ELSEVIER

Journal of Chromatography B, 759 (2001) 73–79

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of residues of enrofloxacin and its metabolite ciprofloxacin in biological materials by capillary electrophoresis ☆

D. Barrón, E. Jiménez-Lozano, J. Cano, J. Barbosa*

Department Analytical Chemistry, University of Barcelona, Avda. Diagonal 647, 08028 Barcelona, Spain

Received 8 November 2000; received in revised form 16 March 2001; accepted 4 April 2001

Abstract

A method for the analysis of enrofloxacin and ciprofloxacin in chicken muscle using marbofloxacin as internal standard is proposed. Clean-up and pre-concentration of the samples are effected by solid-phase extraction and determination is carried out by capillary electrophoresis using a photodiode array detector. The calibration graphs are linear for enrofloxacin and ciprofloxacin from 10 to 300 μg/kg. The method recoveries for enrofloxacin and ciprofloxacin are 74 and 54%, respectively. The limit of detection for the two compounds is lower than 25 μg/kg, which allows the detection of positive muscle samples at the required maximum residue limits.

Keywords: Solid-phase extraction; Enrofloxacin; Ciprofloxacin

1. Introduction

The quinolones are antimicrobial agents used in the treatment of a variety of bacterial infections in human and veterinary medicine. These compounds act directly on bacterial DNA by inhibiting cell reproduction which leads to cell death. They are therefore germicidal [1–3]. In infectious diseases the use of these drugs has become a serious problem, however, as they are substances that leave residues in edible tissue which may be directly toxic or cause resistant human pathogens and possible allergic hypersensitivity reactions in humans [4,5]. To ensure human food safety, the European Union has set tolerance levels for these compounds in animal products. The maximum residue limit (MRL) for enrofloxacin and ciprofloxacin, its major metabolite, is fixed at 100 μg/kg in several edible animal tissues [6]. Therefore, the development or improvement of analytical methods for monitoring their levels in farm animals and their primary products is of increasing interest.

Several methods have been developed using liquid chromatography for the detection of residues of quinolones in biological materials [3,7–9], however the literature contains only a few methods using capillary electrophoresis (CE) to analyse quinolones in pharmaceutical formulations or in body fluids [10,11].

Various CE modes of operation (capillary zone

☆ Presented at the 29th Scientific Meeting of the Spanish Group of Chromatography and Related Techniques, Alcalá de Henares (Madrid), 12–14 July 2000.

* Corresponding author. Tel.: +34-93-402-1279; fax: +34-93-402-1233.

E-mail address: barbosa@apolo.qui.ub.es (J. Barbosa).

0378-4347/01/$ – see front matter
PII: S0378-4347(01)00214-6

electrophoresis, CZE; capillary electrokinetic, MEKC; affinity capillary electrophoresis, ACE) have proven useful in the separation and determination of series of quinolones [12–15]. Yet, while CE offers the high separation efficiency necessary in the analysis of complex mixtures, it is problematic in terms of the concentration limit of detection. In order to determine analytes in a low concentration range, pre-concentration procedures (e.g., off-line solid-phase extraction, SPE) have proved useful in analysing β-receptor agonists, such as terbutaline, in plasma samples, prior to using CE methodologies [16]. Again, however, the literature contains few references to SPE-CE for the analysis of quinolone antibiotics in animal products for human consumption.

The purpose of the present work was to develop a methodology for the determination of enrofloxacin and ciprofloxacin in tissues of chicken using SPE followed by CE with photodiode array detection (PDA). A systematic study of the electrophoretic behaviour of the studied quinolones was also carried out. The detection limits obtained in this work are low enough to determine concentrations below the permissible MRL in animal products [6].

2. Experimental

2.1. Chemicals and reagents

Ciprofloxacin was obtained from LASA Laboratories, S.A., Enrofloxacin from Cenavisa S.A., and Marbofloxacin and Difloxacin were from Vetoquinol Lab. and Abbott S.A., respectively. The structures of the quinolones studied are shown in Fig. 1. All chemicals used in the preparation of buffers and solutions were analytical reagent grade. Diethylmalonic acid was supplied by Aldrich S.A., potassium dihydrogen phosphate, sodium hydroxide, dichloromethane, trifluoroacetic acid, acetonitrile, methanol and hexane were supplied by Merck and acetic acid was obtained from Carlo-Erba. Water, with a resistivity of 18.2 MΩ cm, was obtained using a Milli-Q water purification system (Millipore, Molsheim, France). Bond Elut C_{18} cartridges (500 mg) were obtained from Varian.

2.2. Extraction procedure

Five grams of thawed minced muscle tissue was accurately weighed and placed in a 50 ml centrifuge

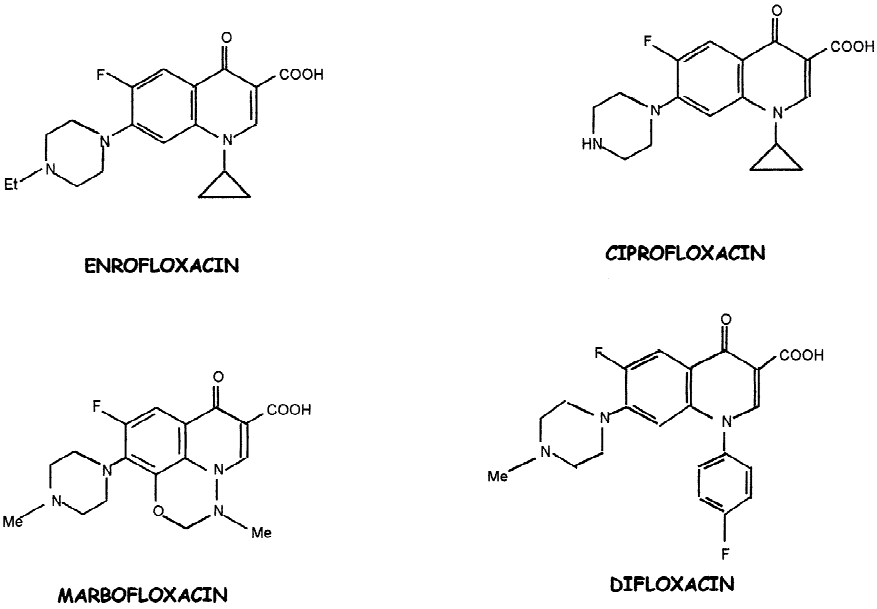

Fig. 1. Structures of the quinolones studied.

tube, and 5 ml of phosphate buffer at pH 7.4 were added to the sample [17]. The homogenate was mixed for 20 min with a mechanical shaker. Dichloromethane (20 ml) was added to the sample in order to extract quinolones [18]. After shaking for 20 min the mixture was centrifuged at 3500 rpm for 10 min. The sample was re-extracted with another portion of 10 ml of dichloromethane. The two organic phases collected were transferred into a 50 ml heart-shaped flask. The dichloromethane was evaporated to dryness under vacuum in a rotary evaporator at room temperature. The extract was redissolved with 5 ml HCl and transferred into a centrifuge tube. The above mixture was defatted with 20 ml of hexane. The aqueous phase was adjusted to pH 7.4 with NaOH and 20 ml of phosphate buffer (pH 7.4) were added.

Solid-phase extraction was performed on a Supelco vacuum tank, by passing the cleaned extract through a C_{18} cartridge that had been activated with 2 ml MeOH, 2 ml water and 2 ml 0.05 *M* phosphate buffer, pH 7.4 [17]. The extract was passed through the cartridge, which was then rinsed with 2 ml of water and 0.5 ml of acetonitrile. The quinolones were eluted with 2 ml of 4% trifluoroacetic acid in water and acetonitrile (25:75), followed by 1 ml of acetonitrile. Fifteen microlitres of a solution of 50 ppm of internal standard were added. The collected eluate was evaporated to dryness under a stream of nitrogen at 50°C. The residue was resuspended in 100 μl of acetonitrile–water (50:50). The resulting solution was injected into the capillary hydrodynamically at 0.5 p.s.i. for 2 s (12.8 nl).

2.3. Fortification

Fortified muscle samples were prepared by spiking 5 g of chicken muscle with an adequate volume of standard solutions of enrofloxacin and ciprofloxacin at 50 ppm to obtain muscle fortified at 10, 25, 50, 75, 100, 150, 200 and 250 μg/kg, respectively, and adding water to obtain a final volume of 1 ml. The samples were allowed to stand for 20 min in the dark before extraction as described above.

2.4. Instrumental parameters

All CZE experiments were performed on a P/ACE System 5500 (Beckman Instruments, Palo Alto, CA, USA) equipped with an autosampler, automatic injector and photodiode array detector. An untreated fused-silica capillary, 47 cm×75 μm I.D., 40 cm from inlet to detector (Polymicro Technologies, Phoenix, AZ, USA), was used. Quantitative analysis was conducted at 20 kV, at 25±0.1°C, in order to keep the total current below 100 μA. Electropherograms were recorded using a computer program (P/ACE Station 1.0 with interface Golden System) supplied by Beckman. Samples were injected hydrodynamically at 0.5 p.s.i. for 2 s. The detection wavelength was set at 275 nm. The pH of the buffer solutions was measured with a Crison 2002 potentiometer (Crison Instruments, Barcelona, Spain) using a Ross electrode 81-02 supplied by Orion Research (Boston, MA, USA). A Supelco vacuum tank was used to perform the SPE procedure. A SBS rotary shaker (SBS Instruments SA) and a macrotronic Selecta centrifuge were used to carry out extractions. A rotary Resona Technics LABO ROTA S300 evaporator was used to remove the extracting solvent.

2.5. Preparation of the capillary

The capillary was purged with 1 *M* sodium hydroxide for 15 min, followed by 20 min with milli-Q water and appropriate buffer electrolyte for 30 min. The final step was the application of a voltage of 20 kV for 20 min with the capillary filled with buffer solution. Each day the system was first purged with 1 *M* NaOH for 5 min followed by water for 15 min and the working buffer solution for 20 min. A voltage of 20 kV was also applied for 10 min with the capillary filled with buffer solution. In order to equilibrate the capillary and, thereby, minimise hysteresis effects, the capillary was flushed between each run with 1 *M* NaOH for 3 min, water for 3 min, followed by the running buffer for 3 min. Capillaries were stored overnight filled with water.

3. Results and discussion

In order to predict the optimal pH for the separation of enrofloxacin and ciprofloxacin, a study of the electrophoretic behaviour vs. pH of these quinolones was carried out. The electrophoretic mobility of a substance is related to the mobility and

molar fraction of its species: $m_e = \Sigma_i x_i m_i$ [19–21], where x_i and m_i correspond to the molar fraction and mobility of each species. The quinolones studied have two relevant ionizable functional groups, which means that their acid–base chemistry includes two protons. Thus, we can write

$$m_e = x_{H_2Z^+} m_{H_2Z^+} + x_{HZ} m_{HZ} + m_{Z^-} m_{Z^-} \quad (1)$$

where H_2Z^+ represents the protonated species, HZ the zwitterionic species and Z^- the fully deprotonated species of the substances. The term corresponding to the intermediate species is considered to be nil because the species HZ has no total charge and migrates with the electroosmotic flow. Considering $m_{H_2Z^+} = m_a$ and $m_{Z^-} = m_b$ and replacing terms $x_{H_2Z^+}$ and x_{Z^-} by their expressions for ampholytes are

$$x_{H_2Z^+} = a^2_{H^+}/(a^2_{H^+} + K_1 a_{H^+} y + K_1 K_2)$$

and

$$x_{Z^-} = K_1 K_2/(a^2_{H^+} + K_1 a_{H^+} y + K_1 K_2)$$

where y is the activity coefficient. The electrophoretic mobility, m_e, can then be obtained:

$$m_e = \frac{a^2_{H^+} m_a + K_1 K_2 m_b}{a^2_{H^+} + K_1 a_{H^+} y + K_1 K_2} \quad (2)$$

where m_b has the opposite sign to m_a, and K_1 and K_2 are the dissociation constants of quinolones and y is the activity coefficient.

The migration of quinolones in CE can be described by Eq. (2), which, at a given pH, relates the electrophoretic behaviour with the mobilities of the fully protonated and deprotonated species, m_a and m_b, and the dissociation constants, K_1 and K_2, taking into account the effect of the activity coefficients. Moreover, Eq. (2) also permits the determination of pK_a values of the substances from a series of m_e–pH data pairs obtained experimentally [22]. Experimental electrophoretic mobility values for the quinolones studied were determined as an average of at least three replicates over the range of pH considered. The electrophoretic mobilities obtained are plotted against the pH of the buffer in Fig. 2. The best fits of the nonlinear regression for each quinolone using Eq. (2) are also shown in Fig. 2, where symbols indicate experimental data and solid lines are the fits of the experimental points in Eq. (2). In order to select the best internal standard, the electrophoretic behaviour

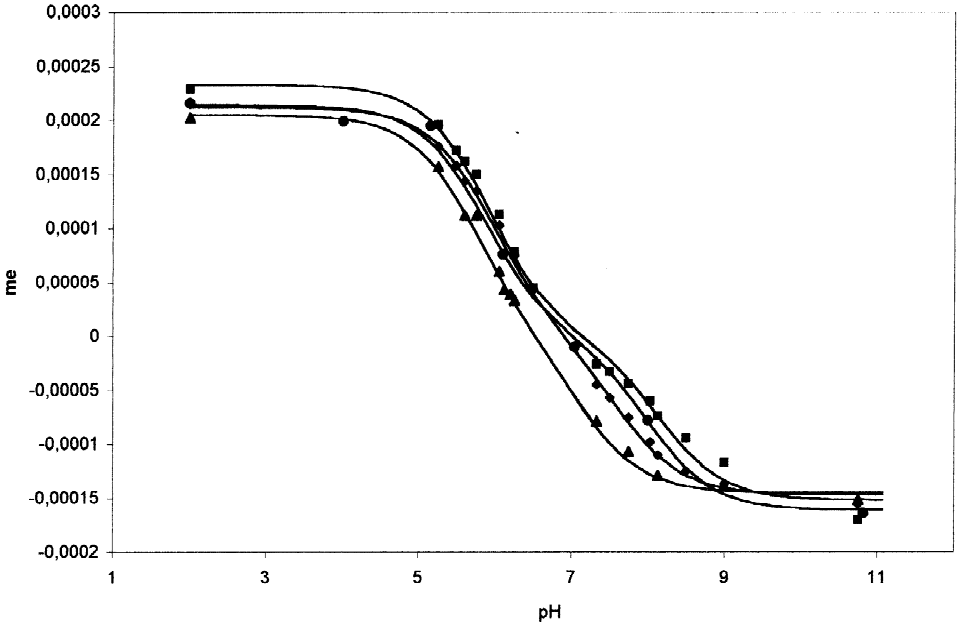

Fig. 2. m_e values vs. pH for the quinolones studied. Experimental values of m_e are represented by symbols, while the fits of the values to Eq. (2) for quinolones are represented by solid lines. Symbols: enrofloxacin (♦), ciprofloxacin (■), marbofloxacin (●), difloxacin (▲).

Table 1
pK_a values of quinolones studied in aqueous media

Quinolone	pK_1	pK_2
Ciprofloxacin	5.88±0.03	7.74±0.03
Enrofloxacin	5.86±0.05	8.24±0.07
Marbofloxacin	5.69±0.10	8.02±0.10
Difloxacin	5.66±0.04	7.24±0.06

of marbofloxacin and difloxacin were also studied. The curves corresponding to marbofloxacin and difloxacin are also included in Fig. 2. The pK_a values for the quinolones [22,23] studied are shown in Table 1.

In order to predict the optimum pH range for the separation of quinolones, it is necessary to identify the pH values at which the differences between the mobilities of the studied substances are greatest and, hence, at which these substances migrate with the greatest separation. From Fig. 2 it is deduced that the best separation is around pH 8, because at this pH the differences between the electrophoretic mobilities are greatest. Besides, these curves allow prediction of the migration order of the substances, since, at a given pH, a quinolone with a higher electrophoretic mobility will be detected earlier, that is to say in less time than one with lower mobility. However, the major or minor separation between two curves of two quinolones at the same pH indicates, in the resulting electropherogram, the major or minor distance between peaks, and the separation from the $m_e = 0$ line indicates the distance from the electroosmotic flow marker.

To verify these predictions, a mixture of standard solutions of the four quinolones was injected in diethylmalonic buffer at pH 8.00, 8.22 and 8.50. Adequate electrophoretic separation was obtained between quinolones at pH 8.22 within 4 min, in agreement with the predictions of the model. This pH allows the substances to migrate at migration times different from the peak of the electroosmotic flow. The migration order was ciprofloxacin, marbofloxacin, enrofloxacin and difloxacin. In our work, marbofloxacin was selected as the internal standard because the peak of difloxacin, the last quinolone migrating in the system, presents a broadening in the width of the peak when a spiked sample of muscle tissue is used and a clean-up procedure is applied. This broadening produces a partial co-migration of difloxacin with enrofloxacin at relatively high quinolone concentrations.

Fig. 3 shows an electropherogram from the analysis of a blank sample of muscle tissue after using the clean-up procedure. As can be observed in Fig. 3, the

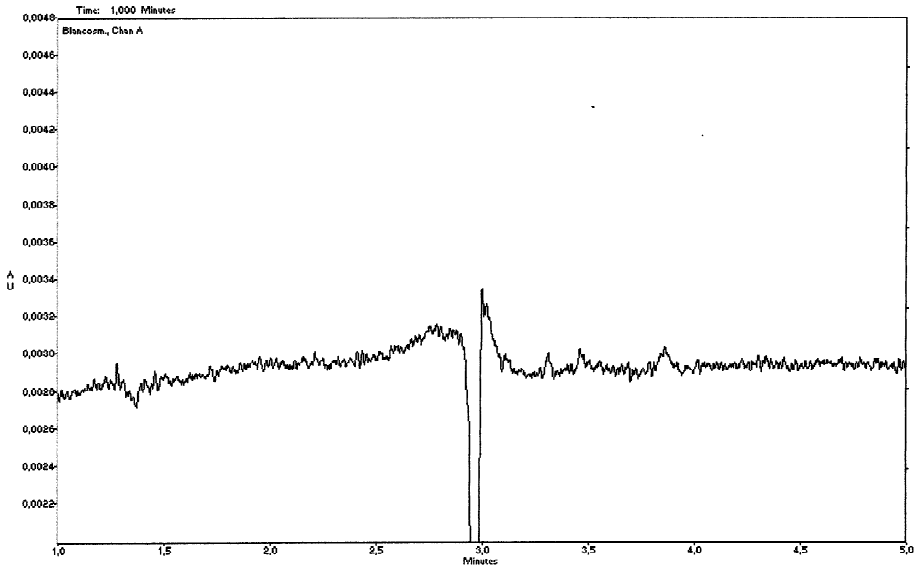

Fig. 3. Electropherogram of a blank sample of chicken muscle.

Table 2
Figures of merit for the total SPE-CE procedure for the determination of quinolone antibiotics

Parameter	Enrofloxacin	Ciprofloxacin
Calibration curves		
Intercept	0.12±0.02	0.14±0.02
Slope	2.27±0.02	1.20±0.02
Correlation coefficient	0.9999	0.9997
Recovery (%)	74	54
Intra-day reproducibility (%)	4.38	3.30
Inter-day reproducibility (%)	5.59	4.31
LOD (μg/kg)	10	25
LOQ (μg/kg)	25	50

blank electropherogram contained no peaks at the retention times corresponding to any of the quinolones tested.

The proposed SPE-CE method was validated for the determination of enrofloxacin and ciprofloxacin in muscle tissue of chicken using marbofloxacin as internal standard. Calibration graphs were from blank sample extracts spiked with an adequate volume of a solution of the quinolone studied and the internal standard. The assays exhibited linearity ($r >$ 0.9997) over the 10–300 μg/kg range for the quinolones studied. The linearity of the curves for enrofloxacin and ciprofloxacin was tested using peak-area ratios between each quinolone and the internal standard [24]. Table 2 shows the correlation coefficient, r, intercept and slope values for each of the quinolones studied.

To evaluate the recovery of ciprofloxacin and enrofloxacin from chicken muscle tissue, the proposed method was applied to the analysis of spiked chicken. Replicate experiments were performed on samples spiked at different levels (from 10 to 250 μg/kg). The mean recoveries for enrofloxacin and ciprofloxacin are shown in Table 2. A typical electropherogram for a spiked muscle sample at 100 μg/kg is shown in Fig. 4. The concentration of marbofloxacin was 150 μg/kg. No interfering peaks from chicken muscle are present at the retention times corresponding to enrofloxacin, ciprofloxacin and marbofloxacin. The data for the intra-day and inter-day reproducibility of the chicken assay procedure are summarized in Table 2. The intra-day accuracy was based on the analysis of three repli-

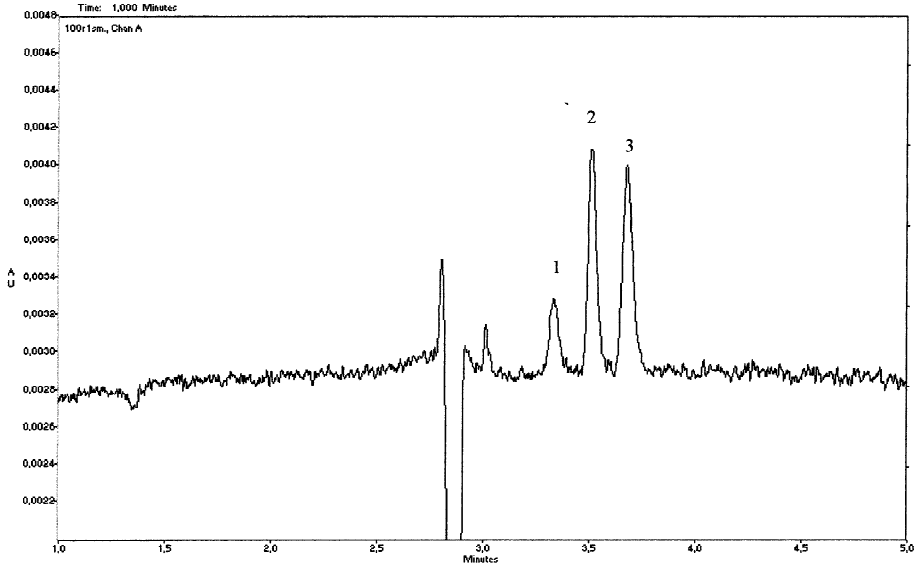

Fig. 4. Electropherogram of chicken muscle spiked with 100 μg/kg for enrofloxacin and ciprofloxacin. The concentration of marbofloxacin (internal standard) is 150 μg/kg. Diethylmalonic buffer at pH 8.22. $\lambda = 275$ nm. Peaks: 1 = ciprofloxacin, 2 = marbofloxacin, 3 = enrofloxacin.

cates of chicken tissues fortified at 100 μg/kg. The inter-day data were obtained from the analysis of samples at a concentration of 100 μg/kg with three assays on separate days.

The limit of detection (LOD) was determined as the sample concentration that produces a peak with a height three times the level of the baseline noise [24], and the limit of quantification was calculated as the sample concentration that produces a peak with a height 10 times the signal-to-noise ratio. For enrofloxacin and ciprofloxacin, the determined LOD values were 10 and 25 μg/kg, while the LOQ was 25 and 50 μg/kg, respectively.

In conclusion, a SPE-CE method for the simultaneous determination of enrofloxacin and ciprofloxacin in chicken muscle tissue has been developed. The results obtained for the limits of detection of the quinolones studied permit the detection of positive muscle samples at the required MLRs.

Acknowledgements

The financial support of DGYCIT of the Spanish Government (project PB98-1174) is gratefully acknowledged.

References

[1] D.C. Hooper, J.S. Wolfson, Quinolone Antimicrobial Agents, 2nd ed., American Society for Microbiology, Washington, DC, 1993.
[2] N. Von Rosenstiel, D. Adam, Drugs 47 (1994) 872.
[3] D.A. Volmer, B. Mansoori, S.J. Locke, Anal. Chem. 69 (1997) 4143.
[4] D.R. Doerge, S. Bajic, Rapid Commun. Mass Spectrom. 9 (1995) 1012.
[5] A. Anadon, M.R. Martínez-Larrañaga, Livest. Prod. Sci. 59 (1999) 183.
[6] EEC Commission Regulation, No. 2377/90, modified in Reg. 508/99, L60/16 of 9/3/99, Brussels, 1999.
[7] F. Belal, A.A. Al-Majed, A.M. Al-Obaid, Talanta 50 (1999) 765.
[8] W.M.A. Niessen, J. Chromatogr. A 812 (1998) 53.
[9] A. Marzo, L.D. Bo, J. Chromatogr. A 812 (1998) 17.
[10] S.W. Sun, A.C. Wu, J. Liq. Chromatogr. Relat. Technol. 22 (1999) 281.
[11] T. Perez-Ruiz, C. Martinez-Lozano, A. Sanz, E. Bravo, Chromatographia 49 (1999) 419.
[12] D. Barrón, A. Irles, J. Barbosa, J. Chromatogr. A 871 (2000) 367.
[13] D. Barrón, E. Jiménez-Lozano, J. Barbosa, Chromatographia 52 (2000) 395.
[14] D. Barrón, E. Jiménez-Lozano, J. Barbosa, Anal. Chim. Acta 415 (2000) 83.
[15] Ph. Schimitt-Kopplin, J. Burhenne, D. Freitag, M. Spiteller, A. Kettrup, J. Chromatogr. A 837 (1999) 253.
[16] M. Petersson, K.G. Wahlund, S. Nilsson, J. Chromatogr. A 841 (1999) 249.
[17] B. Delepine, D. Hurtaud-Pessel, P. Sanders, Analyst 123 (1998) 2743.
[18] A. Anadón, M.R. Martinez-Larrañaga, M.J. Díaz, M.L. Fernández-Cruz, M.A. Martínez, M.T. Frejo, M. Martínez, J. Iturbe, M. Tafur, AJVR 60 (1999) 1377.
[19] S.J. Gluck, K.P. Steele, M.H. Benko, J. Chromatogr. A 745 (1996) 117.
[20] Y. Ishihama, Y. Oda, N. Asakawa, J. Pharm. Sci. 83 (1994) 1500.
[21] J. Barbosa, D. Barrón, E. Jiménez-Lozano, J. Chromatogr. A 839 (1999) 183.
[22] D. Barrón, E. Jiménez-Lozano, A. Irles, J. Barbosa, J. Chromatogr. A 871 (2000) 381.
[23] J. Barbosa, D. Barrón, J. Cano, E. Jiménez-Lozano, V. Sanz-Nebot, I. Toro, J. Pharm. Biomed. Anal. 24 (2001) 1087.
[24] M.G. Khaledi, High Performance Capillary Electrophoresis: Theory, Techniques and Applications, Chemical Analysis, Vol. 146, Wiley, New York, 1998.