ELSEVIER

Journal of Chromatography A, 769 (1997) 293–299

JOURNAL OF CHROMATOGRAPHY A

Determination of heterocyclic aromatic amines by capillary zone electrophoresis in a meat extract

L. Puignou*, J. Casal, F.J. Santos, M.T. Galceran

Departament de Química Analítica, Universitat de Barcelona, Av. Diagonal 647, 08028 Barcelona, Spain

Received 31 October 1996; revised 3 January 1997; accepted 6 January 1997

Abstract

The use of capillary electrophoresis (CE) for the determination of heterocyclic aromatic amines in a complex matrix is reported. This work focuses on the development of a suitable procedure for the quantitation of 2-amino-3,4-dimethylimidazo [4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline ($MeIQ_x$), 2-amino-1-methyl-6-phenylimidazo-[4,5-*b*]pyridine, 3-amino-1-methyl-5H-pyrido[4,3-*b*]indole and 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole at ppb level in a commercial meat extract. The influence of pH and buffer concentration, applied potential, temperature and injection mode were investigated. Several solid-phase extraction steps were performed for the clean-up of the sample, and a 10 m*M* KCl–HCl (pH 2.20) solution was used as running electrolyte for the separation by CE. Furthermore, figures of merit of the method were calculated, and detection limits ranging from 25–50 ng ml^{-1} were obtained. MeIQ and MeIQx were detected in the meat extract at levels of 9.3 ng g^{-1} and 10.4 ng g^{-1}, respectively. The analytical results were verified by a high-performance liquid chromatography method previously established.

Keywords: Food analysis; Amines

1. Introduction

Most human cancers can be attributed to environmental factors such as cigarette smoking, industrial pollutants, radiation and dietary constituents [1]. Thus, identification and control of the causal factors, including exposure to potential environmental carcinogens, could be a way to assess the human cancer risk. A great number of chemicals have been found to be mutagens and potential carcinogens, among them are the heterocyclic aromatic amines (HAAs); all those tested to date are carcinogenic in rodents [2] and candidates to cause human common cancers as well [3,4]. The HAAs constitute a complex group of compounds belonging to two main chemical classes: aminocarbolines and amino-imidazoazaarenes. These compounds are mainly formed at trace quantities (ng g^{-1} level) from protein-rich foods when processed by thermal treatments such as typical cooking practices [5,6]. Previous works have shown that meat extracts and some beef flavours also contain potent mutagenic HAAs [7,8]. The possible formation route of these amines has not been clarified in detail, but different model systems involving several plausible precursors such as creatine and Maillard reaction products originating from sugars and certain amino acids have been suggested [9,10].

The analysis of HAAs in foods and food stuffs requires rather complex analytical methods using a

*Corresponding author.

PII S0021-9673(97)00033-2

variety of liquid–liquid or solid-phase purification techniques, followed by gas or high-performance liquid chromatography [11–13]. In general, the complexity of model reaction mixtures that could be precursors for HAAs resulted in samples of high complexity which demand a rigorous sample clean-up before using an efficient separation technique for the analysis. Capillary electrophoresis (CE) has shown potential to be a powerful technique for the analysis of charged solutes, separation of proteins, peptides, organic and inorganic ions, and chiral separations [14]. The technique provides efficiencies up two orders of magnitude greater than HPLC and is more and more regarded as an alternative separation method capable of faster analysis and higher efficiency than HPLC. In the analysis of real complex samples such as thermally processed foods, CE may become a valuable complementary technique to verify analytical results obtained by chromatographic analysis.

Several amino compounds have been analysed by CE [15], and different substituted amines have been separated and detected using this technique with indirect fluorimetric detection [16]. Recently, CE has been found to be a powerful tool for the separation of Maillard reaction products [17] and several HAAs [18–20], but no work to date has been reported about the determination of HAAs by this technique in meat extracts.

The present work focuses on the development of a suitable method for the determination of 2-amino-3,4-dimethylimidazo[4,5-*f*]quinoline (MeIQ), 2-amino-3,8-dimethylimidazo[4,5-*f*]quinoxaline (MeIQx), 2-amino-1-methyl-6-phenyl-imidazo[4,5-*b*]-pyridine (PhIP), 3-amino-1-methyl-5H-pyrido[4,3-*b*]-indole (Trp-P-2) and 2-amino-6-methyldipyrido[1,2-*a*:3′,2′-*d*]imidazole (Glu-P-1) by CE in a commercial meat extract. We report the results of our investigation on how different variables affect the separation. Capillary conditioning, applied potential, pH and buffer concentration of carrier electrolyte, temperature and injection mode were investigated. The purpose of this study was to determine which variables are critical for successful implementation of the method. Furthermore, figures of merit were calculated and the method was applied to the determination of these compounds in a commercial meat extract. An alternative HPLC method with electrochemical detection previously established [21] was applied to verify the analytical results.

2. Experimental

2.1. Chemicals

MeIQ, MeIQx, PhIP, Trp-P-2 and Glu-P-1 were purchased from Toronto Research Chemicals (Toronto, Canada). All were obtained in the highest purity available, and were used without further purification. Stock standard solutions of 100 μg ml^{-1} in methanol were prepared and used for further dilutions. 1,1′-Ethylene-2,2′-bipyridinium dibromide (diquat) from Chemservice (West Chester, PA, USA) was used as internal standard. HPLC-grade methanol was from Merck (Darmstad, Germany), dichloromethane from Carlo Erba (Milan, Italy) and the water was purified using a Culligan (Barcelona, Spain) system. All the solutions were filtered through a 0.45-μm filter before use.

All other chemicals were analytical grade and purchased from Merck or Carlo Erba. Extrelut extraction cartridges (20 ml) were provided by Merck, Bond-Elut propylsulfonyl silica gel (PRS, 500 mg) and C_{18} (100 mg) cartridges as well as coupling pieces and stopcock were from Analytichem (ICT, Basel, Switzerland). These cartridges were respectively preconditioned with dichloromethane and water–methanol (1+3 ml).

2.2. Instruments

Electrophoretic separations were performed with an Applied Biosystems (Foster City, CA, USA) Model 270A CE system with spectrophotometric detection. Electrophoretic data were processed with a Merck–Hitachi Model 2500 integrator (Darmstadt, Germany). An uncoated fused-silica capillary (Polymicro Technologies, AZ, USA), 72 cm (separation length of 50 cm)×50 μm I.D., was used. A Supelco Visiprep and Visidry SPE vacuum manifold (Supelco, Gland, Switzerland) were used for the clean-up procedure in the meat extract analysis.

2.3. Procedures

Electrolyte running solutions (pH 2.0–4.0) were prepared using low-ionic-strength buffers (I=0.01 M) developed for use in ultraviolet and visible spectroscopy [22]. Temperature was variable between 30 and 45°C, and the applied voltage was between 10 and 30 kV. Before using a new electrolyte running solution, the electrophoretic system was conditioned by flushing the capillary with a fresh 0.10 M sodium hydroxide solution for 30 min. Fresh electrolyte running solution was then flushed through the capillary for 2 h. Migration times and other electrophoretic parameters were determined for at least three consecutive injections of the sample solution. After each run the capillary was washed with 0.01 M sodium hydroxide for 2 min and equilibrated by flushing running electrolyte for 5 min. Working standard solutions in the 0.06–10 $\mu g\,ml^{-1}$ range were used. UV detection was performed at 210 nm.

Injection was performed in the electrokinetic or hydrodynamic (by vacuum) injection mode. In the electrokinetic mode 5 kV was applied for 1–20 s. When the samples were injected by the vacuum technique, 16.9 kPa was applied for 1–20 s. Methanol was used as marker to measure electroosmotic flow.

2.4. Meat extract analysis

Sample preparation and clean-up were carried out according to the method proposed by Gross [8]. A 3–4 g meat extract sample was saponified with 10 ml 1 M NaOH by shaking until dissolution; the alkaline solution obtained was introduced into an Extrelut column coupled to a Bond-Elut PRS column. The analytes adsorbed on the Extrelut packing were extracted using dichloromethane, which was introduced into the PRS column coupled on-line, and the extraction was stopped when 40 ml had passed through the coupling. The Extrelut column was discarded, and the PRS column was successively rinsed with 0.01 M HCl, methanol–0.1 M HCl (4:6, v/v) and water. The PRS column was then coupled to a C_{18} column. This tandem was eluted with 20 ml of 0.5 M ammonium acetate at pH 8.0. The adsorbed HAAs were eluted from the C_{18} column using 0.8 ml of methanol–ammonia (9:1, v/v). The solvent was evaporated with a stream of nitrogen and the analytes were redissolved with 50 μl of the internal standard in methanol.

The HAAs were analysed by CE using 10 mM KCl–HCl (pH 2.20) as running electrolyte. The temperature was held at 30°C and the applied potential was +20 kV. UV detection was performed at 210 nm. Hydrodynamic injection mode (4 s) was used. The compounds were quantified by the standard addition method. Addition of accurately measured amounts of each standard (125, 250 and 500 ng) was performed at the beginning of the clean-up process. Recoveries were estimated from the slope of the regression line obtained from the added amount versus the measured amount.

3. Results and discussion

3.1. CE optimization

The effect of the pH on electrophoretic migration and resolution of the amino compounds was studied. The pH was varied between 2 and 4 using monochloroacetic acid–KOH and formic acid–KOH as buffer solutions at 0.01 M ionic strength. Fig. 1 shows the electropherograms obtained at several pH values, from 2.0 to 3.0. It can be observed that the apparent mobility (μ_{app}) increased because of the increase in electroosmotic flow, although the amines become progressively less ionized. A loss in resolution was observed, which may be due to increasing electroosmosis related to resolution by the selectivity–mobility term $\Delta\mu_{app}/\mu_{app}$. At pH lower than 2.50 the amino compounds were adequately separated with chromatographic resolutions higher than 1.5. However, a pH of 2.20 appeared to be the optimum for separation allowing a run time of 15 min and resolutions better than 1.70.

The effect of the carrier electrolyte composition on selectivity was studied at pH 2.20 using 10 mM glycine–HCl, 10 mM KCl–HCl and 10 mM citric acid–10 mM Na_2HPO_4 as buffer solutions at 0.01 M ionic strength. Using citric acid buffer, Trp-P-2 and Glu-P-1 coeluted and monochloroacetic acid–KOH gave an irregular baseline as can be seen in Fig. 1B. The best results were obtained with glycine–HCl and

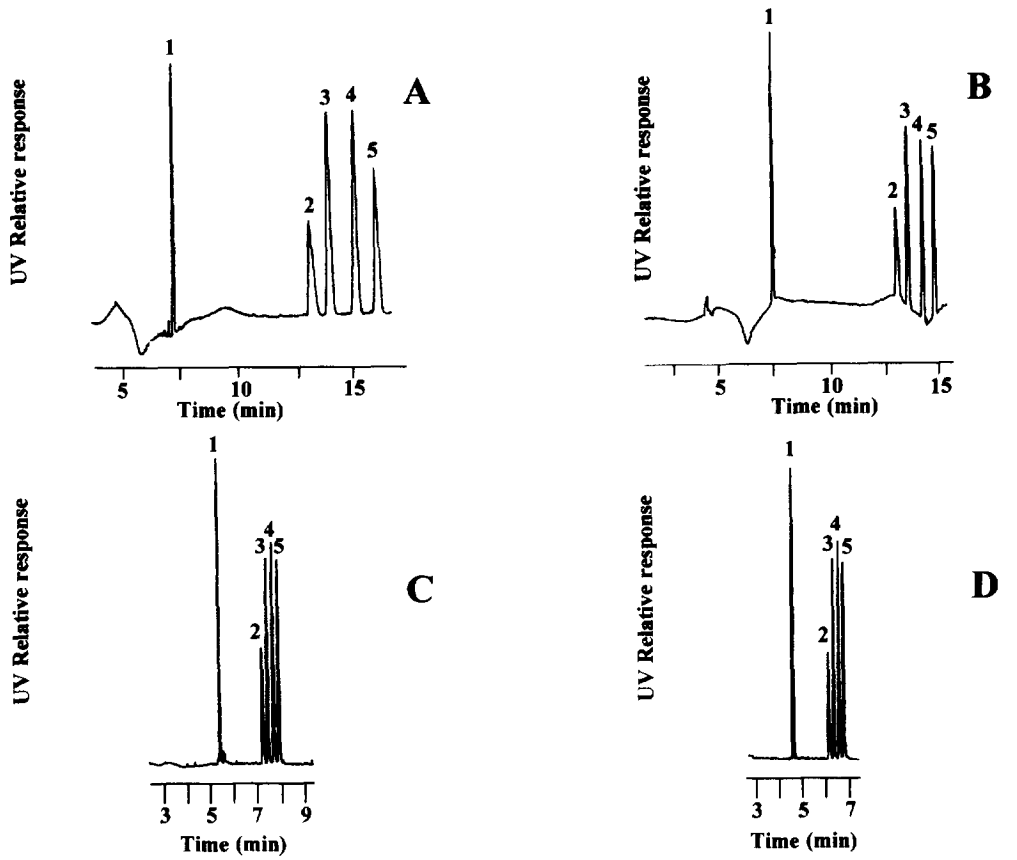

Fig. 1. Electropherograms at different pH of a standard solution containing 5 μg ml^{-1} of each HAA. Carrier electrolyte: monochloroacetic acid–KOH at 10 m*M* of ionic strength; applied voltage, +20 kV; capillary temperature, 30°C; hydrodynamic injection, 3 s; λ 210 nm. (A) pH: 2.00; (B) pH: 2.20; (C) pH:2.60; (D) pH: 3.00. Peaks: 1=MeIQ; 2=Trp-P-2; 3=Glu-P-1; 4=MeIQx; 5=PhIP.

KCl–HCl at pH 2.20. To study the effect of the electrolyte concentration on separation, the pH was maintained at 2.20 and the amount of glycine or KCl was changed. Varying the glycine concentration from 5 to 100 m*M* an increase in migration times occurred, as can be seen in Fig. 2A.1 where apparent mobilities versus electrolyte concentration are given. This fact could be related to a decrease in zeta potential in capillary surface due to the adsorption of glycinium ions on the silica wall. A great improvement on resolution was obtained at 25–50 m*M* but a decrease occurred at higher concentrations. When concentration of KCl was changed from 5–15 m*M* a decrease in run time was observed (Fig. 2A,2). The faster run times at higher buffer concentrations were probably due to a larger cathodic drift. In contrast with the glycinium ion behaviour, potassium ion seems to be non-adsorbed onto the capillary wall, and a slight increase of the mobility and no distortions in the baseline were observed. In both cases resolution increased with concentration (Fig. 2B, 1 and 2), the highest values being obtained between 8 and 12 m*M* for KCl, and between 20 and 50 m*M* for glycine. Slightly higher resolutions were achieved for glycine but higher analysis times also occurred. As a conclusion 10 m*M* KCl was recommended for the CE separation.

The linear velocity of the effluent flow through the column is determined by the electric field strength. Therefore an increase in the voltage enhances the mobility and as a result a decrease in run times occurs. Resolution was rather unaffected by voltage except at high values, the best separation being obtained between 13 and 18 kV. In all cases, the peak

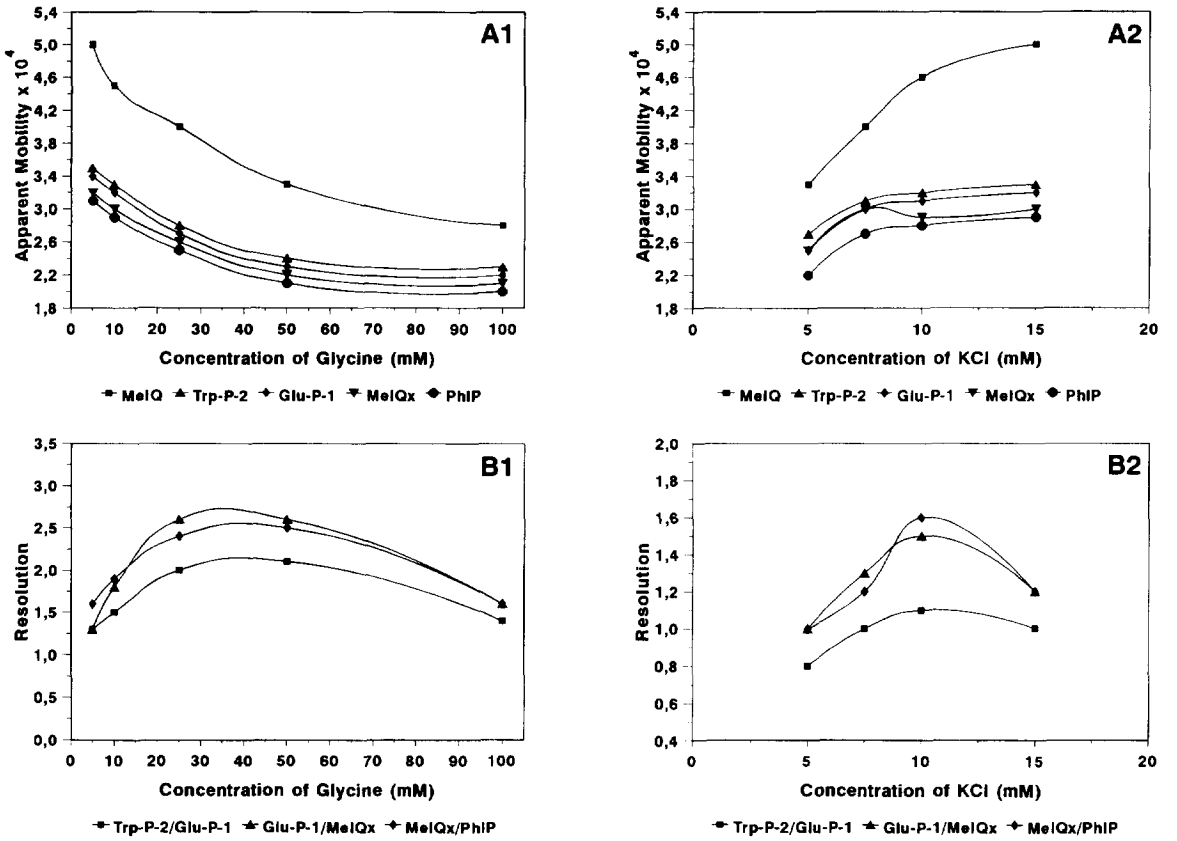

Fig. 2. Effect of carrier electrolyte concentration on (A) apparent mobilities. 1=Glycine; 2=KCl; and (B) resolution. 1= Glycine; 2=KCl. (Experimental conditions as in Fig. 1)

efficiency showed an initial increase till 20–25 kV depending on the solutes, ranging from $7.3 \cdot 10^5$–$12.8 \cdot 10^5$ theoretical plates m^{-1}, but at voltages higher than these values an important decrease was observed. Band dispersion is probably due to thermal effects, an unavoidable factor that contributes to band broadening in CE. A voltage of 20 kV was chosen because high efficiencies were found, resolutions better than 1.5 and run times not higher than 20 min were obtained. Many parameters in CE depend on temperature: viscosity, conductivity and diffusion coefficients and all of them influence the retention time and efficiency, the dependence of mobility and resolution on temperature was checked. As the temperature increased, there was a decrease in retention times and also a decrease in resolution. Since at 30°C good resolution and efficiency were obtained, this operating temperature was selected.

Sample injection using both electrokinetic (EK) and hydrodynamic (HD) injection modes was investigated. As usual, higher responses were achieved for the EK injection mode, but the HD mode gave higher run times and better resolutions, for instance using 4 s resolution between Glu-P-1 and MeIQx was 1.7 for the HD mode and 1.0 for the EK mode. Moreover, the hydrodynamic injection provided better reproducibilities than the electrokinetic one.

3.2. Quality parameters

Detection limits, at λ=210 nm, based on a 3:1 signal-to-background noise ratio ranged from 35 to 50 ng ml^{-1} for HD injection and from 25 to 45 ng ml^{-1} for electrokinetic one. Ten replicate determinations of 1.0 μg ml^{-1} standard solutions of each compound were carried out under the optimum

conditions to determine the run-to-run reproducibilities in both injection modes. Run-to-run R.S.D. in retention times ranged from 0.4 to 1.02%, being smaller for the HD injection mode. For peak areas, standard deviations with HD injection were also lower, between 3.65 and 4.25%.

In order to perform quantitatively, a technique needs to be both linear and reproducible. Calibration curves for HAAs were carried out with concentrations in the range 0.06–10 $\mu g\,ml^{-1}$. Peak area was used as the response. The correlation coefficients of calibration functions were better than 0.999 for all the solutes when HD injection was used.

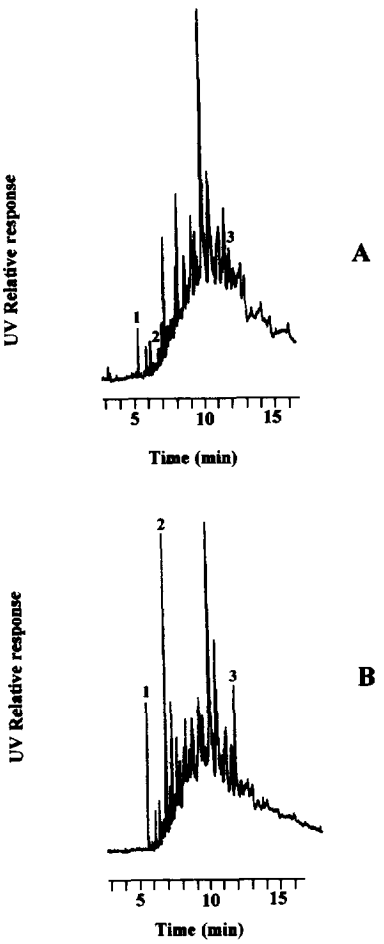

Fig. 3. Electropherograms of a beef sample: (A) extract and (B) extract spiked (250 ng). Carrier electrolyte: 10 m*M* KCl–HCl (pH 2.20); applied voltage, +20 kV; capillary temperature, 30°C; hydrodynamic injection, 4 s; λ 210 nm. Peaks: 1=internal standard; 2=MeIQ; 3=MeIQx.

3.3. Application

The analytical method studied was mainly developed to determine HAAs in processed food samples. So it was applied to the determination of these compounds in a commercial beef extract. Purification of the sample was performed by the method proposed by Gross [8], as described in the Experimental section. MeIQ and MeIQx were identified and quantified in the beef extract, recoveries for MeIQx and MeIQ were 77.7% and 66.6%, respectively, in agreement with the values previously reported [8,13]. The results were 9.3 ng g^{-1} and 10.4 ng g^{-1}, values according with the ones obtained by HPLC [13] for the same sample. Fig. 3 shows the electropherograms of the sample and one spiked sample which confirms the presence of MeIQ and MeIQx. The internal standard used was diquat (DQ) with a run time of 1.3 min where no overlapping with matrix peaks was observed.

4. Conclusions

In this paper conditions for the separation and determination of heterocyclic aromatic amines by CE have been established. The method has been applied to the determination of HAAs in a commercial meat extract, the concentrations of MeIQ and MeIQx in the sample have been found in the range reported by other authors for food-grade beef extracts. The applicability, selectivity and sensitivity of the CE method have been studied showing that CE is a convenient, rapid and efficient technique for small molecule separations, that gives results comparable to HPLC. CE separation has proved to be an efficient technique for the analysis of complex matrices such as beef extracts; more than 25 peaks have been resolved in a very short time, but only MeIQ and MeIQx have been quantified. Selective and sensitive detectors must be used to confirm the identities of resolved peaks. Research is currently in progress to optimize new CE methods using a photodiode array detector and the coupling to a mass spectrometer.

Acknowledgments

The authors gratefully acknowledge the financial support of the C.I.C.YT. to the research project ALI96-0863.

References

[1] F. Perera and P. Boffetta, J. Nat. Cancer Inst., 80 (1988) 1282.
[2] H. Ohgaki, S. Takayama and T. Sugimura, Mutation Res., 259 (1991) 399.
[3] T. Sugimura, M. Nagao and K. Wakabayashi, J. Nat. Cancer Inst., 86 (1994) 2.
[4] D.W. Layton, K.T. Bogen, M.G. Knize, F.T. Hatch, V.M. Johnson and J.S. Felton, Carcinogenesis, 16 (1995) 39.
[5] J.S. Felton and M.G. Knize, Mutation Res., 259 (1991) 205.
[6] K. Skog, Food Chem. Toxicol., 31 (1993) 655.
[7] L.S. Jackson, W.A. Hargraves, W.H. Stroup and G.W. Diachenko, Mutation Res., 320 (1994) 113.
[8] G.A. Gross, Carcinogenesis, 11 (1990) 1597.
[9] M. Jägerstad, K. Skog, S. Grivas and K. Olsson, Mutation Res., 259 (1991) 219.
[10] K. Skog, M.G. Knize, J.S. Felton and M. Jägerstad, Mutation Res., 268 (1992) 191.
[11] M.G. Knize, J.S. Felton and G.A. Gross, J. Chromatogr., 624 (1992) 253.
[12] G.A. Gross, A. Grüter and S. Heyland, Food Chem. Toxicol., 30 (1992) 491.
[13] M.T. Galceran, P. Pais and L. Puignou, J. Chromatogr. A, 719 (1996) 203.
[14] R.L. St.Claire, Anal. Chem., 68 (1996) R569.
[15] S.F.Y. Li, Capillary Electrophoresis: Principles, Practice and Applications, Journal of Chromatography Library, Vol. 52, Elsevier, Amsterdam, 1992.
[16] L. Gross and E.S. Yeung, Anal. Chem., 62 (1990) 427.
[17] A.J. Tomlinson, J.A. Mlotkiewicz and I.A.S. Lewis, Food Chem., 49 (1994) 219.
[18] J. Wu, M.K. Wong, S.F.Y. Li, H.K. Lee and C.N. Ong, J. Chromatogr. A, 709 (1995) 351.
[19] J. Wu, M.K. Wong, H.K. Lee and C.N. Ong, J. Chromatogr. Sci., 34 (1996) 139.
[20] J. Wu, M.K. Wong, H.K. Lee, B.L. Lee, C.Y. Shi and C.N. Ong, Food Addit. Contam., 13 (1996) 851.
[21] M.T. Galceran, P. Pais and L. Puignou, J. Chromatogr. A, 655 (1993) 101.
[22] D.D. Perrin and B. Dempsey, Buffers for pH and Metal Ion Control, Chapman and Hall/Fletcher and Son, Norwich, 1974, p. 44.