ELSEVIER

Journal of Chromatography B, 773 (2002) 17–23

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Separation of new antidepressants and their metabolites by micellar electrokinetic capillary chromatography

L. Labat[a], M. Deveaux[b,*], P. Dallet[a], J.P. Dubost[a]

[a]*Laboratoire de Chimie Analytique, Université de Bordeaux 2, Place de la Victoire, 33076 Bordeaux, France*
[b]*Institut de Médecine Légale, Place Théo Varlet, 59000 Lille, France*

Abstract

Selective serotonin reuptake inhibitors (SSRIs), serotonin noradrenergic reuptake inhibitors (SNaRIs) and noradrenergic and specific serotoninergic antidepressant (NaSSA) are widely used in the treatment of depression. An increase in antidepressant intoxications led to the development of reliable analytical methods for their analysis. A new determination procedure for these compounds (milnacipran, venlafaxine, desmethylvenlafaxine, mirtazapine, desmethylmirtazapine, citalopram, desmethylcitalopram, fluvoxamine, paroxetine, sertraline and fluoxetine) was developed by micellar electrokinetic capillary chromatography (MEKC) with diode array detection (DAD). Separation and determination were optimised on an uncoated fused-silica capillary (600 mm, 75 μm I.D.). The migration buffer consisted of 20 m*M* sodium borate, pH 8.55, with 20 m*M* SDS and 15% isopropanol, at an operating voltage of 25 kV. The column temperature was maintained at 40 °C. Injection in the capillary was performed in the hydrodynamic mode (0.5 p.s.i., 15 s). In these conditions, the migration time of the antidepressants was less than 11 min. In most cases, calibration curves were established for 30–2000 ng/ml ($r>0.995$). The limit of detection and the limit of quantification were ranged between 10 and 20 and between 20 and 30 ng/ml, respectively, for all the molecules. This method allowed the determination of some of these compounds in biological fluids (blood, urine) in post-mortem cases. Samples (1 ml) were extracted with diethyl ether (5 ml) at pH 9.6 and reconstituted in diluted migration buffer. Similar results were obtained by a HPLC–DAD determination, performed as a reference method. These results suggest that this MEKC method can be useful for the determination of new antidepressants in post-mortem cases.

Keywords: Antidepressants

1. Introduction

Before 1980, antidepressant treatment principally consisted of the tricyclics, monoamine oxidase inhibitors and lithium. Since the early 90s, a new generation of compounds is available, having a different pharmacological profile and generally better tolerated adverse effects [1]. The first class introduced was the selective serotonin reuptake inhibitors (SSRIs) which includes fluvoxamine, fluoxetine, sertraline, paroxetine and citalopram. A second class consists of venlafaxine and milnacipran. They have a very similar activity to the SSRIs at low doses where serotonin reuptake inhibition predominates but, at higher doses, noradrenaline reuptake inhibition is prominent and they were called for this reason serotonin noradrenergic reuptake inhibitors (SNaRIs). Mirtazapine belongs to the chemical group

*Corresponding author. Tel.: +33-3-2062-1223; fax: +33-3-2062-1229.

E-mail address: mdeveaux@easynet.fr (M. Deveaux).

1570-0232/02/$ – see front matter
PII: S1570-0232(01)00623-7

of compounds known as piperazinoazepines. It is a noradrenergic and specific serotoninergic antidepressant (NaSSA). New antidepressants offer equivalent or better efficacy than tricyclic class. Even if many of undesirable side-effects have disappeared with these new compounds, they can lead to intoxications [2]. The development of rapid and specific methods allowing the determination of several antidepressants and associated metabolites in biological fluids could be of great interest. Most of the developed methods involve gas chromatography (GC) or high-performance liquid chromatography (HPLC) and allow the determination of one or more of these antidepressants for drug monitoring or toxicological purpose [3–7].

Capillary electrophoresis (CE) has some distinct advantages for identification of unknown toxic compounds, such as high separation efficiency, fast analysis, and a separation mechanism complementary to that of GC and HPLC. In addition, various modes of CE can be performed using the same instrumentation and different experimental conditions, allowing samples to be analysed using different separation mechanisms. To our knowledge, only a few methods have been developed in CE for these compounds [8–10]. Pucci et al. developed several strategies to improve the separation of a mixture of 11 antipsychotics and antidepressants by capillary zone electrophoresis (CZE) [8]. A better separation was obtained at acidic pH with a polymeric pseudo-stationary phase in polyvinylpyrrolidone. Buzinkaiova and Polonsky described a capillary isotachophoresis system with conductivity detection which could provide both qualitative and quantitative analysis of citalopram, fluoxetine, fluvoxamine and sertraline in pharmaceutical drugs [9].

In a previous work, we described separation of new antidepressants with some of their metabolites (milnacipran, venlafaxine, desmethylvenlafaxine, mirtazapine, desmethylmirtazapine, citalopram, desmethylcitalopram, fluvoxamine, paroxetine, sertraline and fluoxetine) in the most widely applied CE mode, the CZE [11,12]. Separation was achieved in 17 min with a borax buffer at pH 9.5 and methanol (84:16, v/v). At this pH, the molecules were poorly ionised and some of them migrate near the electroosmotic flow (EOF), leading for example to the broadening of the peak of mirtazapine [12].

The separation mechanism in CZE is based only on differences in electromigration of charged molecules. Micellar electrokinetic chromatography (MEKC) is an other high resolution technique using surfactant micelles to provide a charged vehicle to perform the electrophoretic separation [13]. The micelles in MEKC add a second separation mechanism based on analyte–micelle interactions. This is used to allow separations of mixtures of analytes, as the partitioning of each molecule will differ depending on its hydrophobic interaction with the micelle.

The purpose of this work was to perform separation of eight antidepressants and three metabolites by a MEKC mode with negatively charged sodium dodecyl sulfate (SDS) micelles. The proposed method was applied to blood and urine in two post mortem cases with suspected intoxication by a new antidepressant.

2. Experimental

2.1. Chemicals

All chemicals and solvents were of analytical or CE grade. Isopropanol and methanol were purchased from Merck (Darmstadt, Germany) and ethyl ether from Fluka (Saint Quentin Fallavier, France). SDS was obtained from Panreac (Barcelona, Spain), sodium borate, boric acid and ammonium chloride from Riedel-de Haën (Saint Quentin Fallavier, France). Water was deionised and glass-distilled prior to use.

Citalopram (HBr) and desmethylcitalopram (HBr) were kindly donated by Lundbeck (Copenhagen, Denmark), fluvoxamine (maleate) by Solvay Pharma (Suresnes, France), milnacipran (HCl) by Pierre Fabre (Castres, France), mirtazapine and desmethylmirtazapine by Organon (Oss, The Netherlands), paroxetine (HCl) by Smith Kline Beecham (Nanterre, France), sertraline by Pfizer (Amboise, France), venlafaxine (HCl) and desmethylvenlafaxine by Lederle (Pearl River, NY, USA). Fluoxetine (HCl) was purchased from Sigma (Saint Quentin Fallavier, France). For quantitative determination, the molecule F2570 kindly donated by Pierre Fabre was used as internal standard.

Individual stock solutions of each antidepressant were prepared at a concentration of 1 mg/ml in methanol and stored at −20 °C. Working solutions

were prepared by two successive dilutions of the stock solutions in migrate buffer (1/20) and in water to a final concentration of 25 μg/ml. All the solutions were filtered through a 0.45-μm nylon filter before injection.

2.2. Instrumentation and electrophoretic conditions

A Beckman P/ACE MDQ capillary electrophoresis system (Beckman, Fullerton, CA, USA) with diode array detector was used to conduct the electrophoretic experiments. The uncoated fused-silica capillary, purchased from Beckman, had an inner diameter of 75 μm and a total length of 600 mm (508 mm effective length). The temperature of the capillary was controlled at 40 °C. A constant operating voltage of 25 kV was used for the separation. Samples were injected with hydrodynamic mode into the capillary for 15 s by applying 0.5 p.s.i. at the anodic end of the capillary. The migration buffer consisted of 20 m*M* sodium borate (adjusted to pH 8.55 with boric acid 20 m*M*) with 20 m*M* SDS and 15% isopropanol. It was filtered through a 0.45-μm membrane filter, prior to use.

Each day before starting analysis, the capillary column needed a conditioning in different steps [14]. Before each injection, the capillary was washed with 0.1 *M* NaOH (2 min) and migration buffer (2 min).

2.3. Extraction procedure

To 1 ml of standard solution (water-spiked sample) or sample (blood, urine), were added 1 μg of internal standard (F2570), saturated ammonium chloride (pH 9.6), and 5 ml of diethyl ether. After stirring for 20 min and centrifugation (10 min, 1000 *g*), the organic layer was separated and evaporated under nitrogen to dryness. The residue was reconstituted in 100 μl migration buffer and water (50:50, v/v) and transferred to autosampler vials.

3. Results and discussion

3.1. Optimisation of MEKC separation

For the determination of the antidepressants by MEKC, factors affecting the separation of the eight drugs and their metabolites were examined. The influence of the chemical properties and the concentration of organic solvents in running buffer on the electrophoretic separation was the first studied. It has not been possible to achieve separation of all the antidepressants using unmodified buffers MEKC (buffers without organic modifiers). The addition of acetonitrile or methanol to the running buffer were investigated. Both the EOF and the analyte migration times increase as the organic solvent content increases. However, elevated concentrations of solvents (>20%) may break down the micelles [15]. This phenomenon was more pronounced for acetonitrile than for methanol. Then, addition of these two organic modifiers at concentrations less than 20% did not allow the separation of all the molecules. Baseline resolution of all the antidepressants without splitting of the peaks was only achieved with isopropanol. The use of this organic modifier at a concentration of 20% in the migration buffer contributed to the extension of the migration–time window and allowed to optimise the selectivity of the MEKC separation.

The SDS micelles were found to be suitable for the separation of antidepressants. In a recent study, Boone et al. [16] compared MEKC procedures for the analysis of acidic, basic and neutral drugs. The study showed that only MEKC method with cationic micelles was suitable for the analysis of basic compound. The SDS micelles were not found suitable for the separation of the protonated basic compounds which interacted too strongly with the negatively charged micelles and then all migrated around the same migration time [16]. In this study, we showed that the addition of organic modifier allowed the separation of these basic molecules.

The influence of SDS concentration (5–50 m*M*) on migration times and separation efficiencies was investigated. In order to separate these molecules in a short time, a relatively low concentration of 20 m*M* of SDS was used in the running buffer, in combination with 20 m*M* borate (pH 8.55) as the background electrolyte. Consequently, a migration time of less than 11 min could be obtained for all the antidepressants at a run potential of 25 kV and a temperature held at 40 °C. A typical electropherogram is shown in Fig. 1: the molecules migrated forming well shaped, symmetrical single peaks, well separated from the EOF. Under these optimised conditions, the

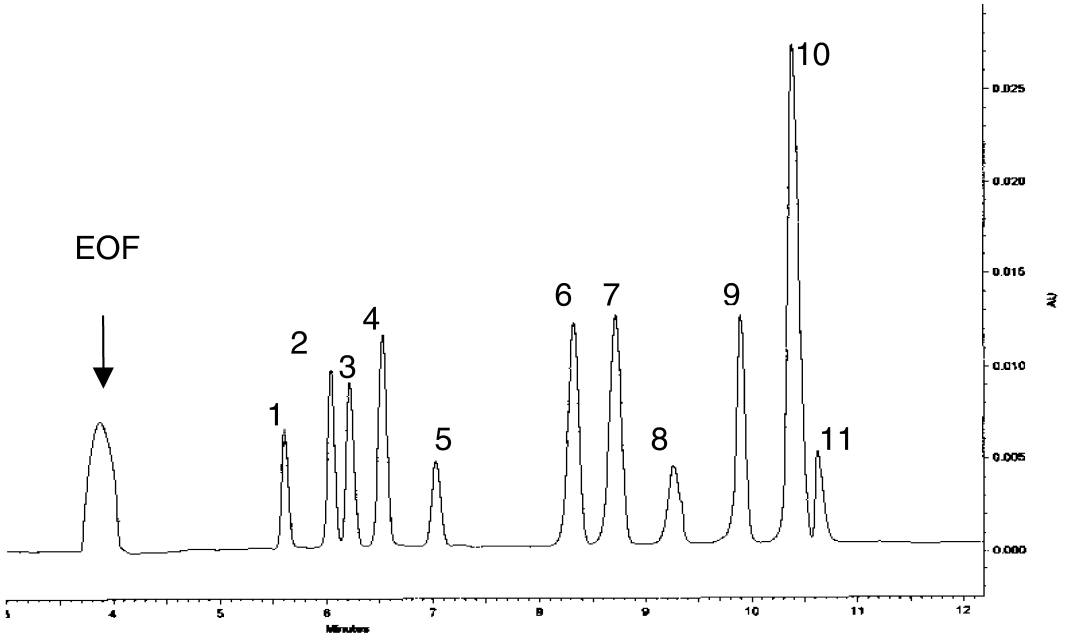

Fig. 1. Electropherogram of a standard mixture of eight antidepressants and three metabolites under the described MEKC conditions (with (1) desmethylvenlafaxine, (2) milnacipran, (3) desmethylmirtazapine, (4) venlafaxine, (5) mirtazapine, (6) desmethylcitalopram, (7) citalopram, (8) fluvoxamine, (9) paroxetine, (10) sertraline, (11) fluoxetine).

migration time of a neutral marker of the EOF (methanol) was 3.88 min.

Hydrodynamic injection is often preferred to electrokinetic injection, especially when analysing biological matrices like blood with varying composition and conductivity. Hydrodynamic injection is more precise and robust than electrokinetic mode. We have studied influence of injection time (between 10 and 30 s) and injection pressure (between 0.5 and 1.2 p.s.i.) on quantitative determination. The choice of hydrodynamic injection during 15 s at 0.5 p.s.i. improved quantitative values in term of reproducibility (corrected peak area RSD$<$0.5% for all the molecules) and sensitivity.

3.2. Analysis by MEKC

The repeatability (RSD) of both the migration time and the corrected peak area was determined over five replicate injections with the method developed (see electrophoretic conditions). The data indicates that the repeatability of the migration times (Table 1) was characterized with RSD values ranged from 6.10 to 13.32%. The migration time in CE is known to have poor reproducibility, mainly caused by fluctuations in the EOF, but also by wall interactions of the analytes and temperature fluctuations [17]. The effective mobility (μ_{eff}) is often preferred as an identification parameter, since it is independent of the EOF and, therefore, much more reproducible than migration time. Then, repeatability of the effective mobility of the eight antidepressants and three metabolites were satisfactory with RSD less than 4.93%. For the corrected peak area (see Table 1), RSD less than 1.21% indicate good precision for all the molecules.

A liquid–liquid extraction was chosen for the analysis of blood samples in terms of convenience and simplicity. Ethyl ether was selected as extracting solvent because it has little prone to emulsion formation and generally provides satisfactory recoveries with basic molecules.

For quantification of the 11 molecules, an internal standard (F2570) was used with a migration time of 7.60 min ($\mu_{\text{eff}}=-2.53\times10^{-8}$ m^2/Vs). Linear calibration curves were obtained over the working

Table 1
Migration time (t_M), effective mobility (μ_{eff}) and repeatability data (%RSD) for t_M, μ_{eff} and corrected peak area of 11 antidepressants ($n=6$)

Molecules	t_M mean (min)	t_M RSD	μ_{eff}[a] mean (10^{-8} m^2/Vs)	μ_{eff} RSD	Corrected peak area RSD
Desmethylvenlafaxine	5.61	9.13	−1.62	2.21	1.22
Milnacipran	6.05	8.54	−1.88	1.84	0.37
Desmethylmirtazapine	6.22	9.21	−1.97	1.85	0.32
Venlafaxine	6.53	12.30	−2.13	1.48	0.29
Mirtazapine	7.03	6.10	−2.35	2.38	0.87
Desmethylcitalopram	8.33	11.51	−2.80	4.13	0.33
Citalopram	8.72	13.32	−2.91	4.93	0.54
Fluvoxamine	9.26	11.08	−3.04	4.55	1.21
Paroxetine	9.90	7.37	−3.19	2.77	0.38
Sertraline	10.38	9.60	−3.28	2.47	0.86
Fluoxetine	10.63	9.52	−3.33	1.05	1.01

[a] With μ_{eff} calculated using: $\mu_{eff}=\mu_{app}-\mu_{EOF}=l\cdot L/V\,(1/t_M-1/t_{EOF})$, where μ_{app} is the apparent mobility and μ_{EOF} is the mobility of the EOF, which are calculated using the capillary length to the detection window (l), the total capillary length (L), the applied voltage (V), the migration time (t_M) and the migration time of a neutral marker that moves at a velocity equal to EOF (t_{EOF}).

concentration range 30–2000 ng/ml. Adequate concentrations of each molecule were performed in water-spiked samples. All these samples were extracted and analysed by the MEKC method. For each molecule, the antidepressant/internal standard normalized area ratios (i.e., area/migration time) [14] were plotted as a function of antidepressant concentration. Correlation coefficients obtained using the least-squares regression method for the linear fits are higher than 0.995. The correlation coefficients (r) are listed in Table 2. Results indicate a good linearity between the responses and the antidepressant concentrations. The method's precision was evaluated by injecting the antidepressant mixture (1000 ng/ml) for the 11 compounds, six times. The repeatability (intra-day) and intermediate precision (inter-day) assays gave satisfactory RSD% values, which are shown in Table 2. Limits of detection (LOD) and limit of quantification (LOQ) estimated at 3.3 and 10 times the background noise, respectively, are sum-

Table 2
MEKC method precision (repeatability and intermediate precision), regression coefficient of the equations of the calibration curves, LOD and LOQ of the 11 antidepressants

Molecules	Repeatability[a] RSD (%) ($n=6$)	Intermediate precision[a] RSD (%) ($n=6$)	Regression coefficient (r^2)	LOD (ng/ml)	LOQ (ng/ml)
Desmethylvenlafaxine	3.8	6.4	0.996	20	30
Milnacipran	3.8	4.0	0.999	10	20
Desmethylmirtazapine	3.6	4.8	0.996	10	20
Venlafaxine	1.8	3.9	0.996	10	20
Mirtazapine	4.0	2.5	0.998	20	30
Desmethylcitalopram	2.9	2.6	0.997	10	20
Citalopram	2.9	3.3	0.997	10	20
Fluvoxamine	4.7	6.8	0.995	20	30
Paroxetine	4.1	5.1	0.999	10	20
Sertraline	3.7	5.8	0.998	10	20
Fluoxetine	4.3	4.9	0.997	20	30

[a] Determination in water-spiked samples (1000 ng/ml).

marized in Table 2. The LOD were ranged between 10 and 20 ng/ml and the LOQ were ranged between 20 and 30 ng/ml for all the molecules.

The MEKC method described was used in two fatal cases with antidepressant intoxication primarily analysed by a HPLC–DAD reference method [18,19]. The first case was a paroxetine intoxication with Deroxat®. The second case was a venlafaxine intoxication with Effexor®. Heart blood in both two cases and urine in the second case were submitted for toxicology evaluation. The results obtained in HPLC–DAD and in MEKC method are summarized in Table 3. The same liquid–liquid extraction procedure was used prior to analysis of the biological samples by both separation methods.

With the exception of paroxetine, all of the SSRIs have active metabolites which are selective for serotonin reuptake inhibition [2]. It was therefore important to consider venlafaxine and *O*-desmethylvenlafaxine metabolite concentration in the second fatal case.

Similar results were obtained with the MEKC and the HPLC–DAD determination, performed as a reference method. In the first case, the paroxetine concentration was higher than the previously reported levels (0.40 μg/ml) described as lethal concentration in the absence of other risk factors [2]. Levine et al. [20] described distribution of venlafaxine in three post mortem cases in which the cause of death was venlafaxine or multiple drug intoxication. The concentrations of venlafaxine and *O*-desmethylvenlafaxine were in these cases in the same order with the results found in the second case described.

The two cases described have shown that the MEKC method can be useful for the determination of paroxetine, venlafaxine and *O*-desmethylvenlafaxine in post mortem cases. LOD for these three molecules were adequate for the determination in the biological fluids and in each case, the analyses were performed in less than 11 min.

4. Conclusion

To our knowledge, we describe the first procedure in MEKC allowing simultaneous detection of eight antidepressants (SSRIs and NaSSA) and three of their metabolites. This investigation demonstrates that MEKC method allows the separation of a mixture of numerous structurally similar molecules. The addition of isopropanol as organic modifier to the MEKC electrolyte buffer lengthened the migration time and resulted in the best separation of all the antidepressants in the test mixture used in this work.

Calculating effective mobilities better than measuring migration time enhances the reproducibility: together with UV spectrum, it is a significant parameter to identify the different antidepressants in the toxicological screening [17,21].

The two fatal cases described in this study were due to an acute overdose of an SSRI on its own. The MEKC method allowed the determination of the compounds in a biological sample with sufficient sensitivity and without interferences. Capillary electrophoresis is not yet widely used in analytical toxicology due to lack of sensitivity. But, this first approach in MEKC coupled with DAD is very attractive. Extraction procedure and injection procedure through field amplified stacking injection [22] may be improved in the future to obtain an useful procedure in systematic toxicological analysis.

Table 3
Determination by HPLC and MEKC of antidepressants in biological fluids in two post mortem cases

	Concentration (μg/ml)	
	HPLC–DAD	MEKC-DAD
Case no. 1	(B) Paroxetine = 1.02 μg/ml	(B) Paroxetine = 1.00 μg/ml
Case no. 2	(B) Venlafaxine = 4.90 μg/ml	(B) Venlafaxine = 5.04 μg/ml
	(B) *O*-Desmethylvenlafaxine = 5.20 μg/ml	(B) *O*-Desmethylvenlafaxine = 4.95 μg/ml
	(U) Venlafaxine = 9.43 μg/ml	(U) Venlafaxine = 9.01 μg/ml
	(U) *O*-Desmethylvenlafaxine = 7.60 μg/ml	(U) *O*-Desmethylvenlafaxine = 7.32 μg/ml

B, blood; U, urine.

References

[1] J.M. Kent, Lancet 355 (2000) 911.
[2] K.E. Goeringer, L. Raymon, G.D. Christian, B.K. Logan, J. Forensic Sci. 45 (2000) 633.
[3] E. Lacassie, J.M. Gaulier, P. Marquet, J.F. Rabatel, G. Lachatre, J. Chromatogr. B 742 (2000) 229.
[4] C.B. Eap, P. Baumann, J. Chromatogr. B 686 (1996) 51.
[5] H.H. Maurer, J. Bickeboeller-Friedrich, J. Anal. Toxicol. 24 (2000) 340.
[6] L. Kristoffersen, A. Bugge, E. Lundanes, L. Slordal, J. Chromatogr. B 734 (1999) 229.
[7] P. Dallet, L. Labat, M. Richard, M.H. Langlois, J.P. Dubost, J. Liq. Chromatogr. Rel. Technol. (2002) in press.
[8] V. Pucci, M. Raggi, E. Kenndler, J. Chromatogr. B 728 (1999) 263.
[9] T. Buzinkaiova, J. Polonsky, Electrophoresis 21 (2000) 2839.
[10] A. Aumatell, R.J. Wells, J. Chromatogr. B 669 (1995) 331.
[11] L. Labat, M. Deveaux, V. Dumestre-Toulet, P. Dallet, E. Kummer, J.P. Dubost, Eur. J. Emerg. Med. 8 (2001) 67.
[12] L. Labat, P. Dallet, M. Deveaux, J.P. Dubost, Ann. Toxicol. Anal. 13 (2001) 104.
[13] A. E Bretnall, G.S. Clarke, J. Chromatogr. A 716 (1995) 49.
[14] L. Labat, M. Deveaux, J.P. Dubost, Ann. Toxicol. Anal. 12 (2000) 179.
[15] J.C. Jacquier, P.L. Desbène, J. Chromatogr. A 743 (1996) 307.
[16] C.M. Boone, J.W. Douma, J.P. Franke, R.A. de Zeeuw, K. Ensing, Forensic Sci. Int. 121 (2001) 89.
[17] C.M. Boone, J.P. Franke, R.A. de Zeeuw, K. Ensing, Electrophoresis 21 (2000) 1545.
[18] Y. Gaillard, G. Pepin, J. Chomatogr. A 763 (1997) 149.
[19] L. Labat, M. Deveaux, J.P. Dubost, Presented at the 39th Annual Meeting of The International Association of Forensic Toxicologists, Prague, 2001.
[20] B. Levine, A. Jenkins, M. Queen, R. Jufer, J. Smialek, J. Anal. Toxicol. 20 (1996) 502.
[21] C.M. Boone, J.P. Franke, R.A. de Zeeuw, K. Ensing, J. Chromatogr. A 838 (1999) 259.
[22] B.X. Mayer, J. Chromatogr. A 907 (2001) 21.