ELSEVIER

Journal of Chromatography A, 855 (1999) 247–253

JOURNAL OF
CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Application of ion-exchange cartridge clean-up in food analysis II. Determination of benzylpenicillin, phenoxymethylpenicillin, oxacillin, cloxacillin, nafcillin and dicloxacillin in meat using liquid chromatography with ultraviolet detection

Yuko Ito[a,*], Yoshitomo Ikai[a], Hisao Oka[a], Tadaaki Kagami[a], Kazue Takeba[b]

[a]*Aichi Prefectural Institute of Public Health, Tsuji-machi, Kita-ku, Nagoya 462-8576, Japan*

[b]*The Tokyo Metropolitan Research Laboratory of Public Health, Hyakunin-cho, Shinjuku-ku, Tokyo 169-0073, Japan*

Received 19 October 1998; received in revised form 25 May 1999; accepted 2 June 1999

Abstract

A multiresidue analytical method was developed for the simultaneous determination of benzylpenicillin (PCG), phenoxymethylpenicillin (PCV), oxacillin (MPIPC), cloxacillin (MCIPC), nafcillin (NFPC) and dicloxacillin (MDIPC) in meat. The method involves the use of an ion-exchange cartridge for sample clean-up followed by ion-pair high-performance liquid chromatography with ultraviolet detection. The recoveries of PCG, PCV, MPIPC, MCIPC, NFPC and MDIPC from pork muscle spiked at levels of 0.5, 0.1 and 0.05 mg/kg were in the range of 77–90, 73–95 and 80–93% with coefficients of variation of 0.5–1.7, 1.6–4.4 and 3.2–6.6%, respectively. For beef muscle spiked at levels of 0.5, 0.1 and 0.05 mg/kg, the recoveries of these compounds were 83–92, 71–86 and 77–90% with coefficients of variation of 1.7–4.4, 2.6–7.0 and 3.9–6.4%, respectively. The detection limits for each penicillin were 0.02 mg/kg in meat. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Antibiotics; Benzylpenicillin; Phenoxymethylpenicillin; Oxacillin; Cloxacillin; Nafcillin; Dicloxacillin

1. Introduction

Penicillin antibiotics have been widely used for livestock as veterinary drugs to prevent and treat infectious diseases. Such usage may lead to problems with residues in livestock products. One of the major roles as public health agencies is to provide safe products for consumers through quantification of these residues in livestock products.

Although bioassays are the most commonly employed methods for determining the contents of penicillins in livestock products, they are complicated, time consuming, and non-specific. On the other hand, as high-performance liquid chromatography (HPLC) is a fast and reliable technique with high sensitivity, many HPLC methods have been reported for the analysis of residual penicillins in milk [1–7]. However, only a few methods have been reported for the simultaneous analysis of penicillins in meat [8–13] and most of them require derivatization using toxic reagents or special instruments, because penicillins do not have any specific strong ultraviolet (UV) absorption. There is, there-

*Corresponding author. Tel.: +81-52-911-3111; fax: +81-52-913-3641.

0021-9673/99/$ – see front matter © 1999 Elsevier Science B.V. All rights reserved.
PII: S0021-9673(99)00695-0

fore, a great need for a simple analytical method which permits the simultaneous determination of penicillins in meat.

We have reported in our previous studies [14–18] the applicability of sample clean-up by an ion-exchange cartridge in combination with ion-pair HPLC for the analysis of ionable compounds. The objective of this study was to develop an analytical method for the quantitative determination of benzylpenicillin (PCG), phenoxymethylpenicillin (PCV), oxacillin (MPIPC), cloxacillin (MCIPC), nafcillin (NFPC) and dicloxacillin (MDIPC), all of which are ionable substances.

This paper describes a sample clean-up procedure using an ion-exchange cartridge followed by ion-pairing HPLC determination with UV detection for the simultaneous analysis of the penicillins in meat.

2. Experimental

2.1. Chemicals and reagents

Methanol, acetonitrile and distilled water (H_2O) were reagents of HPLC grade. Sodium chloride (NaCl), phosphoric acid, disodium hydrogenphosphate dodecahydrate, sodium dihydrogenphosphate dihydrate and other chemical reagents were of analytical reagent grade. Cetyltrimethylammonium chloride, tetra-*n*-butylammonium bromide and *n*-dodecyltrimethylammonium chloride were ion-pairing reagents from Tokyo Kasei Kogyo (Tokyo, Japan). Hyflo Super-Cell was obtained from Johns-Manville (Denver, CO, USA).

Bond Elut C18 (Lot No. 070070, 071070, 071251 and 071540), Bond Elut PSA (Lot No. 120598), Bond Elut SAX (Lot No. 183537) and Bond Elut DEA (Lot No. 232198) were purchased from Varian (Harbor City, CA, USA). Sep-Pak Accell Plus QMA (Lot No. T6305G1, T7125B1, T7023G1 and W8117J1) and Sep-Pak Vac NH2 (Lot No.T5276G1) were obtained from Waters (Milford, MA, USA). All cartridges were of 3 ml capacity packed with a 500 mg solid-phase.

PCG potassium salt, PCV potassium salt, MPIPC sodium salt, MCIPC sodium salt, NFPC sodium salt and MDIPC sodium salt were purchased from Sigma (St. Louis, MO, USA). Separate stock solutions of each penicillin were prepared by dissolving 50 mg of compound in 50 ml of H_2O. Subsequent dilutions were made using the mobile phase of the HPLC. All the working standards were prepared fresh daily.

2.2. Apparatus

The HPLC system consisted of a LC-10AD pump, a SIL-10A$_{XL}$ auto injector, a SCL-10A system controller, a SPD-10A UV–Vis absorbance detector, and a CR-6A recorder (Shimadzu, Kyoto, Japan).

2.3. Chromatographic conditions

The separation was performed on a TSKgel ODS-80Ts column (5 μm, 150×4.6 mm, I.D.; TOSOH, Tokyo, Japan) at 30°C. The mobile phase consisted of acetonitrile–0.02 *M* phosphate buffer pH 6.2 (4.3:5.7, v/v) containing 12 m*M* cetyltrimethylammonium chloride. The flow-rate of the mobile phase was 0.8 ml/min and detection was carried out at 220 nm.

2.4. Analytical procedure

2.4.1. Preparation of crude extracts of pork and beef muscles

Pork muscle was sliced and homogenized with a mixer. An aliquot (5-g) of a homogenized sample was weighed in a 50-ml centrifuge tube and blended with 30 ml of H_2O using a high speed blender. After centrifugation (3100 rpm, 15 min), the supernatant was filtered through a glass microfiber filter into a 100-ml Erlenmeyer flask. The residual plug was reextracted twice with another 20 ml of H_2O, combining the supernatants in the Erlenmeyer flask.

Beef muscle was homogenized and extracted as described above using a 5-g sample with 60 ml of 2% NaCl aqueous solution. The supernatant was filtered through a glass microfiber filter with about 1.5 g of Hyflo Super-Cell. The residual plug was reextracted twice with 40 ml of 2% NaCl aqueous solution, combining the supernatants in the Erlenmeyer flask.

2.4.2. Purification of crude extract using preclean-up cartridge (Bond Elut C18)

The crude extract collected in the Erlenmeyer flask

(about 70 ml for pork and 140 ml for beef) was passed through a Bond Elut C18 cartridge preconditioned with 5 ml of methanol followed by 5 ml of H_2O at a flow-rate of ca. 1 ml/min. The Bond Elut C18 cartridge was washed with 10 ml of H_2O, 5 ml of 15% methanol containing 2% NaCl, and 5 ml of H_2O, and then air-dried under vacuum for 10 min. The elution was carried out with 5 ml of 55% methanol.

2.4.3. Purification of eluate from Bond Elut C18 by clean-up cartridge (Sep-Pak Accell Plus QMA)

The eluate from the Bond Elut C18 cartridge was applied to a Sep-Pak Accell Plus QMA cartridge preconditioned with 5 ml of methanol, 5 ml of H_2O, and 5 ml of 55% methanol. The Sep-Pak Accell Plus QMA cartridge was washed with 3 ml of 55% methanol followed by 3 ml of H_2O, and then air-dried under vacuum for 10 min. The retained penicillins were eluted with 2 ml of the HPLC mobile phase. A 20-μl aliquot of the eluate was injected into the HPLC.

2.5. Quantitation

Calibration curves were obtained by plotting the absolute peak heights of the penicillins versus the concentration of the standard solutions. Quantification of the penicillins in meat samples was calculated from the calibration curves and reported in grams of sample weight (mg/kg). Recoveries were calculated as the ratio of the peak heights of the analytes from the fortified samples to the peak heights of the standard solutions. The calibration curves were linear over the ranges of 0.01–2 μg/ml (for PCG, PCV and MDIPC) and 0.005–1 μg/ml (for MPIPC, MCIPC and NFPC) with correlation coefficients over 0.999.

3. Results and discussion

3.1. Optimization of HPLC conditions

3.1.1. Effect of ion-pair reagent

It is difficult to determine penicillins on the same chromatogram using isocratic HPLC due to the significant difference in their polarities. So, the ion-pair technique was applied in the present study. The polarities of the penicillins are higher in the following order, MDIPC, NFPC, MCIPC, MPIPC, PCV and PCG. PCG and PCV showed a weaker retention on the C18 column than the other penicillins did. In order to increase the retention of PCG and PCV on the HPLC column, the following ion-pair reagents were added to the mobile phase to evaluate the effect of these chemicals on the retention of these penicillins: cetyltrimethylammonium chloride, *n*-dodecyltrimethylammonium chloride and tetra-*n*-butylammonium bromide. Among these chemicals, cetyltrimethylammonium chloride gave the most favorable effect on the retention of PCG and PCV on the column resulting in a complete separation of all of the penicillins examined. Accordingly, cetyltrimethylammonium chloride was chosen as the ion-pair reagent.

In addition, selected concentrations of this ion-pair reagent (cetyltrimethylammonium chloride) ranging from 0 to 14 m*M* were examined to obtain the optimal separation of the penicillins. With an increase in the concentration of this reagent, the retention of each penicillin was improved (Fig. 1). Because the best result was achieved with concentrations between 10–14 m*M*, 12 m*M* was selected as the concentration of the ion-pair reagent for the determination of the penicillins.

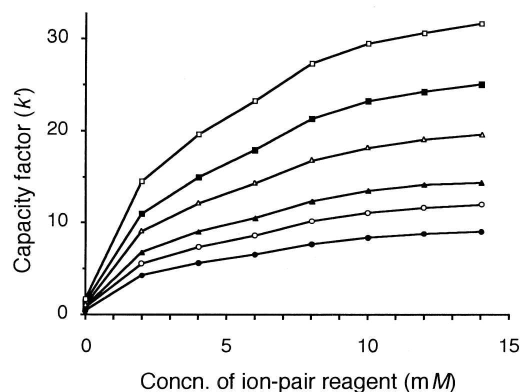

Fig. 1. Effect of the concentration of ion-pair reagent in the mobile phase on the capacity factor (k') of penicillins. Operating conditions, column: TSKgel ODS-80Ts (150×4.6 mm I.D.); mobile phase: acetonitrile–0.02 *M* phosphate buffer, pH 6.2 (4.3:5.7, v/v) containing 0–14 m*M* cetyltrimethylammonium chloride; flow-rate: 0.8 ml/min; detector: UV 220 nm; column temp.: 30°C. Results of three replicates. (●) PCG, (○) PCV, (▲) MPIPC, (△) MCIPC, (■) NFPC, (□) MDIPC.

3.1.2. The pH of the phosphate buffer

Effects of pH of the phosphate buffer were examined between pH 5.6 and 7.0. When the pH value was higher than 6.7, separation of PCV, MPIPC and co-existence substance from the samples was insufficient. With the pH value of 5.6, the capacity factor of MDIPC was too large ($k' > 40$). A pH of 6.2 provided a good separation with adequate capacity factors for each penicillin, therefore, the pH value of the mobile phase was adjusted to 6.2 in this study.

Thus, a satisfactory separation of the six penicillins was obtained using the TSKgel ODS-80Ts (5 μm, 150×4.6 mm, I.D.) column and acetonitrile–0.02 *M* phosphate buffer, pH 6.2, (4.3:5.7, v/v) containing 12 m*M* of cetyltrimethylammonium chloride as the mobile phase (Fig. 2).

3.2. Sample extraction

Although the repeated (three times) extraction of the penicillins with water (30, 20, 20 ml) from pork muscle (5-g of homogenate) was very simple and gave a satisfactory extraction efficiency (recoveries of 90–95%), this extraction procedure could not be applied to beef muscle because of the poor efficiency (recoveries less than 75%) for PCG and PCV. After a series of various examinations of extraction procedure, we found that repeated (three times) extraction with 2% NaCl aqueous solution (60, 40, 40 ml) gave a satisfactory efficiency (recoveries of 90–95%) for beef muscle (5-g of homogenate). Based on these preliminary experiments, we selected repeated extraction procedures with water (30, 20, 20 ml) and 2% NaCl aqueous solution (60, 40, 40 ml) for 5-g each of pork and beef muscle homogenate, respectively.

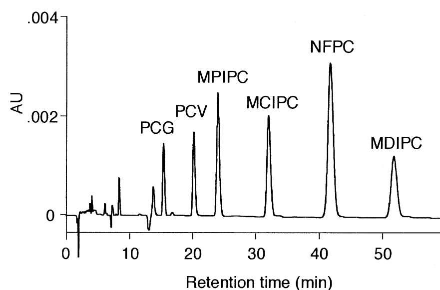

Fig. 2. Typical HPLC chromatogram of penicillins. Mobile phase: acetonitrile–0.02 *M* phosphate buffer, pH 6.2 (4.3:5.7, v/v) containing 12 m*M* cetyltrimethylammonium chloride. Other operating conditions as in Fig. 1.

3.3. Purification of crude extracts by double cartridges

3.3.1. Preclean-up method with C18 cartridge

Because muscle extract contains many substances interfering with the ion-exchange capacity of the cartridge, the ion-exchange cartridge could not retain the penicillins when the crude muscle extract was directly applied to the cartridge. Accordingly, a preclean-up method was needed prior to the application of the extract to the cartridge for clean-up. It had been reported that washing of a crude extract loaded C18 cartridge with an aqueous methanol solution containing NaCl was effective as a preclean-up of samples for the determination of PCG [19]. We, therefore, investigated the preclean-up method using the Bond Elut C18 cartridge.

C18 cartridge preclean-up without the washing described above did not give a sufficient effect, namely, the ion-exchange cartridge could not retain PCG and PCV, because many substances interfering with the ion-exchange capacity still remain in the eluate from the C18 cartridge. Accordingly, the effect of washing solvents on the recoveries of the penicillins was examined. The C18 cartridge was loaded with pork muscle extract spiked with all of the penicillins (0.5 mg/kg), and then washed with 10 ml of water and 5 ml of selected concentrations (0 to 25%) of methanol containing 2% NaCl. The penicillins were eluted with 5 ml of methanol. When the concentration of methanol was lower than 15%, recoveries of the penicillins were higher than 90%, however, the effect of clean-up was decreased with more interfering substances eluted. Judging from the results of these preliminary experiments, we decided to use 15% methanol as the washing solution to obtain an optimal effect of the clean-up and the recovery.

Next, the effect of methanol concentration in the elution solvent for the C18 cartridge was investigated using the washing solvent described above. A methanol concentration greater than 45% gave recoveries of all penicillins over 95%, however, the higher the

concentrations of methanol became, the more interfering substances were eluted. Accordingly, 55% methanol was chosen as the elution solvent because of the good recoveries of the penicillins with substantially small amount of interfering substances eluted. Finally, the optimal volume of the elution solvent applied to the C18 cartridge was determined. The penicillins were eluted with the elution solvent after being retained (2.5 μg each) on the C18 cartridge. Every 1 ml of the eluate was collected for the HPLC determination of penicillins, and it was found that all of the six penicillins were recovered after applying 5 ml of elution solvent to the cartridge.

3.3.2. Ion-exchange cartridge clean-up method

It is desirable to use the same solution for the HPLC mobile phase and for the elution solvent from the clean-up cartridges to assure good reproducibility of the HPLC determination of the penicillins. We investigated the suitability of the selected ion-exchange cartridges (Sep-Pak Accell Plus QMA, Sep-Pak Vac NH2, Bond Elut PSA, Bond Elut SAX and Bond Elut DEA) using the above described mobile phase as the elution solvent. After 10 ml of water spiked with the six penicillins (2.5 μg each) was loaded on the cartridges, the penicillins were eluted with 5 ml of the HPLC mobile phase from the cartridge. Among the five cartridges examined, Sep-Pak Accell Plus QMA (QMA) was found to provide the best results with all of the penicillins completely eluted out. Accordingly, Sep-Pak Accell Plus QMA was chosen as a clean-up cartridge in the subsequent studies.

Next, the optimal volume of the elution solvent applied to the QMA cartridge was investigated using the same procedure as in the case for the C18 cartridge described above. All kinds and amounts of the penicillins loaded on the cartridge were found to be eluted after application of 2 ml of the elution solvent. We, therefore, applied 2 ml of the elution solvent to the QMA cartridge.

Even after this QMA cartridge clean-up procedure with the C18 cartridge preclean-up, the samples were not cleaned up enough for precise determination of all of the penicillins leaving a few interfering peaks appearing around the same retention time as that of PCG on the chromatogram. We, therefore, tried to wash the QMA cartridge to improve the clean-up effect. After the application of the QMA cartridge clean-up procedure, the cartridge was further washed with 3 ml of selected solvents (ethyl acetate, acetonitrile, water, and selected concentrations (0–25%) of methanol aqueous solutions), and then the penicillins were eluted with 2 ml of the mobile phase. Among these solvents, only water gave satisfactory results; the disappearance of an interfering peak at the PCG retention time.

3.4. Recoveries

Pork and beef muscle samples were fortified with the six penicillins (0.5 or 0.1 or 0.05 mg/kg each), and analyzed according to the procedure described in Section 2.4 in this paper. The recoveries and corresponding coefficients of variation (C.V.) are listed in Table 1. The average recoveries for the penicillins from pork muscle ranged from 73 to 95% with the C.V. ranging from 0.5 to 6.6%. For beef muscle, the average recoveries ranged from 71 to 92% with the C.V. ranging from 1.7 to 7.0%. Figs. 3 and 4 show typical chromatograms of the fortified pork and beef muscle samples (b) and their corresponding controls (a), respectively. The detection limit for each penicillin was 0.02 mg/kg in the meat (S/N ratio=3). In order to prove the clean-up effect of the present method, we analyzed ten muscle samples (five each of pork and beef) which were previously found to be negative for penicillins by bioassay screening. The chromatgrams were obtained almost the same as in Figs. 3(a) and 4(a) except for the shorter retention time region of 10 min. These results clearly indicate that the method proposed here has satisfactory reproducibility, recovery and accuracy for the simultaneous determination of PCG, PCV, MPIPC, MCIPC, NFPC and MDIPC in meat.

Pork and beef samples on the market (15 pork and 10 beef) were analyzed according to the present method, all of which were not detected any of the six penicillins and interfering peaks on the chromatograms. The present method was successfully applied to a positive beef muscle sample obtained from a slaughterhouse, which was previously found to be positive for penicillins by bioassay screening, and 0.06 mg/kg of PCG was detected. Analysis time required is about 3.5 h for a pork muscle sample and

Table 1
Recoveries of penicillins from pork and beef muscles

Penicillins	Pork muscle			Beef muscle		
	Added (mg/kg)	Recovery[a] (%)	C.V.[b] (%)	Added (mg/kg)	Recovery[a] (%)	C.V.[b] (%)
Benzylpenicillin	0.5	85	1.4	0.5	92	2.9
	0.1	80	4.4	0.1	83	7.0
	0.05	84	6.6	0.05	77	6.4
Phenoxymethylpenicillin	0.5	90	0.5	0.5	90	2.4
	0.1	89	2.3	0.1	82	4.8
	0.05	89	3.3	0.05	84	5.4
Oxacillin	0.5	89	0.5	0.5	86	1.9
	0.1	95	2.6	0.1	74	3.5
	0.05	82	3.2	0.05	80	3.9
Cloxacillin	0.5	85	0.5	0.5	85	1.8
	0.1	85	2.6	0.1	86	3.1
	0.05	93	3.7	0.05	82	4.0
Nafcillin	0.5	86	1.5	0.5	89	1.7
	0.1	86	3.3	0.1	85	2.6
	0.05	93	4.2	0.05	90	5.2
Dicloxacillin	0.5	77	1.7	0.5	83	4.4
	0.1	73	1.6	0.1	71	2.6
	0.05	80	5.7	0.05	79	6.4

[a] Average of five trials.
[b] C.V.: coefficient of variation.

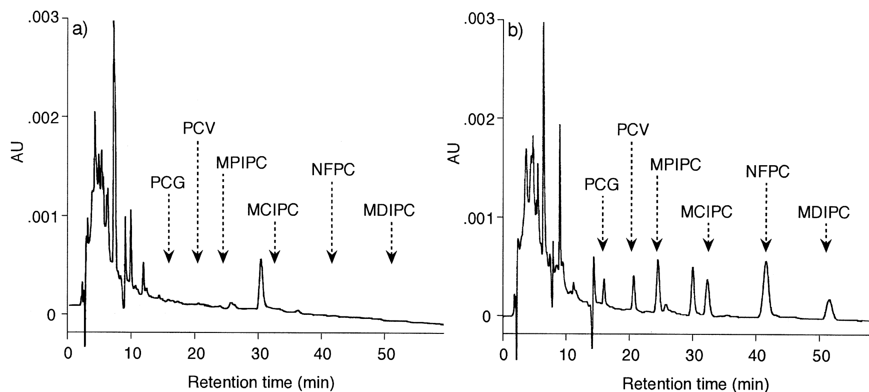

Fig. 3. Typical HPLC chromatograms of pork muscle samples. (a) Pork muscle (control); (b) added at a level of 0.1 mg/kg each of penicillins.

5 h for a beef muscle sample, and the time is quite shorter than the bioassay and combination analysis of individual chemical analysis. We consider that the present method is suitable for the determination of the penicillins for a positive sample by bioassay screening.

4. Conclusions

A high-performance ion-pair chromatographic method with UV detection for the separation and determination of PCG, PCV, MPIPC, MCIPC, NFPC and MDIPC in meat in a single assay was developed.

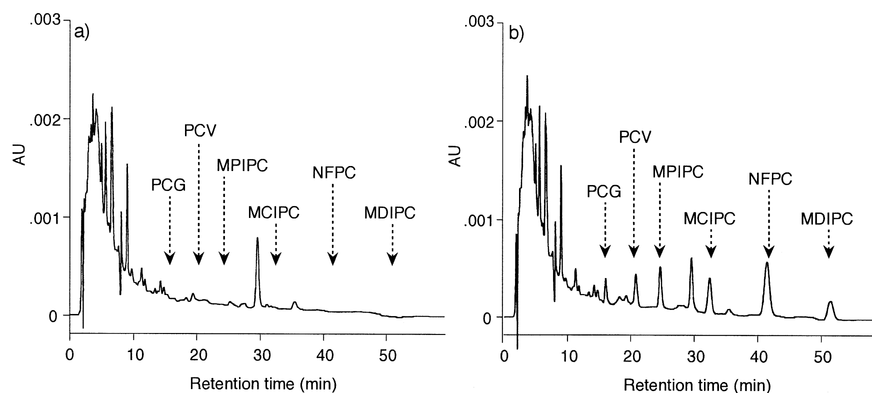

Fig. 4. Typical HPLC chromatograms of beef muscle samples. (a) Beef muscle (control); (b) added at a level of 0.1 mg/kg each of penicillins.

The obtained results indicate that the combination of an ion-exchange cartridge clean-up following the C18 cartridge clean-up, and an ion-pair HPLC can provide sufficient sensitivity and quantitativity for the measurement of these penicillins. We, therefore, recommend the present analytical method for the simultaneous determination of PCG, PCV, MPIPC, MCIPC, NFPC and MDIPC in meat.

Acknowledgements

We thank Dr. Y. Miyazaki, Director of Aichi Prefectural Institute of Public Health, for his encouragement.

References

[1] E. Verdon, P. Couedor, J. Chromatogr. B 705 (1998) 71.
[2] B. Perez, C. Prats, E. Castells, M. Arboix, J. Chromatogr. B 698 (1997) 155.
[3] W. Luo, E.B. Hansen Jr., C.Y.W. Ang, J. Deck, J.P. Freeman, H.C. Thompson Jr., J. Agric. Food Chem. 45 (1997) 1264.
[4] C.Y.W. Ang, W. Luo, J. Assoc. Off. Anal. Chem. 80 (1997) 25.
[5] L.K. Sorensen, B.M. Rasmussen, J.O. Boison, L. Keng, J. Chromatogr. B 694 (1997) 383.
[6] W.A. Moats, R.H. Khan, J. Assoc. Off. Anal. Chem. 78 (1995) 49.
[7] K.L. Tyczkowska, R.D. Voyksner, R.F. Straub, A.L. Aronson, J. Assoc. Off. Anal. Chem. 77 (1994) 1122.
[8] H.E. Gee, K.B. Ho, J. Toothill, J. Assoc. Off. Anal. Chem. 79 (1996) 640.
[9] S. Lihl, A. Rehorek, M. Petz, J. Chromatogr. A 729 (1996) 229.
[10] W.J. Blanchflower, S.A. Hewitt, D.G. Kennedy, Analyst 119 (1994) 2595.
[11] N. Snippe, N.C. van de Merbel, F.P.M. Ruiter, O.M. Steijger, H. Lingeman, U.A.Th. Brinkman, J. Chromatogr. B 662 (1994) 61.
[12] W.A. Moats, J. Chromatogr. 593 (1992) 15.
[13] W.A. Moats, J. Chromatogr. 317 (1984) 311.
[14] Y. Ito, Y. Ikai, H. Oka, J. Hayakawa, T. Kagami, J. Chromatogr. A 810 (1998) 81.
[15] H. Oka, Y. Ikai, N. Kawamura, K. Uno, M. Yamada, J. Chromatogr. 389 (1987) 417.
[16] H. Oka, Y. Ikai, N. Kawamura, K. Uno, M. Yamada, J. Chromatogr. 400 (1987) 253.
[17] Y. Ikai, H. Oka, N. Kawamura, M. Yamada, J. Chromatogr. 477 (1989) 397.
[18] Y. Ikai, H. Oka, N. Kawamura, J. Hayakawa, M. Yamada, J. Chromatogr. 541 (1991) 393.
[19] H. Terada, M. Asanoma, Y. Sakabe, J. Chromatogr. 318 (1985) 299.