibitor cocktail (product number P8340) and crystal violet are from Sigma Aldrich (St. Louis, MO, USA).

2.2 Equipment and Techniques

2.2.1 Large volume cEE set-up

Voltage and pressure application during cEE experiments was achieved with an Agilent Technologies (Santa Clara, CA, USA) 1600 series CE apparatus, using LC Certified 12x32mm screw thread autosampler vials with black 8 mm white silicone/red PTFE screw caps from Grace (Deerfield, IL, USA) and tubing and capillaries were purchased from Inacom Instruments (Overberg, the Netherlands). The 10-port 2-way switching valve with polyaryletherketone (PAEK) stator including actuator (Cheminert® HPLC injection valve model C2H-2340) was purchased at Valco Instruments (Houston, TX, USA). This valve is bio-compatible and the liquid has no contact with metal parts, preventing short circuiting and unsafe working conditions. For injection of the aqueous and organic phases, the flush function of the CE apparatus was used.

In Fig. 4.1 all the major components are depicted and in section 3.1 the procedure is described. During the whole procedure, the start signals of the CE apparatus is controlling the other components.

2.2.2 Mass spectrometry

Mass spectrometric experiments were carried out on a Bruker Daltonics MicrOTOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Spray settings were: end-plate offset -529 V, capillary voltage -2000 V, nebuliser gas pressure 0.4 bar, dry gas flow 4.0 L/min and dry temperature 200 °C. Identification of the test peptides took place by searching for the known peptide masses.

2.2.3 Liquid Chromatography

LC was carried out with an Agilent Technologies 1200 series µHPLC apparatus, equipped with a ZORBAX SB-C18 (5 µm, 150 x 0.5 mm) capillary HPLC column, also purchased from Agilent. A 20 µL/min gradient elution was employed with 0.1 % FA in water as solvent A and 0.08% FA in ACN as solvent B, starting at 0% B and ending after 30 min at 35%. In plasma analysis, the gradient ended after 40 min. Post conditioning concerned 5 min 95% solvent B and 5 min 100% solvent A. On measurement days, a blank run was performed first.

2.3 Sample pretreatment

Plasma (EDTA, citrated, and heparin) was purchased from Richmond Pharmacology (London, Great-Britain. Ultracentrifugation took place in Microcon mass centrifugal filter devices (MW cut-off 30 kDa; Millipore, Billerica, MA, USA) using an Eppendorf 5415R centrifuge (Eppendorf AG, Hamburg, Germany) and for SPE an Oasis HLB µElution plate was used (30 µm, Waters Corporation, Milford, MI, USA).

To break interactions between peptides and plasma proteins [17], plasma (300 µL) was 5 times diluted with 5% DMSO. Then the sample was ultracentrifuged (mass cut-off 30 kDa, 14000g, 4°C). After 50 min, 100 µL 5% DMSO was added and the sample was centrifuged for another 50 min. The filtrate was acidified with TFA (final concentration 1%), after which RP-SPE was carried out. The SPE material was wetted with ACN, equilibrated with 1% TFA, after which the sample was applied. Elution took place with 75 µL 100% ACN, which was directly mixed with 450 µL EtOAc for consecutive large volume cEE, by pipetting up and down. In one cEE experiment, 100 µL EtOAc is extracted, representing a plasma aliquot of 66.7 µL.

In the first instance, it was noticed that the peptide spikes, especially bradykinin, were degraded, resulting in unsatisfactory results. After adding a protease inhibitor mix (Sigma Aldrich, product number P8340 (St. Louis, MO, USA)), most of the degradation was avoided and results improved significantly.

3. Results and discussion

3.1 Description of the EE-LC-MS procedure

In Fig. 4.1, a scheme of the switching valve and the 4 main steps of the EE-LC-MS procedure are depicted. When the flush function of the CE apparatus is used, a pressure of approximately 950 mbar is applied on the inlet vial. This function was chosen to be able to quickly fill the large-volume EE capillary (part B in Fig. 4.1). The flow was restricted via the inlet capillary (part A in Fig. 4.1). The EE capillary, valve and outlet capillary had such a high inner diameter that virtual no back pressure was generated. By choosing optimal dimensions for the inlet capillary, a controlled flow was generated that allowed for precise injections within a reasonable time span. It was found that an inlet capillary with a length of 25 cm and an ID of 75 µm delivered just the right flow to obtain reasonable injection times (less than 3 min). To investigate whether this injection method was reliable, fixed-time injections were carried out. The vials were weighed prior and after each injection period, to

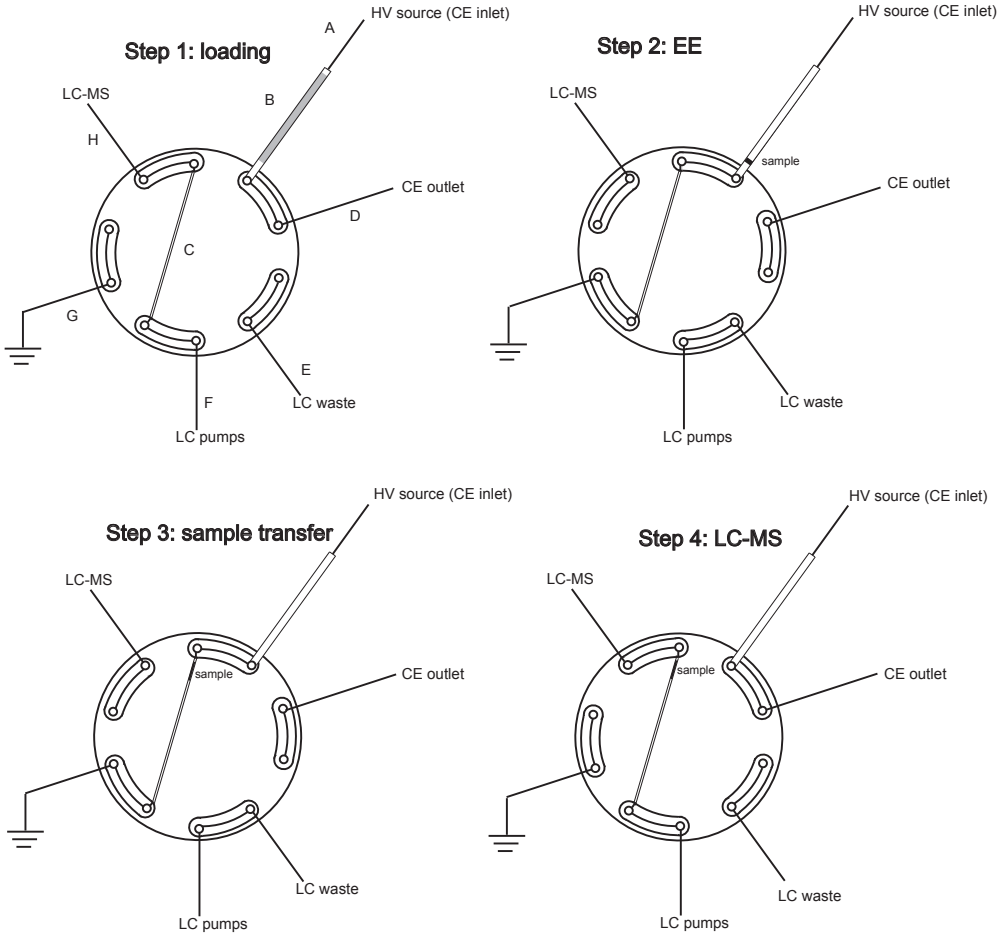

Figure 4.1 Valve set-up for interfacing large volume cEE with LC. A) inlet capillary (fused silica, L = 25 cm, OD = 365 μm, ID = 75 μm), B) EE capillary (PEEK, L = 20 cm, OD = 1.59 mm, ID = 1 mm), C) sample loop (PEEK, L = 20 cm, OD = 1.59 mm, ID = 0.5 mm) D) outlet capillary (fused silica, L = 75 cm, OD = 365 μm, ID = 200 μm) E) LC waste (PEEK, L = 100 cm, OD = 1.59 mm, ID = 0.5 mm), F) LC pump tubing (PEEK, L = 30 cm, OD = 1.59 mm, ID = 50 μm), G) tubing to earth (PEEK, L = 20 cm, OD = 1.59 mm, ID = 0.5 mm). H) tubing to LC-MS (PEEK, L = 20 cm, OD = 1.59 mm, ID = 50 μm) Step 1: situation after all phases have been loaded. The gray zone in the EE-capillary depicts the organic phase with analytes. Before EE starts, the valve is switched. Step 2: situation after EE is finished; the small black zone depicts the concentrated analytes. Step 3: situation after the sample has been transferred into the sample loop by applying pressure. After sample transfer, the valve is switched back. Step 4: situation where the sample zone is being injected into the LC-MS system.

be able to determine the injection volume.

During these experiments it was noticed that room temperature fluctuations significantly

influenced the injection volume; this is because temperature significantly influence the viscosity. This is undesirable, because when the location of the liquid-liquid interface is not well-controlled, the sample may not reach the sample loop completely or, on the other hand, be (partly) pushed beyond the loop. Therefore, the auto-sampler of the CE apparatus was cooled with streaming tap water, which controlled the temperature to 16.0-16.3 °C. Then, the injection method appeared to be highly repeatable for both the organic (40.5 ± 0.23 µL/min) and the aqueous phase (18.8 ± 0.054 µL/min). In summary, the location of the liquid-liquid interface and therefore also the injection volume could be controlled very accurately. During step 1 of the cEE-LC-MS procedure (Fig. 4.1), the EE capillary (B) was filled with the aqueous and organic phases via the inlet capillary (A). First, 0.1% FA in water was injected, which was the mobile phase used in LC. Then, EtOAc, containing the analytes, was injected and finally the second aqueous phase, containing 5% FA. Optimisation of the composition of the liquid phases will be discussed in section 3.3.2.1 and 3.3.2.2. After the loading step was finished, the valve was switched and cEE started (step 2). During cEE, the electrical circuit was grounded via an external vial containing 0.1% FA. As soon as the positive voltage was applied, the cationic analytes migrating towards the ground electrode (earth) were extracted and concentrated into a small zone. At the beginning of each measurement day, the tubing to earth (part G in Fig. 4.1) was flushed with 0.1 % FA to ensure good connection with earth. When cEE was finished, the sample zone was transferred into the sample loop using hydrodynamic pressure (step 3) after which the valve was switched back and the sample was injected in the LC-MS system and consecutive LC-MS analysis took place (step 4). The whole large volume cEE-LC-MS procedure was automated and was able to run without human interference for at least 18 hours, performing 24 experiments in a row.

The location of the liquid-liquid interface in the system is of crucial importance for the success of the experiments. In the first instance, it was attempted to carry out cEE in the port-to-port volume (internal loop volume) of the valve. However, this appeared to result in current leakage through the valve, due to the very low conductivity of the large EtOAc zone. Therefore, ideally, the interface is just in front of the valve. Then, after cEE, only a short pressure application step is needed to push the sample into the sample loop.

At first, a series of feasibility experiments was carried out in which 0.5 µM crystal violet was extracted from EtOAc into 0.1 % FA in a transparent EE capillary (PTFE, 20 cm x 0.8 mm) to be able to visually follow the progress of cEE (Fig. 4.2). On top of this, the current behaviour during cEE was monitored. The purpose of these experiments was to globally characterise how the liquid-liquid interface behaved and to study the cEE process

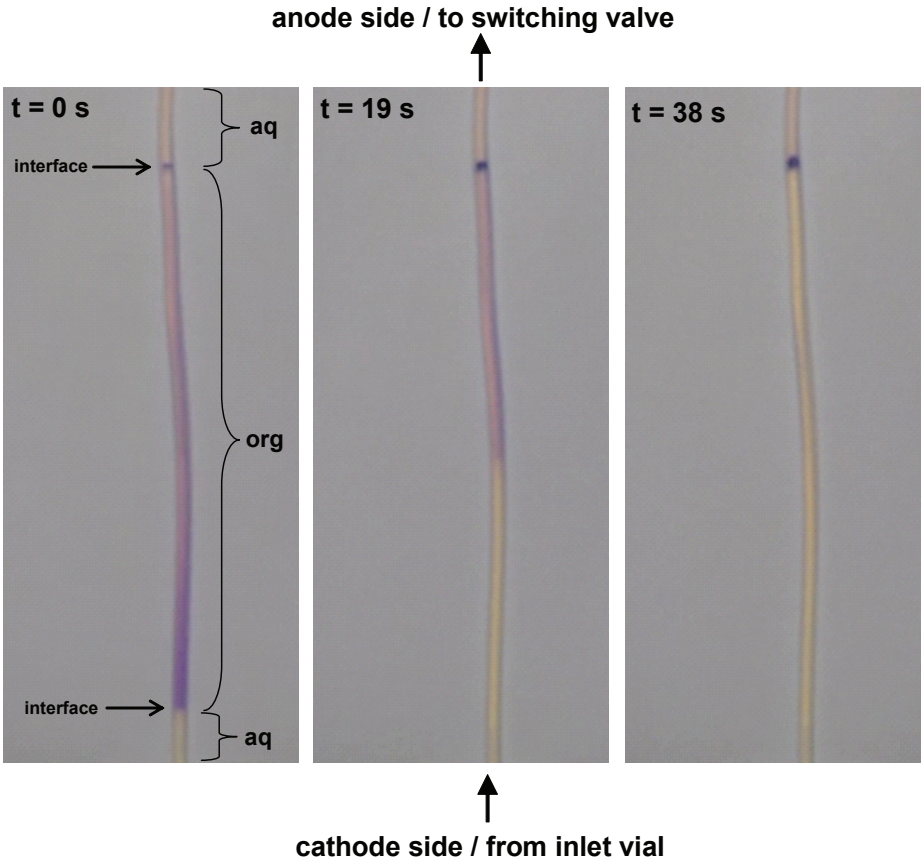

Figure 4.2 Video stills of cEE of the purple dye crystal violet in a 0.8 mm ID PTFE capillary. The organic phase consisted of EtOAc containing 0.5 µM crystal violet; the aqueous phase at the anode side of 0.1% FA and the aqueous phase at the cathode side of 5% FA.

It was observed that when a voltage higher than 1 kV was immediately applied, the cEE process was halted due to bubble formation. When the voltage was kept at 1 kV or lower during the whole experiment, extraction times became unfavourably long (> 10 min). However, it was found that when the voltage was increased gradually after applying a start voltage of 1kV no bubble formation occurred and complete extraction could be achieved within a minute. As shown in [14], the current is maximal at the start of cEE, after which it decreases exponentially. When the extraction voltage is low at the start of cEE, excessive Joule heating which results in bubble formation can be avoided.

It was also observed that during the loading procedure water droplets sometimes occurred in the organic phase. Despite the fact that these artefacts in the organic phase did not prevent cEE from taking place, it took longer and the current profile became unpredictable. However, it was found that when some ACN, which is miscible with both water and EtOAc, was injected before and after the organic phase, no water droplets occurred and cEE performance was significantly improved. The effect of injecting similar amounts of methanol, ethanol and isopropanol at the liquid-liquid interfaces were studied as well and showed similar effects. The volume of ACN that was injected was varied as well. When 1 µL ACN was used, cEE stability and speed was observed to be optimal (data not shown). Apparently, the presence of a small quantity of polar organic solvent at the interface that is soluble in both liquid phases is beneficial for cEE. The explanation for these observations is a subject for ongoing research.

It was also observed that increasing the FA concentration of the aqueous phase increased the extraction speed dramatically. This can be explained by the larger electric field strength that will be in the organic phase when the conductivity of the aqueous phase increases.

3.2 Application to peptides

3.2.1 System alterations

After the feasibility experiments, the method was coupled to LC-MS and the extraction of 5 test peptides was optimised. In these experiments, a PEEK capillary (20 cm x 1 mm ID) was used instead of the 20 cm x 0.8 mm ID PTFE capillary mentioned in the previous section, to increase the extraction volume. PTFE tubing of 1 mm ID appeared to be too difficult to connect properly due to the softness of the material; therefore a PEEK capillary was used. The organic sample volume was 100 µL for this set-up. Chromatographic peak area was used as measure for the extraction efficiency. The loading procedure in all experiments was as follows: 1) 10 min 950 mbar with 0.1% FA 2) 24 s 50 mbar ACN 3) 2.66 min 950 mbar EtOAc with analytes (= 100 µL) 4) 24 s 50 mbar ACN 5) 3.2 min 950 mbar with 5% FA. Then, the cEE process was started. After cEE was finished, the generated sample plug was pushed into the injection loop with 0.95 min 950 mbar, after which the valve was switched and the sample was injected into the LC-MS system. The resulting chromatograms showed good separation, an example of which can be seen in Fig. 4.4.

3.2.2 Organic phase optimisation

When only test peptides were present in the EtOAc, almost no current was observed

and the resulting chromatogram contained only low peptide peaks. This is not surprising, since there is a zone with very low conductivity with a length of 12.7 cm present that acts as insulator. To improve extraction, the conductivity of the EtOAc zone was increased. The addition of a small amount of pure FA (p*Ka* 3.77), acetic acid (p*Ka* 4.76) or TFA (p*Ka* 0.23) was investigated. First, a series of experiments (n=3, EE time 4 min, EE voltage gradient 1 to 15 kV) was carried out with 0.1% or 1% of one of the three acids present in the organic phase. From these experiments it became clear that, while all three acids caused an increased, comparable current, TFA enhanced the peptide extraction by far the best (Fig. 4.3A). Moreover, 1% acid had a greater effect than 0.1% in the case of all three studied acids. TFA is the strongest acid of the three acids studied and an acidic environment is obviously favourable for peptides to be present in the cationic form. However, more research will be required to assign the effects of adding acids.

Next, the TFA concentration was optimised. A series of experiments (n = 3, equal EE conditions) was carried out in which the amount of TFA was varied (0.2% to 1.8% in steps of 0.2%). The results of these experiments indicated that the TFA concentration was not of great influence on the peak area (data not shown). At the highest TFA concentration the peptide peak areas were slightly larger, while the overall relative standard deviation improved also slightly. When TFA concentrations above 1.8% were used, the current became so high that too much Joule heating took place leading to bubble formation and consequently decreased cEE performance or even failure of experiments. In the rest of the experiments, 1.8% TFA was present in the organic phase.

3.2.3 Aqueous phase optimisation

The aqueous phase into which the peptides were extracted had to be injected into the LC. To avoid sample solvent related problems during LC separation, this aqueous phase was chosen to be mobile phase A, i.e. 0.1% FA, and not further optimised.

The composition of the aqueous phase at the other side of the organic phase, however, was optimised. In cEE experiments with crystal violet, it was noticed that when a higher FA concentration was used, cEE proceeded faster (see section 3.1). Since the conductivity of the aqueous phase increases at increased FA percentage, the electric field strength decreases. This causes a higher electric field strength in the organic phase and therefore faster ion migration in the organic phase. In a series of experiments (n = 3, EE time 4 min, EE voltage 15 kV, 1.8% TFA in organic phase), the FA concentration was varied and the relative peak area increased from 0.1% to 2% FA after which it remained rather constant with a slight optimum at 5% FA (Fig. 4.3B).

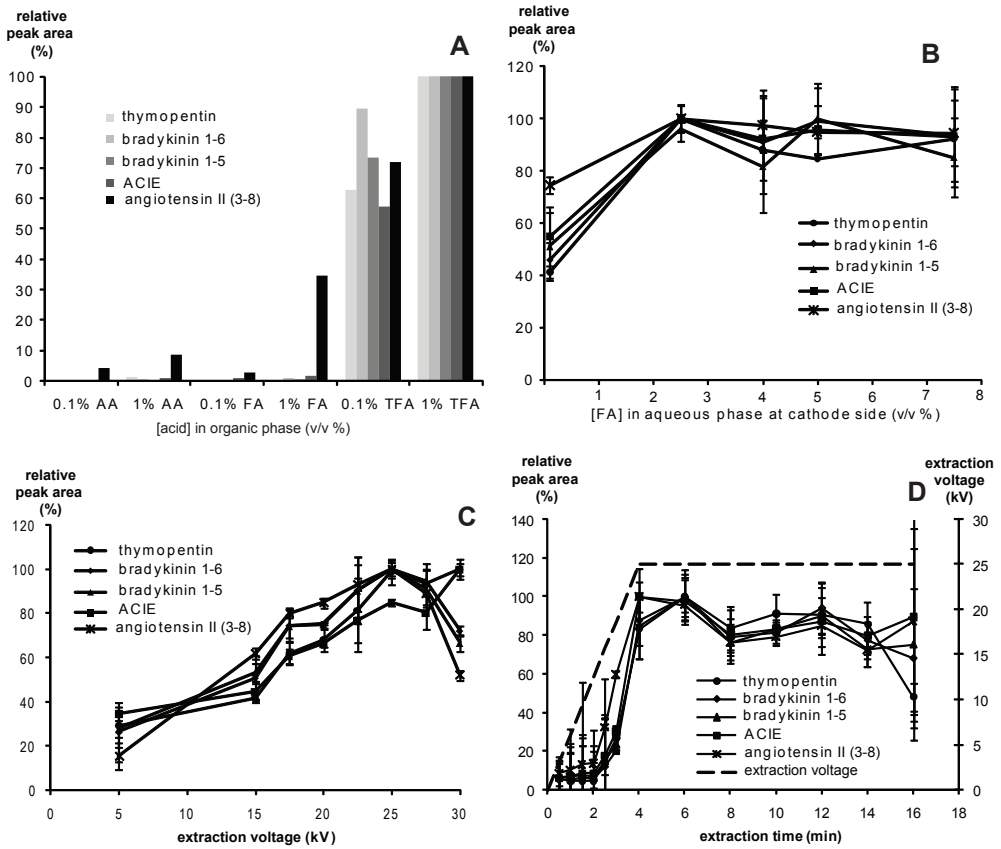

Figure 4.3 A) influence of presence of two concentration levels of acetic acid (AA), FA (FA) and TFA (TFA) on the extraction of 5 test peptides from EtOAc, B) optimisation of FA in aqueous phase C) optimisation of extraction voltage D) optimisation of extraction time. Relative peak area is calculated by setting the highest obtained value at 100%.

3.2.4 Extraction voltage and time optimisation

During several series of experiments, all carried out in triplicate, the optimal HV and extraction time were determined (other conditions: 1.8% TFA in organic phase, 5% FA the aqueous phase at the inlet side, 0.1 % FA at the outlet side). It was studied how fast the HV could be increased and how long it should be applied after it reached its maximum. As can be seen in Fig. 4.3C, the optimal HV appeared to be 25 kV. The optimal time to increase the HV from 0 to 25 kV was 4 min, i.e. 6.25 kV/min. When faster HV gradients were applied, current profiles became instable and lower peaks were obtained (data not shown). When the

maximal HV of 25 kV was reached, prolonging the extraction time did not increase peptide extraction (Fig. 4.3D). All peptides showed the same pattern in these voltage and time optimisation series of experiments, indicating that differences in electrophoretic mobility have no influence on the extraction success at this timescale.

In summary, the optimal cEE voltage procedure was to start at 0 kV and increase with 6.25 kV/min to the maximum voltage, being 25 kV.

3.2.5 Enrichment effect

To determine the enrichment factor that the optimised cEE procedure can achieve, chromatographic peak areas resulting from large volume cEE-LC-MS were compared with conventional 0.1 µL LC-MS injections (the maximal injection volume advised by the column manufacturer when the sample is not in water) of the sample. The starting concentration of the peptides was equal in cEE-LC-MS and LC-MS experiments. In Fig. 4.4, a comparison between a conventional LC-MS injection of 0.1 µL EtOAc and a cEE-LC-MS injection of 100 µL EtOAc containing peptides is shown. Based on this comparison, enrichment factors could be determined to be 570 times for thymopentin, 840 times for bradykinin (1-6), 990 times for bradykinin (1-5), 830 times for ACIE and 590 times for angiotensin 2 (3-8).

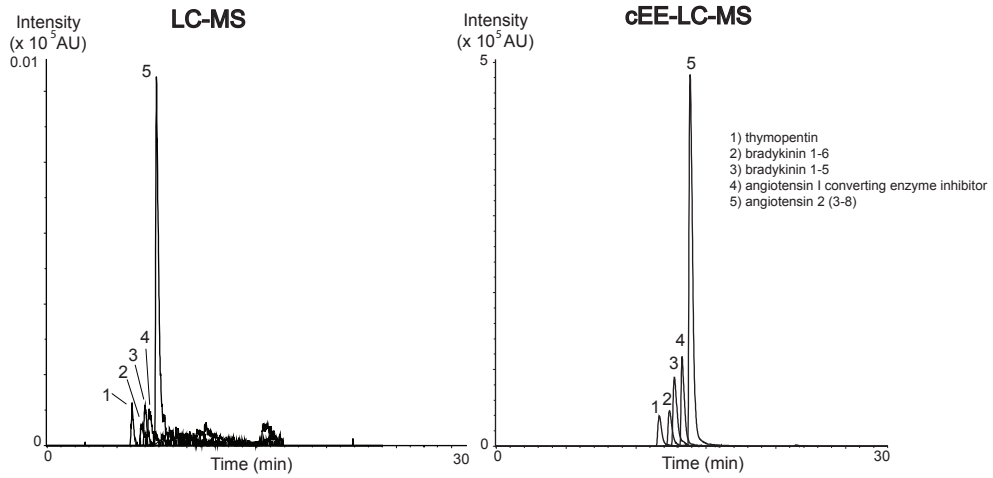

Figure 4.4 Comparison of chromatograms obtained from LC and large volume cEE-LC-MS analysis. After an EE step of only 4 min, peaks are considerably higher. Injection volume in LC was 0.1 µL EtOAc; extracted organic phase in EE was 100 µL. The initial peptide concentration was equal.

This is a significant improvement with respect to the previously described cEE method, where enrichment factors of ~100 were obtained [14]. Whereas the maximum possible enrichment factor is 1000 times, the obtained enrichment factors are lower. During the

optimisation, it was shown that prolonging the extraction time does not result in increased peptide peak areas (Fig. 4.3D). A possible explanation for the sub-maximal enrichment factors is peptide loss due to wall adsorption, a phenomenon that has been reported previously in literature [18, 19].

3.2.6 Linearity and repeatability

When constructing calibration curves, it was noticed that at low analyte concentrations, the peak area of the internal standard (bradykinin) decreased, even though the concentration of it was equal in all calibration samples. This affected the linearity of the calibration curves (data not shown). As mentioned in section 3.3.2.4, a probable reason is adsorption of the peptides to the wall. As a remedy, 2 μM substance P was added to all samples to suppress analyte and internal standard adsorption by competition. This resulted in equal internal standard peak areas in all calibration measurements. Satisfactory calibration curves (range 0.2 - 1000 nM in water) of the 5 test peptides could be constructed (n=3), of which the regression data is shown in Table 4.1. Regression analysis showed that the curves had no significant intercept with the y-axis ($P < 0.05$ at a confidence level of 95%), which indicated that the lines could be drawn through origin. The repeatability and linearity were good and LOD values were between 0.5 and 10 nM

Table 4.1 Analytical performance characteristics of cEE-LC-MS analysis of test solutions of model peptides

peptide	slope ± stdev	standard error	# data points	correlation coefficent	range (uM)	LOD (nM)
thymopentin	0.56 ± 0.072	0.034	18	0.985	0.01-1	10
bradykinin 1-6	0.43 ± 0.022	0.0094	21	0.998	0.005-1	5
bradykinin 1-5	0.77 ± 0.041	0.017	21	0.997	0.005-1	5
ACIE	1.22 ± 0.067	0.025	30	0.997	0.0005-1	0.5
angiotension 2 (3-8)	4.39 ± 0.28	0.1	30	0.996	0.0005-1	0.5

3.3 Application to plasma analysis

3.3.1 Sample pretreatment prior to plasma analysis

When untreated, i.e. when no SPE or ultrafiltration was applied and plasma was directly mixed with EtOAc prior to cEE, low peptide recovery was obtained and the EE process was unreliable. Therefore, plasma samples were deproteinated with ultrafiltration and desalted with RP-SPE. The trapped peptides were eluted from the cartridge with ACN. Instead of evaporating the SPE eluent and reconstitution in water before injection into the RP-

LC system, which is common practice in peptide analysis [20, 21], the eluent was mixed with EtOAc by pipetting up and down and cEE was performed. This required less sample handling; therefore it is less labour-intensive, less susceptible to experimental errors and easier to automate. Test runs with EDTA, heparinated and citrated plasma showed similar results and EDTA plasma was chosen for all further experiments.

3.3.2 Quantitative aspects

Contrary to test mixtures, a multitude of charged compounds can be expected to be present in plasma analysis, even after desalting. This will likely influence the EE process and therefore, cEE voltage and time were re-optimised. It was found that above 15 kV, the liquid-liquid interface became instable and that when 15 kV was used as maximal voltage, the extraction time had to be prolonged with 2 min (data not shown). In summary, the voltage was increased in 4 min from 0 to 15 kV (3.75 kV/min) and then 15 kV was maintained for 2 more min. Under these circumstances, similar enrichment factors (500-1000 times) as with the academical mixtures were achieved (data not shown).

The recovery of the ultrafiltration and SPE procedure was studied. Large volume cEE-LC-MS peak areas resulting from samples spiked prior to and after ultrafiltration and SPE were compared. The recoveries were determined to be 87 ± 2% for angiotensin 2 (3-8), 82 ± 17% for angiotensin 1, 70 ± 10% for bradykinin, 62 ± 11% for angiotensin 2, and lower than 5% for thymopentin, bradykinin (1-5) and bradykinin (1-6) (n=3). The low recoveries obtained for both thymopentin, bradykinin (1-5) and bradykinin (1-6) fragments are not caused by cEE (as shown in section 3.3.2.4, large volume cEE of these peptides is highly feasible) but can be explained by the fact that the SPE conditions were not optimal for these rather hydrophilic peptides.

Then, calibration curves of the relevant plasma peptides angiotensin 1 and 2 as well as angiotensin 2 (3-8) spiked to plasma were constructed, using bradykinin as internal standard, and measured in triplicate (Fig. 4.5). Regression analysis showed that the curves had no significant intercept with the y-axis ($P < 0.05$ at a confidence level of 95%), which indicated that the lines could be drawn through origin. Linearity was good to excellent, R^2 0.990 - 0.998, and mean relative standard deviation of the slope satisfactory between 1 % and 18%. LOD values were 10-50 nM (Table 4.2).

Large volume cEE-LC-MS experiments performed on unspiked plasma resulted in around 60 putative endogenous peptides (Fig. 4.6).

Table 4.2 Analytical performance characteristics of large volume cEE-LC-MS of three peptides spiked to plasma

peptide	slope ± stdev	standard error	# data points	correlation efficient	range (uM)	LOD (nM)
angiotensin 2	6.36 ± 0.086	0.31	18	0.991	0.005-1	10
angiotension 2 (3-8)	0.18 ± 0.00054	0.03	18	0.994	0.05-5	50
angiotensin 1	0.76 ± 0.10	0.23	18	0.984	0.05-5	50

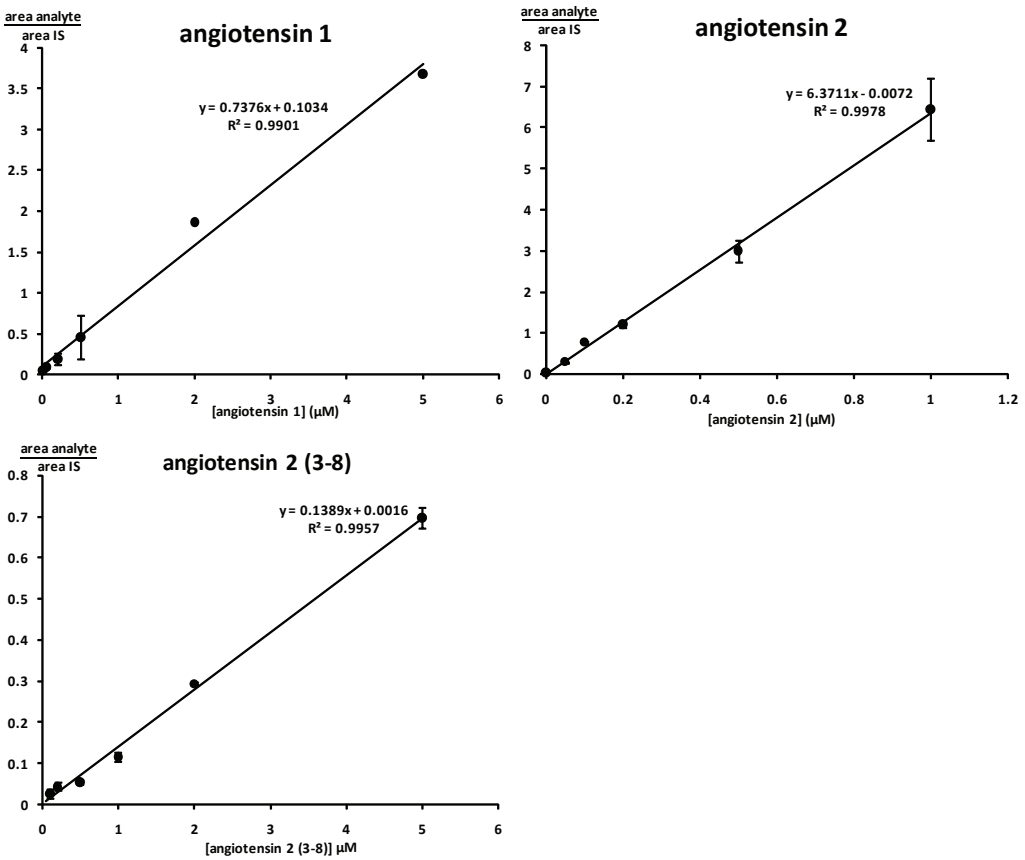

Figure 4.5 Calibration curves and data of angiotensin 1, angiotensin 2 and angiotensin 2 (3-8) in plasma (n = 3).

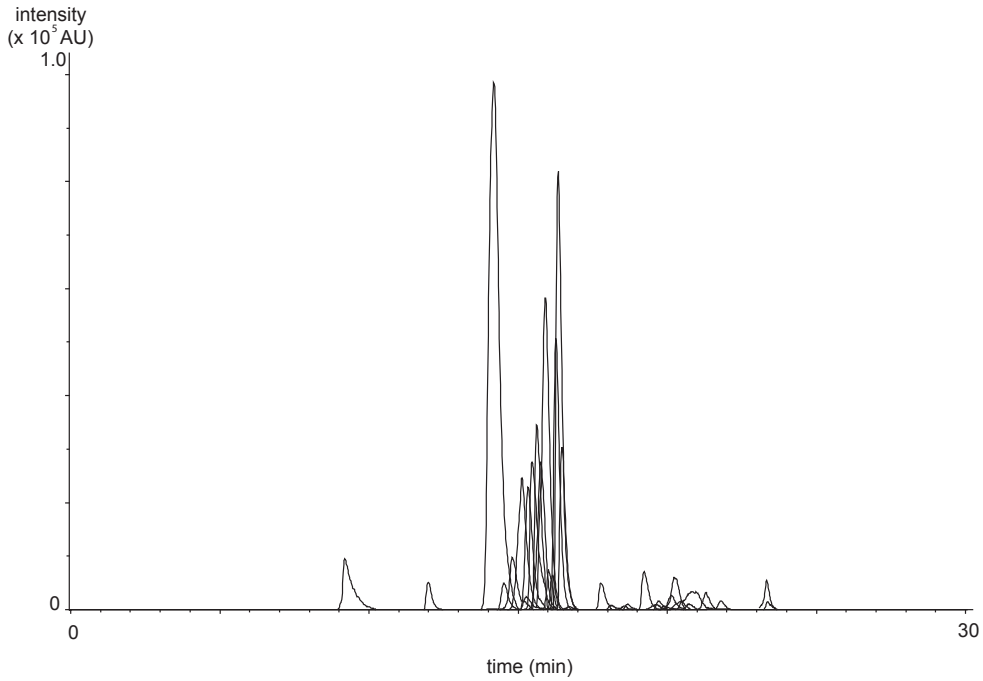

Figure 4.6 Large volume cEE-LC-MS chromatogram of unspiked EDTA plasma. The injected plasma aliquot was 67 μL.

4. Conclusions

We described the set up, optimisation and biological application of large volume cEE-LC-MS of peptides. The method presented in this chapter is capable of on-line enrichment of peptides within 4 min, resulting in significantly increased peak heights compared to conventional LC injection. Enrichment factors were between 570 and 990 times when compared with a conventional LC injection. In comparison with the previously described cEE set-up [14], the method is capable of extracting larger volumes, resulting in significantly higher enrichment factors, and it is faster. The addition of TFA improved EE dramatically. The whole procedure has been automated and could be used routinely for plasma. Linearity and repeatability were excellent between 0.2 nM and 1000 nM. The described method is fully automated and minimises sample handling after SPE (i.e. evaporation and reconstitution steps). Therefore, it can be an important step in full automation of analytical procedures. Future research will be devoted to coupling of SPE directly to large volume cEE, to coupling of large volume cEE to other separation methods than RP-HPLC and to analysis of other compounds than peptides.

5. References

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