Author: Lindenburg, Petrus Wilhelmus
Title: New electromigration-driven enrichment techniques for peptidomics and metabolomics
Date: 2012-06-05

Chapter 5

Feasibility of electroextraction as versatile sample concentrating pretreatment for fast analysis of low abundant urine metabolites and its application to acylcarnitines

Based on
Lindenburg P.W., Tjaden U.R., Van der Greef, J., Hankemeier, T.
Feasibilityof electroextraction as versatile sample concentrating pretreatment forfast analysis of low abundant urine metabolites and its application to acylcarnitines.
Submitted to *Electrophoresis.*

Abstract

In this work we demonstrate the applicability of electroextraction (EE) to urine metabolites. To investigate which urine metabolite classes are susceptible to EE, off-line EE experiments were carried out with a prototype device, in which urine metabolites were electroextracted from ethyl acetate (EtOAc) into water. The obtained extracts were examined with direct infusion MS and the results demonstrated that several compound classes could be extracted, amongst which amino acids and acylcarnitines. Acylcarnitines were selected for evaluation of the performance of EE. For this, the EE set-up was adapted to capillary EE (cEE) to be able to analyze large urine sample series, and it was coupled on-line to LC-MS. cEE-LC-MS of acylcarnitines was optimised and characterised. The recovery, linearity, repeatability and limit of detection of the cEE-LC-MS method was good to excellent. To demonstrate the versatility of EE for sample preparation in analytical procedures, extracts were injected into a CZE-MS system, resulting in detection of the acylcarnitines along with more than 100 presumed metabolite peaks. The results presented here indicate that EE can be used as a fast sample preconcentration technique of low abundant urine metabolites, in combination with both LC and CZE.

1. Introduction

Metabolomics studies typically involve the measurement of large sample sets. To be able to gain more insight into important metabolic processes, as many metabolites as possible should be measured, including the low-abundant ones that often are of high biological relevance. Therefore, fast analytical techniques are required that are capable of measuring many low-abundant compounds.

Sample pretreatment procedures that are based on electromigration are very promising, because they meet the requirements mentioned above in several ways. First, a considerable part of the metabolome consists of compounds that are or can be charged and so are suitable for an electrophoretic approach, such as fatty acids, carbohydrates, nucleotides, amino acids and acylcarnitines [1]. Furthermore, electromigration can be used to concentrate analytes. Despite the fact that this approach is well-known in CE-based techniques [2-4], relatively few examples of the application of electromigration-based sample pretreatment to large sample volumes can be found in literature. One example is electrodialysis, which has been applied as a fast and selective sample pretreatment method to LC-MS analysis of low-abundant peptides in synovial fluid [5]. Other examples of electromigration-based sample pretreatment techniques that have been applied to complex samples are preparative isotachophoresis [6-8] and isoelectric focusing [8].

Another electromigration-based analyte preconcentration technique is electroextraction (EE), which is capable of concentrating analytes two-three orders of magnitude within several minutes, resulting in improved LOD [9, 10]. EE takes place when an electric field is applied to a two-phase liquid-liquid system consisting of an aqueous and an organic phase. Ions in the organic phase are subjected to a very high electric field strength, due to the very low conductivity. As a consequence, they migrate at full speed towards the liquid-liquid interface, to be concentrated in the aqueous phase directly after they pass the interface, because the electric field strength is so much lower there [9, 10]. Initially, EE has been developed as a purification technique in the field of chemical engineering, to enhance product yields resulting from solvent extraction [11-16]. However, EE can also be used to enhance the sample aliquot that is injected into analytical methods. In the 1990s Van der Vlis *et al.* adapted EE for analytical purposes, using EtOAc as organic phase and performing EE of test solutions in capillaries (cEE) [17-19]. Recently, we showed the successful application of cEE coupled to LC-MS to the analysis of peptides in urine [9]. Then, we further improved cEE to large volume cEE and showed extraction of plasma peptides from large sample volumes (up to 100 µL) coupled to LC-MS [10].

So far, cEE has been reported for some β-agonists, leukotrienes and catecholamines from test mixtures [17-19] as well as cEE of peptides in urine and plasma [9, 10]. The goal of this research paper is to demonstrate that cEE is applicable to a wider range of compound classes in complex samples than has been demonstrated so far. Therefore, the performance of EE as sample pretreatment of human urine samples was investigated. In a quantitative screening experiment using a prototype EE device (Fig. 5.1), it was investigated which metabolite classes are susceptible to EE. Due to its biological relevance [20], in further experiments the focus was put on analysis of acylcarnitines. The performance of EE of acylcarnitines in urine was evaluated with consecutive on-line LC-MS analysis. On-line large volume cEE as described in [10] was optimised, then calibration curves of 4 acylcarnitines spiked to urine were generated and endogenous levels were determined by the standard addition method.

Finally, to assess how versatile EE is as a generic sample pretreatment technique, EE was coupled off-line to CZE-MS instead of LC-MS.

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. Acetonitrile (ACN), EtOAc, formic acid (FA), ammonium hydroxide, sodium hydroxide and TFA were obtained from Biosolve (Valkenswaard, The Netherlands). The acyl-L-carnitine standards were from Sigma (St. Louis, MO, USA).

2.2 Sample pretreatment

L-acylcarnitine stock solutions of 5 mM were prepared in 1:1 water/methanol (v/v), and were stored at -20° C. Stock solutions of 0.5 mM and 0.05 mM were made by dilution with 1:1 water/methanol (v/v). Calibration solutions were made by adding various amounts of the stock solutions to water or urine. Urine was collected from 3 healthy volunteers, pooled, centrifuged and stored at -80° C in aliquots of 1 mL. This urine was used in all experiments.

2.3 Equipment and techniques

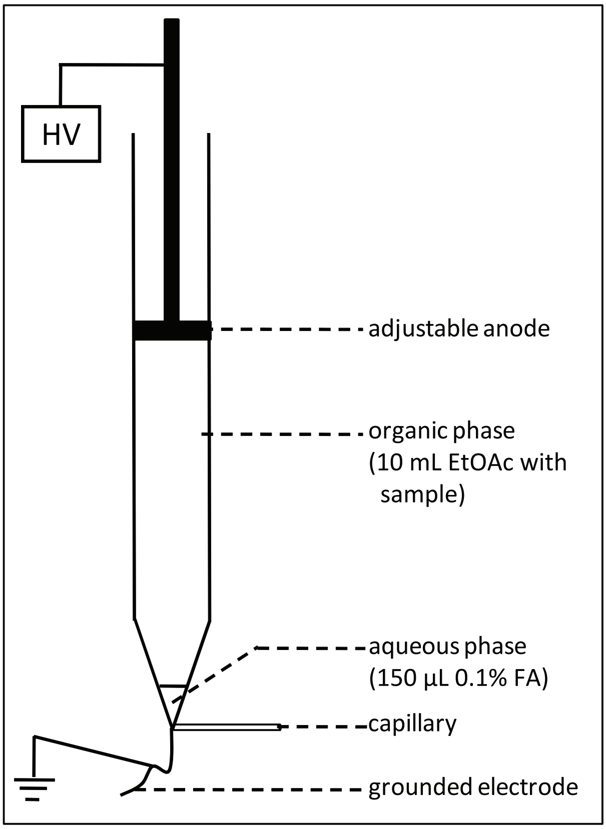


Figure 5.1 Schematic drawing of the off-line EE prototype device

2.3.1 Screening experiment with off-line EE set-up

The prototype EE device (Fig. 5.1) has been described in detail in [9]. In short, the device consisted of a polychlorotrifluorethylene (KelF) vial with a pointed bottom, where a capillary and a platinum grounding electrode were located. The capillary served to inject and remove aqueous phase underneath the organic phase, in the pointed bottom of the device. The electric circuit was closed by using a piston-like gold coated electrode as anode fitting exactly in the vial, thus contacting exactly the whole surface of the organic phase to ensure that all charged molecules in the organic phase were in the electric field. High voltage (HV) was applied with a Spellman HV Power Supply (Spellman, Hauppauge, New York, USA). Urine was brought to 2% FA and 20 µL was added to 10 mL EtOAc. The sample was centrifuged 5 min at 15000 g to remove insoluble particles, after which EE was performed on the supernatant at 2.5 kV for 10 min, with 150 µL 0.1% FA as aqueous acceptor phase.

The extracts were infused into a linear triple quad - ion cyclotron resonance - Fourier transform - mass spectrometer (LTQ-FTICR-MS) (Thermo Fisher Scientific, San Jose, CA, USA) to obtain accurate mass values of the extracted compounds. Direct infusion mass spectra were measured, at a resolution of 100000. To get an impression of which metabolite classes were extracted, the most intense recorded masses (mass range 50-300 m/z) were matched with a database (www.hmdb.ca, [1]), taking 0.06 Dalton as maximal mass difference for identification.

2.3.2 On-line large volume cEE-LC-MS set-up

The on-line large volume cEE-LC-MS set-up (Fig. 5.2) has been described in detail recently [10]. In short, aqueous and organic phases were injected into a capillary (part B in Fig. 5.2) and the location of the interface was chosen to be just in front of the valve (step 1). Then, the valve was switched and the extraction voltage was applied. When cEE is finished (step 2), the concentrated sample plug was transferred into the sample loop (step 3) by applying pressure, after which the valve was switched back to inject the sample into the LC-MS system (step 4). Voltage and pressure application during cEE experiments were done with an Agilent Technologies (Santa Clara, CA, USA) 1600 series CE apparatus. The start signal of the CE apparatus was used as a trigger pulse for the valve, LC and MS.

LC was carried out with an Agilent Technologies 1200 series µHPLC apparatus, equipped with a ZORBAX SB-C18 capillary HPLC column (5 µm, 150 x 0.5 mm; Agilent Technologies). A 20 µL/min gradient elution was employed with 0.1 % FA in water as solvent A and 0.1% FA in ACN as solvent B and consisted of 5 steps: 1) 1 min 100% A, 2) decrease to 20% in 14 min 3) 6 min at 20% A, 4) 5 min at 0% A for cleaning and 5) 5 min at 100% A for reconditioning. On each measurement day, a blank run was performed first.

Detection was achieved with a Bruker Daltonics MicrOTOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Mass spectra from the time-of-flight MS (TOF-MS) were internally calibrated using a sodium formate adduct plug (10 mM NaOH, 0.1 FA in 1:1 methanol/water) that was infused (using a Pharmacia LKB HPLC Pump 250 (Uppsala, Sweden)) at the end of every chromatogram via the switching valve on the MS.

2.3.3 CZE-MS

Experimental conditions of CZE experiments were based on [21] and were carried out using an Agilent Technologies 1600 series CE apparatus. An untreated fused capillary was used (length 80 cm, ID 75 µm) and hydrodynamic injection was employed (2s, 50 mbar). Separation took place in 0.8% FA as background electrolyte under a separation voltage of 30

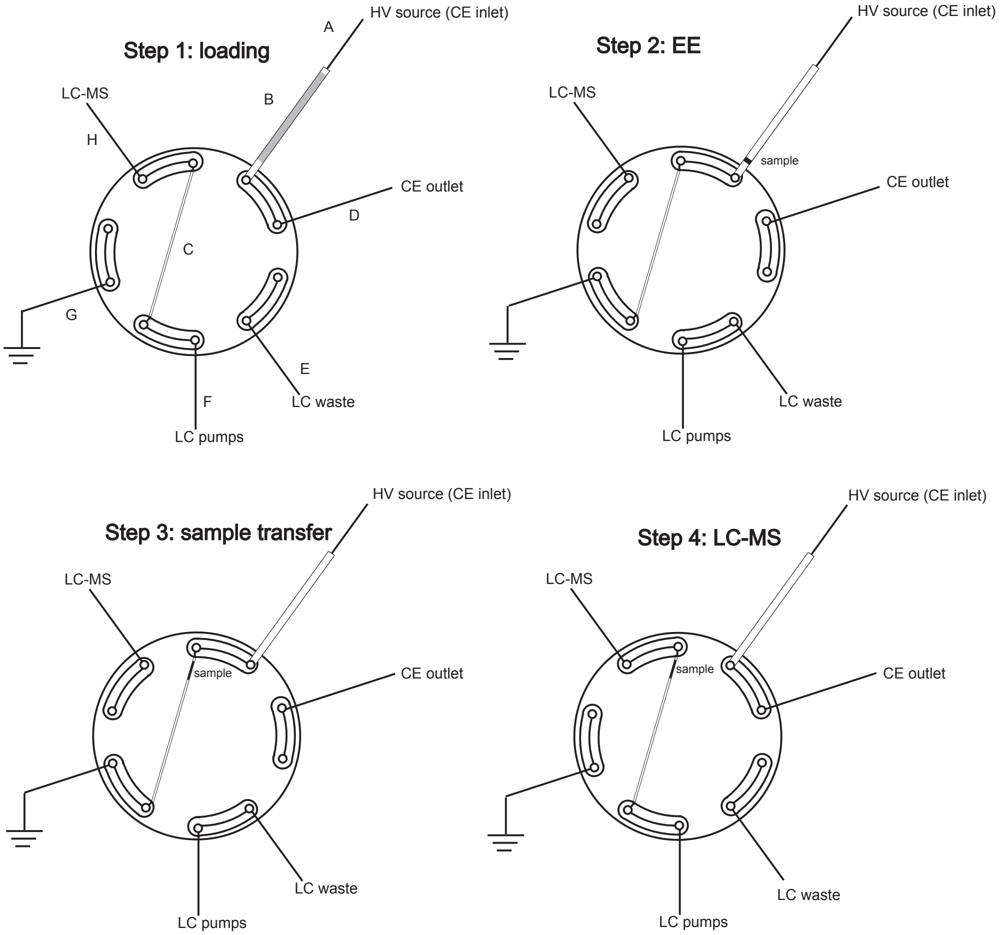


Figure 5.2 Valve set-up for interfacing large volume cEE with LC. A) inlet capillary (fused silica, L = 25 cm, OD = 365 µm, ID = 75 µm), B) EE capillary (PEEK, L = 20 cm, OD = 1.59 mm, ID = 1 mm), C) sample loop (PEEK, L = 20 cm, OD = 1.59 mm, ID = 0.5 mm) D) outlet capillary (fused silica, L = 75 cm, 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.

kV. Between measurements, the capillary was reconditioned by flushing 5 min with distilled water, 5 min with 1 M ammonium hydroxide and 5 min with background electrolyte. The CZE system was coupled to an Agilent Technologies 6530 QTOF-MS, using a CE-MS sheath-liquid assisted sprayer which was also from Agilent Technologies (part number 1607-60001). The sheath liquid consisted of 50/50% methanol/ 0.1% acetic acid in distilled water and was delivered with an Agilent Technologies 1100 series pump at a rate of 10 µL/ min.

3. Results and discussion

3.1 Off-line EE

The purpose of this screening experiment was to quickly get a rough idea which metabolite classes in urine are susceptible to EE. For this, electroextracts from urine were infused into an LTQ-FTICR-MS for accurate mass determination and, using the human metabolome database, 49 masses could be putatively attributed to metabolites that were electroextracted into the aqueous phase. The number of putatively identified metabolites can be expected to be higher, since the mass spectra contained hundreds of peaks. However, the purpose of this experiment was, as mentioned above, to obtain a rough impression of which metabolite classes can be electroextracted from urine. Therefore, no further effort was made to annotate all the mass peaks. Definitive identification of the metabolites requires MS/MS of MS^n analysis of the observed masses, which is beyond the scope of this study.

Many of the supposed identified metabolites were amino acids and acylcarnitines (Table 5.1). To optimise the cEE procedure, to validate with cEE-LC-MS and to demonstrate the potential of the cEE approach, acylcarnitines were chosen as metabolite class of interest. Acylcarnitines (carnitine esters) are conjugates of carnitine and fatty acids that can function as biomarkers for various inborn metabolism errors; many genetic and acquired disorders affect the acylcarnitine levels in biological fluids [20].

The prototype device is useful for a quick experiment, but for the analysis of a large series of samples, the set-up of the method has to be automated and coupled on-line. Therefore, an on-line large volume cEE set-up was optimised for acylcarnitines and coupled to LC-MS.

3.2 On-line large volume cEE of acylcarnitines in urine

The cEE-LC-MS set-up that has proven its potential for peptides [10] was now optimised for acylcarnitines. In a series of experiments, the extraction voltage and TFA concentration (0, 0.1, 0.5, 1, 1.5 and 2%) were optimised using test solutions of EtOAc that was saturated with acylcarnitines in water. The injected volume of organic phase extracted was 100 µL. The extraction procedure was as follows: in 4 min the voltage was gradually increased from

1 kV to the maximum voltage (1, 15 or 30 kV), after which the maximum voltage was maintained for 2 additional min. As can be seen in Fig. 5.3A, 15 kV was sufficient and it was therefore selected for subsequent experiments.

Table 5.1 List of classes of putatively assigned compounds detected in urine by off-line EE/DI-LTQ-FT-MS compounds classes and detected number of compounds per class

compound class	# detected analytes
Amino acids	19
Acylcarnitines	6
Amino ketones	3
Acyl glycines	2
Amino alcohols	2
Aromatic acids	2
Fatty acids	2
Nucleosides	2
Aliphatic amines	1
Catecholamines	1
Cyclic amines	1
Dicarboxylic acids	1
Dipeptides	1
Hydroxy acids	1
Indoles	1
Keto acids	1
Peptides	1
Polyphenols	1
Purines	1
Total	49

Apart from a blank, 5 different TFA concentrations were studied (Fig. 5.3B). In [10], it was found that the addition of TFA to the organic phase improved cEE of peptides dramatically. Between 0.1% and 2% TFA, the results were satisfactory for all acylcarnitines, except for stearoylcarnitine. The unpredictable results of stearoylcarnitine are possibly due to its low solubility in water. However, when no TFA was added, peak areas were significantly lower and not reproducible. There were no marked differences between 0.5%, 1% and 1.5% TFA. The lowest suitable concentration, 0.5% TFA, was chosen for the remainder of the experiments to avoid risk of excessive Joule heating caused by the increased conductivity. As the results obtained for HV and TFA optimisation were very similar to the results obtained in peptide analysis [10], the same extraction time and aqueous phase composition were selected.

Summarising, the final cEE procedure was a HV gradient of 4 min from 1 to 15 kV, followed by 2 more min of 15 kV. The TFA concentration was 0.5%, the aqueous phase at the cathode side 5% FA and at the anodic side 0.1% FA (i.e. the LC mobile phase).

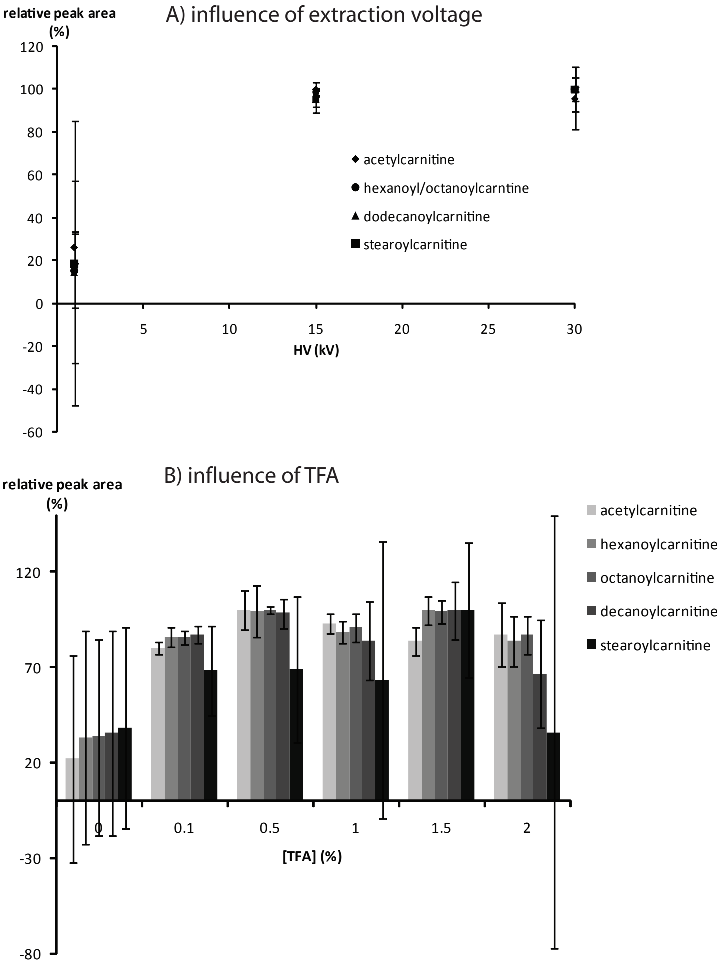


Figure 5.3 Optimisation of A) extraction voltage and B) TFA concentration. Relative peak area was calculated per acylcarnitine, by setting the highest obtained peak area per acylcarnitine to 100%

Peak areas of chromatograms resulting from on-line large volume cEE-LC-MS (100 μL) and LC-MS (0.1 μL) of EtOAc saturated with acidified urine were compared (Fig. 5.4) and it was found that the peak area of hexanoylcarnitine was increased 970 times, decanoylcarnitine 920 times, octanoylcarnitine 790 times, stearoylcarnitine 640 times and acetylcarnitine 175 times. Based on the corresponding volume ratios, the enhancement can amount to maximally 1000 times, which demonstrates that the cEE of hexanoylcarnitine and decanoylcarnitine is virtually complete, while some loss of octanoylcarnitine and stearoylcarnitine occurs. The low recovery of acetylcarnitine might be explained by the fact that it elutes from LC with the void volume in 100 % solvent A (0.1% FA), which makes it difficult to compare the conventional LC injection with the cEE-LC injection, due to possible ion suppression by matrix compounds.

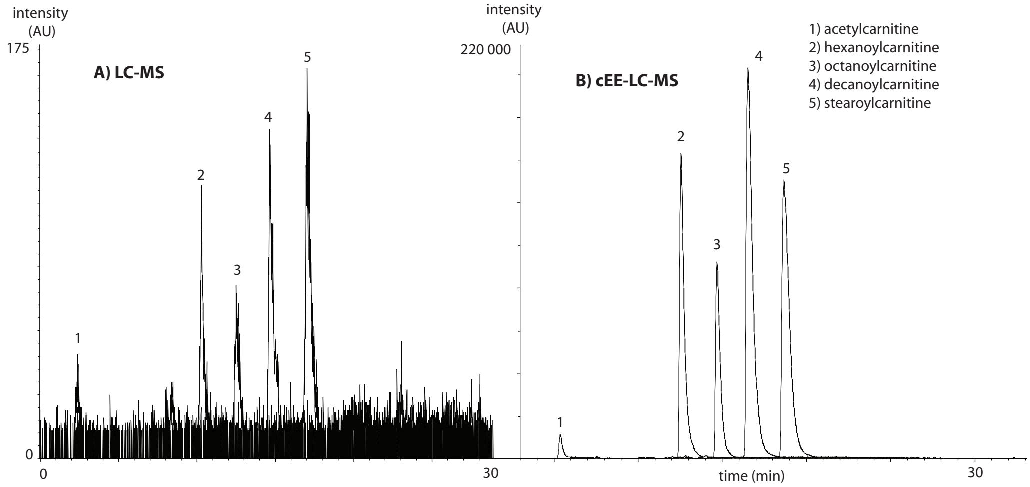


Figure 5.4 Comparison of chromatograms obtained from LC and cEE-LC-MS analysis of acylcarnitines spiked to urine. After a cEE step of only 6 min, peaks are considerably higher. Injection volume in LC was 0.1 μL; extracted organic phase in cEE was 100 μL. The initial acylcarnitine concentration in urine was equal (octanoylcarnitine 20 μM, other acylcarnitines 50 μM).

After optimisation experiments with acylcarnitine test solutions, acylcarnitines spiked to urine were analyzed. In one single run, an aliquot of 2 μL urine was analyzed, i.e. 100 μL EtOAc saturated with urine was electroextracted. Sample pretreatment of the urine only required acidification with TFA, mixing with EtOAc and centrifugation prior to transfer into an autosampler vial for subsequent cEE-LC-MS analysis. In Fig. 5.5, the calibration curves obtained for acetylcarnitine, hexanoylcarnitine, decanoylcarnitine and stearoylcarnitine spiked to urine are shown. For the calibration curve of acetylcarnitine, a separate series of measurements was carried out, because its high endogenous concentration required a different calibration range and internal standard concentration. Octanoylcarnitine was used as internal standard (20 μM in the case of acetylcarnitine, 2 μM in the remainder). Concentrations for construction of the calibration curves were 0, 1, 2, 5, 10, 20, and 50 µM in the case of acetylcarnitine and 0, 0.05, 0.1, 0.2, 0.5, 1, 2, and 5 µM in the other acylcarnitines.

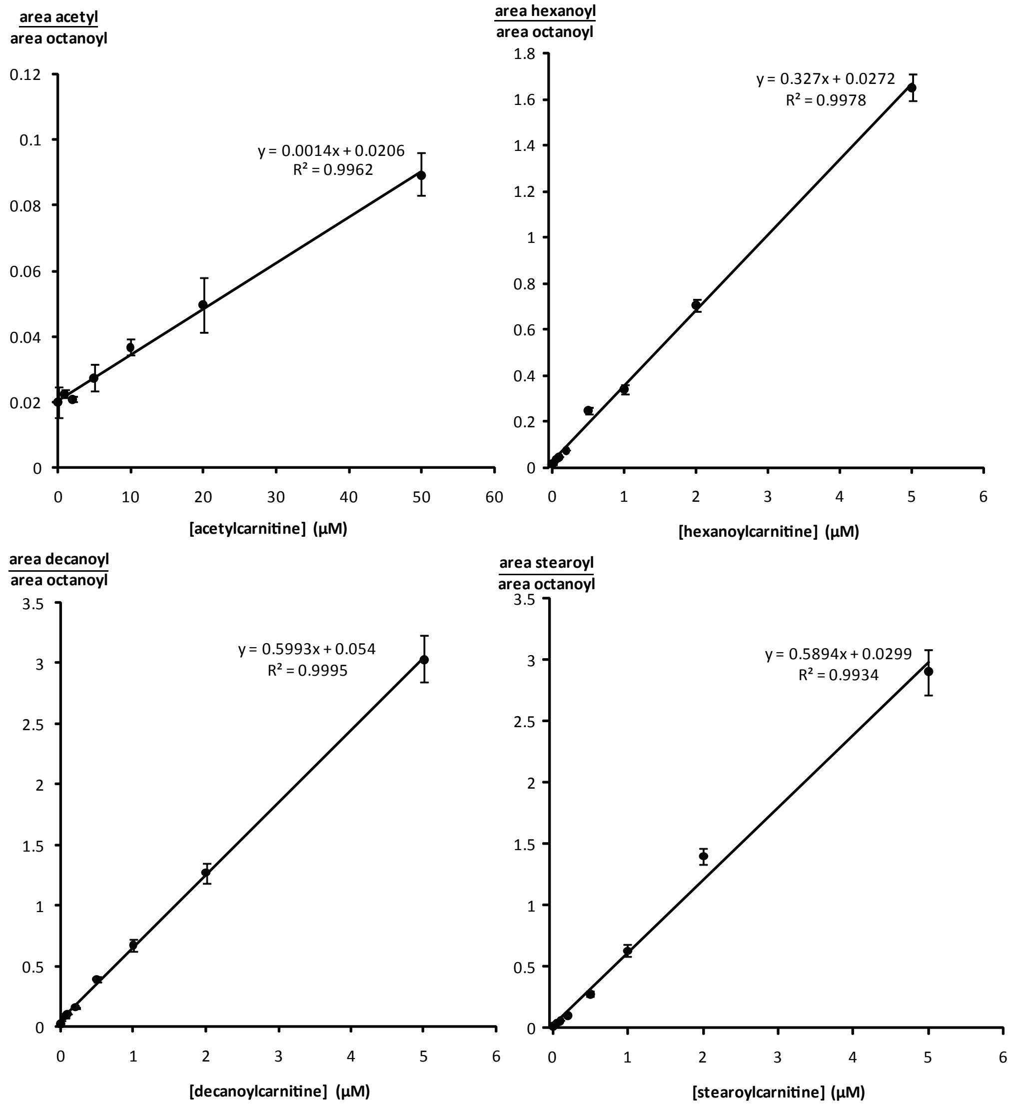


Figure 5.5 cEE-LC-MS calibration curves of acetylcarnitine, hexanoylcarnitine, decanoylcarnitine and stearoylcarnitine in urine

Repeatability (characterised by the analysis of three calibration sample sets) and linearity are excellent in the whole range (n = 3). In the blank urine samples, acetylcarnitine, hexanoylcarnitine and stearoylcarnitine peaks were detected (Fig. 5.6). The signal-to-noise ratio of the endogenous peaks was 25-112, which was sufficient for quantification, and the endogenous concentrations that were found are given in Table 5.2. Based on the signal-to noise ratios of the endogenous peaks, the LOD could be estimated to be 0.18 µM for acetylcarnitine, 0.008 µM for hexanoylcarnitine and 0.007 µM for stearoylcarnitine, corresponding to on-column injected amounts of 1 around pmol, 50 amol and 20 amol (based on the injection volume of 2 µL). In the case of hexanoylcarnitine and stearoylcarnitine, these values are roughly one order of magnitude more favourable than methods described in the literature [21, 22], describing sample pretreatment involving multiple steps. In [23], acylcarnitines were also observed in urine with LC-MS. When taking into account the different column dimensions (2 mm in [23], 0.5 mm here), the injected aliquot of urine is increased 16 times. An advantage of on-line large volume cEE is the minimal sample handling; after having mixed the urine with EtOAc, centrifugation and transferred it to an autosampler, the whole analysis takes place without further human interference. In consequence, the method is not labour-intensive and no errors and sample losses inevitably associated with each extra sample handling, such as evaporation and reconstitution [21, 22], are introduced. For example, in [22], urine was first diluted 5 times with water, then acidified with HCl (1 M) and finally subjected to strong cation exchange SPE. The eluent was evaporated to dryness, reconstituted in 1 mL methanol, re-evaporated and then reconstituted in methanol-water, prior to injection into the LC-MS system. In [21], a study was made of SPE as well as LLE as sample pretreatment prior to CZE-MS. In SPE, urine was evaporated to dryness, reconstituted in methanol, centrifuged and applied to a silica gel column. The eluent was again evaporated to dryness and reconstituted prior to injection. In LLE, 500 µL EtOAc/ACN (9:1) was used to extract acylcarnitines from 500 µL urine. The organic layer was evaporated and the residue was reconstituted in 200 µL 1.5% FA in methanol, which was used for injection.

Table 5.2 validation results for cEE-LC-MS of several acylcarnitines in urine

Analyte	R^2 value	Mean relative standard deviation (%)	Between day mean relative standard deviation (%)	Endogenous concentration (µM)	S/N ratio endogenous peak	Recovery (%)
acetyl	0.9962	11.3	11.8	14.7	112	52
hexanoyl	0.9978	5.6	7.3	0.083	23	84
decanoyl	0.9995	4.7	7.2	-	-	96
stearoyl	0.9934	7.4	10.8	0.051	23	90

(*: concentrations obtained from standard addition calibration curves of Fig. 5.5)

The recovery of cEE-LC-MS was calculated by determining the ratio of the slopes of the calibration curves from urine and water and expressed as percentages. The results (Table 5.2) indicate that cEE of hexanoylcarnitine, decanoylcarnitine and stearoylcarnitine are affected only to a limited extent by the urine matrix, or hardly at all (decanoylcarnitine). The recovery of acetylcarnitine is rather low; this may be explained, as mentioned above, by the fact that it co-elutes in the void volume with various other compounds in LC, leading to less favourable electrospray conditions (ion suppression).

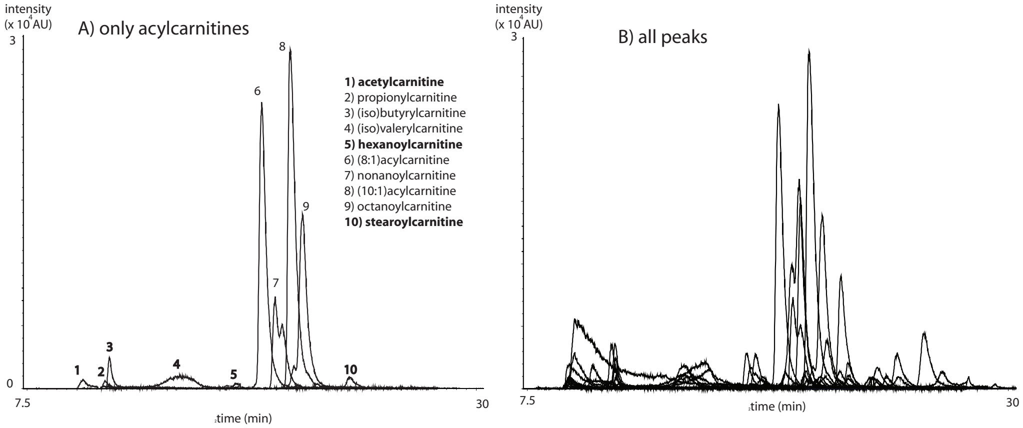
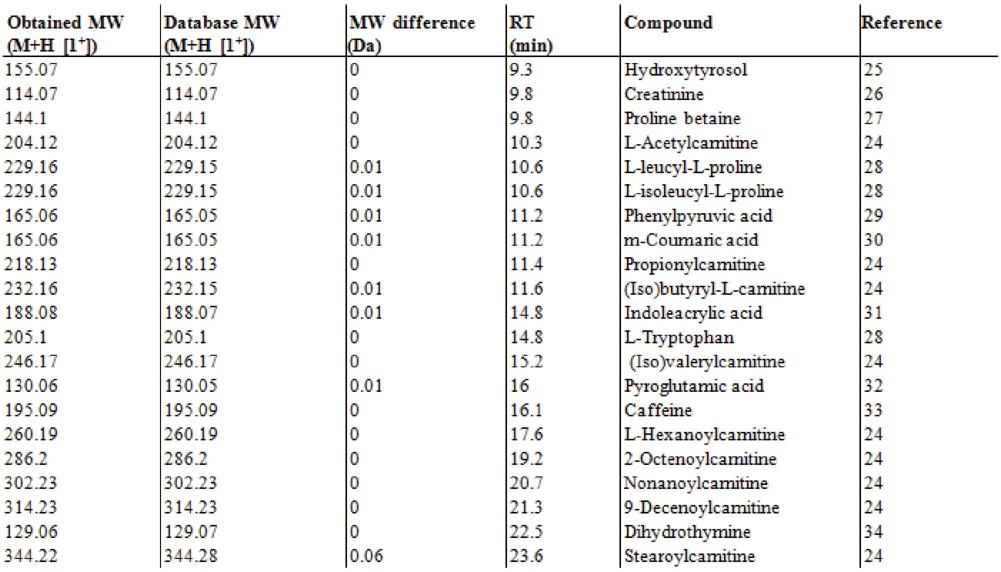


Figure 5.6 cEE-LC-MS chromatogram of A) reconstructed ion chromatograms of endogenous acylcarnitines, except for the added spike octanoyl carnitine, in urine; acylcarnitines mentioned in bold are identified by adding standards, other acetyl carnitines are putative; (8:1) means the acyl group contains 8 C atoms and one double bond, 10:1 means 10 C atoms and one double bond; and B) reconstructed ion chromatograms of all detected metabolite peaks

In total, more than 60 peaks have been detected in cEE-LC-MS of urine with the internal standard octanoylcarnitine as the only spike. Of these, 17 masses could be putatively annotated using the accurate mass and elemental composition (determined with the SmartFormula function of the Bruker Daltonics DataAnalysis software) in combination with the Human Metabolome Database (www.hmdb.ca) and a literature search (see references in Table 5.3). Interestingly, apart from the three acylcarnitines already described, strong indications of the presence of five more endogenous acylcarnitines were found. In Fig. 5.6A, a reconstructed cEE-LC-MS chromatogram with only the acylcarnitines peaks is shown and in Fig. 5.6B, all peaks are shown. To be conclusive about the identity of the 5 acylcarnitines not used for validation, standards of these acylcarnitines should be added. The retention order of these acylcarnitines is supporting the identification; it corresponds well with the expectations based on their polarity and with what has been reported previously [22]. To elucidate the exact structure of (iso)butyrylcarnitine, (iso)valerylcarnitine, octenoylcarnitine (8:1 carnitine) and decenoylcarnitine (10:1), MS/MS work should be done. In the Human Metabolome Database and in [24] only 9-decenoylcarnitine and 2-octenoylcarnitine are mentioned, indicating that these are the found acylcarnitine species.

In [22], it is stated that a typical LC-MS chromatogram (150 x 2.0 mm C_8 column) of healthy urine shows an acetylcarnitine peak, and depending on the urine concentration, also propionylcarnitine and (iso-)valerylcarnitine can be observed. In [21], only acetylcarnitine could be quantified with CZE-MS, the other acylcarnitine being detectable but could not be quantified. With the cEE-LC-MS method developed, combining the electrophoretic with the chromatographic approach, 7 acylcarnitines peaks that were high enough for quantitation were found and of three of them, trace concentrations could actually be determined in only 2 µL urine.

Table 5.3 Putatively identified metabolites in 2 µL urine, analyzed with cEE-LC-MS



Obtained MW (M+H $[1^+]$)	Database MW (M+H $[1^+]$)	MW difference (Da)	RT (min)	Compound	Reference
155.07	155.07	0	9.3	Hydroxytyrosol	25
114.07	114.07	0	9.8	Creatinine	26
144.1	144.1	0	9.8	Proline betaine	27
204.12	204.12	0	10.3	L-Acetylcarnitine	24
229.16	229.15	0.01	10.6	L-leucyl-L-proline	28
229.16	229.15	0.01	10.6	L-isoleucyl-L-proline	28
165.06	165.05	0.01	11.2	Phenylpyruvic acid	29
165.06	165.05	0.01	11.2	m-Coumaric acid	30
218.13	218.13	0	11.4	Propionylcarnitine	24
232.16	232.15	0.01	11.6	(Iso)butyryl-L-carnitine	24
188.08	188.07	0.01	14.8	Indoleacrylic acid	31
205.1	205.1	0	14.8	L-Tryptophan	28
246.17	246.17	0	15.2	(Iso)valerylcarnitine	24
130.06	130.05	0.01	16	Pyroglutamic acid	32
195.09	195.09	0	16.1	Caffeine	33
260.19	260.19	0	17.6	L-Hexanoylcarnitine	24
286.2	286.2	0	19.2	2-Octenoylcarnitine	24
302.23	302.23	0	20.7	Nonanoylcarnitine	24
314.23	314.23	0	21.3	9-Decenoylcarnitine	24
129.06	129.07	0	22.5	Dihydrothymine	34
344.22	344.28	0.06	23.6	Stearoylcarnitine	24

3.3 EE as sample pretreatment prior to CZE

The combination of EE with LC offers selectivity, since the electrophoretic as well as the chromatographic mechanism is employed during analysis. For example, when EE is coupled to RP-LC, as is carried out in [10] and in this work, the system is suitable for the analysis of charged, rather apolar compounds, such as peptides, and acylcarnitines. The charge of the analytes makes them susceptible to the electric field strength that is employed during EE, while their apolar character ensures retention on the RP column. For a more comprehensive (i.e. less selective and therefore less discriminative) approach desirable in metabolomics analysis, the combination of EE with CZE is attractive, since both techniques are electromigration techniques and all compounds that are feasible in EE should be feasible in CZE, regardless their polarity.

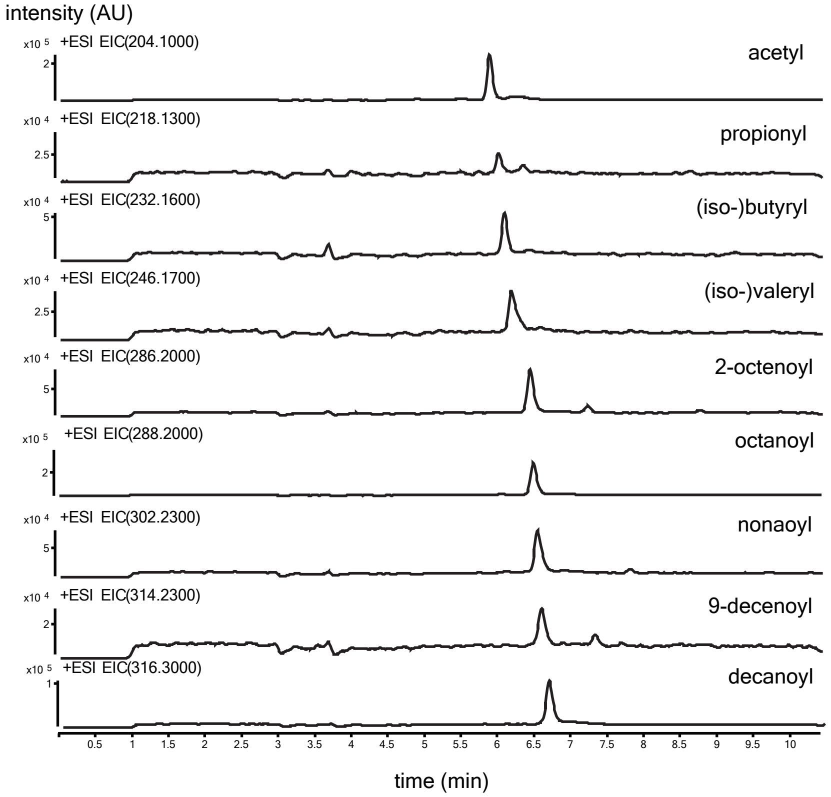


Figure 5.7 Results from off-line EE-CZE-MS. Acetylcarnitine, octanoylcarnitine and decanoylcarnitine were spiked to urine (2 μM each) prior to EE, the other detected acylcarnitines are endogenous.

To explore the applicability of EE prior to CZE, an off-line EE experiment of urine spiked with acetylcarnitine, octanoylcarnitine and decanoylcarnitine (2 μM each) prior to EE, was carried out, after which the extract was injected into a CZE-MS system. The resulting electropherogram (Fig. 5.7) showed good separation of all endogenous acylcarnitines that were found using on-line cEE-LC-MS, except for hexanoylcarnitine, which was not present. These first results indicate that the high salt concentration in urine does not have a negative effect on the CZE separation, a well-known complication in CZE analysis of biological samples [35]. Possibly, the salt precipitates when the urine is mixed with EtOAc. Future experiments are directed at investigating this process. Moreover, almost twice as many peaks were observed as with cEE-LC-MS, as a matter of fact more than 100. The combination of large volume EE with CZE has great potential and the direct on-line coupling of EE to CZE has our current attention.

4. Conclusions

The intention of this study was to demonstrate the great potential of EE in metabolite analysis. Our results indicate that electromigration-based sample pretreatment techniques for bio-analysis deserve more attention. We successfully demonstrated the applicability of EE as sample pretreatment of low-abundant urine metabolites in urine. Also, we showed that many compounds, belonging to several classes, can be extracted from untreated urine samples with EE. Using acylcarnitines as an example in on-line cEE-LC-MS, it was shown that EE is linear, sensitive and has a high recovery. In aliquots of only 2 µL urine, 10 endogenous acylcarnitines could be found. Three of them were quantified with the standard addition method. In an off-line EE/CZE-MS experiment, the same acylcarnitines were found, along with around 100 other putative metabolites. This demonstrates that EE is a versatile technique and that combining EE with CZE, has great potential. Further work will be directed to the on-line coupling of large volume cEE to CZE-MS and identification of all extracted metabolites.

5. Ackowledgements

Jeroen van Vonderen is acknowledged for his help with the CZE experiments.

6. References

[1] Galperin, M.Y., Cochrane G.R., *Nucleic Acids Res*, **2009**, *37*, D1-D4

[2] Chien, R-L., Burgi, D.S., *J Chromatogr,* **1991**, *559,* 141-152

[3] Britz-Kibbin, P., Terabe, S., *J Chromatogr A*, **2003**, *1000,* 917-934

[4] Breadmore, B.C., Thabano, J.R.E., Dawod, M., Kazarian, A.A., Quirino, J.P., Guijt, R.M., *Electrophoresis,* **2009**, *30,* 230-248

[5] Kamphorst, J.J., Tjaden, U.R., Van der Heijden, R., De Groot, J., Van der Greef, J., Hankemeier, T., *Electrophoresis*, **2009**, *30*, 2284-2292

[6] Hutta, M., Kaniansky, D., Kovalčíková, E., Marák. J., Chalányová, M., Madajová V., Šimuničová, E., *J Chromatogr A*, **1995**, *689*, 123-133

[7] Wang, T., Qin, Y., He, H., Lv, J., Fan, Y., *J Chromatogr A*, **2011**, *1218*, 185-189

[8] Bier, M., *Electrophoresis*, **1998**, 19, 1057-1063

[9] Lindenburg P.W., Seitzinger R., Tempels F.W.A., Tjaden U.R., Van der Greef, J., Hankemeier, T. *Electrophoresis,* **2010**, *31*, 3903-3912

[10] Lindenburg, P.W., Tempels, F.W.A., Tjaden, U.R., Van der Greef, J., Hankemeier, T. *On-line large-volume electroextraction coupled to LC-MS to improve determination limits of peptides*. Accepted for publication in J Chromatogr A

[11] Zhai, S. L., Luo, G. S., Liu, J. G., *Sep Purif Technol,* **2001**, *21*, 197-203

[12] Zhai, S. L., Luo, G. S., Liu, J. G., *Chem Eng J,* **2001**, *83*, 55-59

[13] Theos, C. W., Clark, W. M., *Appl Biochem and Biotechnol,* **1995**, *54*, 143-157

[14] Luo, G. S., Shan, X. Y., Qi, X., Lu, Y. C., *Sep Purif Technol,* **2004**, *38*, 265-271

[15] Bailes, P. J., *Ind Eng Chem Process Des Dev,* **1981**, *20*, 564-570

[16] Scott, T. C., *Sep Purif Methods,* **1989**, *18*, 65-109

[17] Van der Vlis, E., Mazereeuw, M., Tjaden, U. R., Irth, H., Van der Greef, J., *J Chromatogr A,* **1994**, *687*, 333-341

[18] Van der Vlis, E., Mazereeuw, M., Tjaden, U. R., Irth, H., Van der Greef, J., *J Chromatogr A,* **1995**, *712*, 227-234

[19] Van der Vlis, E., Mazereeuw, M., Tjaden, U. R., Irth, H., Van der Greef, J., *J Chromatogr A,* **1996**, *741*, 13-21

[20] Longo, N., di San Filippo, C.A., Pasquali, M., *Am J Med Genet Part C Med Genet,* **2006***, 142c,* 77-85

[21] Heinig, K., Henion, J., *J Chromatogr B*, **1999**, *735,* 171-188

[22] Vernez, L., Hopfgartner, G., Wenk M., Krähenbühl., *J Chromatogr A,* **2003**, *984,* 203-213

[23] Kloet, F.M., Tempels, F.W.A., Ismail, N., Van der Heijden, R., Kapser, P.T., Rojas-Cherto, M., Van Doorn, R., Spijksma, G., Koek, M., Van der Greef, J., Mäkinen, V.P., Forsblom., Holthöfer, H., Groop, P.H., Reijmers, T.H., Hankemeier, T., *Metabolomics*, **2012**, *8(1)*, 109-119

[24] Montgomery, J. A., Mamer, O. A., *Anal Biochem*, **1989**, *176,* 85-95

[25] Miró-Casas, E., Farré Albaladejo, M., Covas, M-I., Ortuño Rodriguez J., Menoyo Colomer, E., Lamuela Raventós, R. M., De la Torre, R., *Anal Biochem*, **2001**, *296*, 63-72

[26] Clark, L. C., Thompson, H. L., *Anal Chem*, **1949**, *21(10)*, 1218-1221

[27] Chambers, S. T., Kunin, C. M., *J Clin Invest*, **1987**, *79,* 731-737

[28] Guo, K., Li, L., *Anal Chem,* **2009**, *81,* 3919-3932

[29] Lee, S. H., Kim, S. O., Chung, B. C., *J Chromatogr B Biomed Sci Appl,* **1998**, *719 (1-2)*, 1-7

[30] Ito, H., Gonthier, M-P., Manach, C., Morand, C., Mennen, L., Rémésy, C., Scalbert, A., *Br J Nutr*, **2005**, *94,* 500-509

[31] Greenberg, A. J., Ketcham, R., *J Pharm Sci*, **1978**, *4,* 478-480

[32] Bishop, M. J., Crow, B. S., Kovalcik, K. D., George, J., Bralley, J. A., *J Chromatogr B,* **2007**, *848,* 303-310

[33] Zydroń, M., Baranowski, J, Baranowska, I., *J Sep Sci*, **2004**, *27,* 1166-1172

[34] Hofmann, U., Schwab, M., Seefried, S., Marx, C., Zanger, U. M., Eichelbaum, M., Mürdter, T. E., *J Chromatogr B*, **2003**, *791*, 371-380

[35] Veraart, J.R., Lingeman, H., Brinkman, U.A.Th., *J Chromatogr A*, **1999**, *856,* 483-514