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Chiral separation of homocysteine by derivatization with 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole followed by capillary electrophoresis using γ-cyclodextrin

Ick-Joong Kim, Soo-Jin Park, Hie-Joon Kim*

School of Chemistry & Molecular Engineering, Seoul National University, Seoul 151-742, South Korea

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Abstract

Homocysteine was derivatized with 4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole (ABD-F) to form an inclusion complex with cyclodextrin and to facilitate UV detection. ABD-homocysteine showed interaction with β- and γ-cyclodextrin in capillary electrophoresis at pH 2.25 as indicated by the decreased migration time. However, chiral separation of D,L-ABD-homocysteine was observed using γ-CD only. Optimal separation was obtained at pH 2.25, 50 m*M* γ-CD concentration, and 20 kV applied voltage. L-ABD-Homocysteine migrated faster than the D-isomer as demonstrated by a spiking experiment using dithiothreitol-reduced L-homocystine.

Keywords: Enantiomer separation; Derivatization, electrophoresis; Homocysteine; Aminosulfonylfluorobenzoxadiazole; Cyclodextrins; Amino acids

1. Introduction

Homocysteine is a known risk factor for atherothrombotic disease [1,2] and, therefore, total plasma homocysteine level is often monitored. Several high-performance liquid chromatographic (HPLC) methods for homocysteine have been reported since 1987 [3]. Recently, Fermo et al. reported determination of total plasma homocysteine levels by HPLC after derivatization with SBD-F (ammonium-7-fluorobenzo -2-oxa-1,3-diazole-4-sulfonate) [4].

Capillary electrophoresis (CE) has also been used for homocysteine analysis. Kang et al. determined homocysteine and other thiols in human plasma by CE after derivatization with ABD-F (4-aminosulfonyl-7-fluoro-2,1,3-benzoxadiazole) [5]. Gotti et al. derivatized cysteine, homocysteine, penicillamine, and reduced glutathione by ESB {1,1′-[ethenylidenebis-(sulfonyl)]bis-benzene} and separated the derivatized compounds by CE [6]. Vecchione et al. [7] and Causse et al. [8] reported determination of homocysteine by high-performance CE with laser-induced fluorescence detection. However, the chiral separation of D,L-homocysteine has not been reported. Plasma homocysteine level is increased after oral administration of L-methionine [4]. Therefore, one might suspect that L-homocysteine is probably involved in adverse reactions in atherothrombosis, and it would be desirable to determine L-homocysteine separately from D-homocysteine.

*Corresponding author.

E-mail address: hjkim1@snu.ac.kr (H.-J. Kim)

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Direct chiral separation of thiol compounds, such as cysteine, homocysteine, and reduced glutathione is difficult, because they have limited interaction with such chiral selectors as cyclodextrin (CD). Easy oxidation to disulfide and lack of chromophore for detection pose additional difficulties.

In the past, derivatization of the sulfhydryl group using ESB, ABD-F and SBD-F has been utilized to facilitate separation of thiols and their detection in HPLC and CE [9–14]. It appeared to us that derivatization of homocysteine by ABD-F might open up a possibility of chiral separation using CD as chiral selector in CE. The nonpolar ABD portion might fit the cavity of CD and make an inclusion complex. The chiral center would be near the opening of the cavity where enantiospecific interactions could occur with the hydroxyl groups of the CD through hydrogen bonding. Of course, the ABD moiety would make UV detection possible. In this paper, we demonstrate that chiral separation of ABD-F derivatized homocysteine can be achieved by CE using γ-CD as chiral selector.

2. Experimental

2.1. Materials

D,L-Homocysteine, L-homocystine, dithiothreitol (DTT), and α-, β-, γ-CD were purchased from Sigma (St. Louis, MO, USA). ABD-F was from Dojindo Labs. (Kumamoto, Japan). Other chemicals were reagent grade. HPLC-grade methanol and acetonitrile were used in the eluent. Distilled water was prepared using a NANOpure II system (Barnstead, Dubuque, IA, USA).

2.2. Reduction of L-homocystine

A 0.5-ml aliquot of 1 m*M* DTT solution in deionized water was mixed with an equal volume of 0.1 m*M* solution of L-homocystine in 0.1 *M* borate buffer, pH 8, with 2 m*M* EDTA. Reduction was carried out for 60 min at 50°C.

2.3. Derivatization procedure

Homocysteine was derivatized with ABD-F according to the method of Toyo'oka and Imai [15]. A 0.2 m*M* solution of D,L-homocysteine was prepared in 0.1 *M* borate buffer (pH 8) containing 2 m*M* EDTA. A 1 m*M* ABD-F solution was also prepared in 0.1 *M* borate buffer. Aliquots (0.5 ml) of the two solutions were mixed in a 1.5-ml Eppendorf vial and the vial was heated at 50°C for 10 min using a heating block. The reaction was terminated by immersing the vial in ice water and adding 0.25 ml of 0.1 *M* HCl. The vial was wrapped in aluminum foil to protect from light and stored in a refrigerator. L-Homocysteine resulting from reduction of L-homocystine by DTT was derivatized similarly.

2.4. Capillary electrophoresis

A Beckman P/ACE 5000 series CE system consisting of an automatic injector, a fluid-cooled column cartridge, and a filter-type UV detector module (Beckman Instruments, Fullerton, CA, USA) was used. A fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) of 57 cm×50 μm I.D. was used. Sample was injected hydrodynamically for 10 s at 3.5 kPa. The temperature of the capillary was maintained at 15°C. Detection was at 50 cm from the inlet. UV detection at 220 nm was used.

Before every injection, the capillary was washed for 3 min with 0.1 *M* NaOH solution, for 2 min with distilled water, and for 2 min with the running buffer. Chiral selector was dissolved directly in 50 m*M* sodium phosphate running buffer at different pH, and the running buffer was filtered through a 0.45-μm membrane filter. Applied voltage was either 20 or 25 kV.

2.5. HPLC

A Hewlett-Packard 1100 Series HPLC system consisting of a quartenary pump, an on-line vacuum degasser, a Rheodyne 7725i injector with a 20-μl loop, and a diode array detector was used. A Nova-Pak C_{18} column (150×3.9 mm; Waters, Milford, MA, USA) was used to monitor the derivatization reaction. The eluent was 20% acetonitrile and 0.1% trifluoroacetic acid in water. Flow-rate was 1 ml/min. UV detection at 254 nm was used.

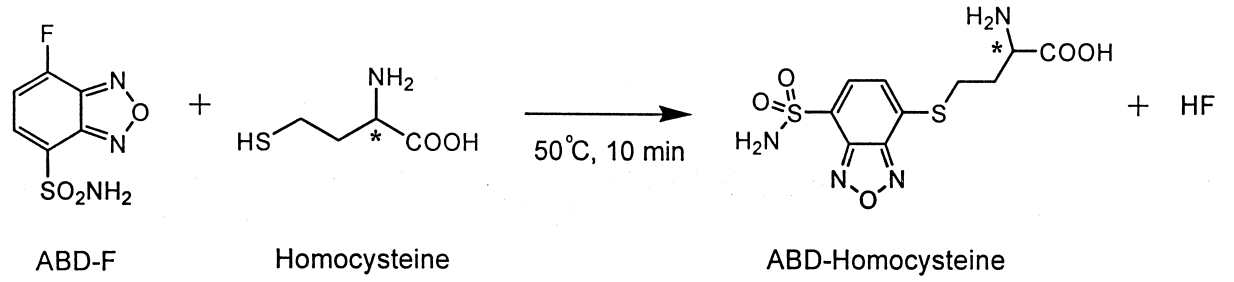

Fig. 1. Derivatization of homocysteine with ABD-F.

3. Results and discussion

3.1. Derivatization reaction

In the derivatization reaction, the nucleophilic thiolate substitutes for the fluorine in ABD-F (Fig. 1). Progress of the derivatization reaction was monitored by HPLC. As shown in Fig. 2, the chromatogram at 254 nm showed only one peak at 3.6 min in addition to the EDTA peak at 1.2 min and the ABD-F peak at 7.1 min. The UV absorption maximum of ABD-F is shifted from 320 nm to 380 nm upon reaction with homocysteine (Fig. 2, inset) as reported previously [16].

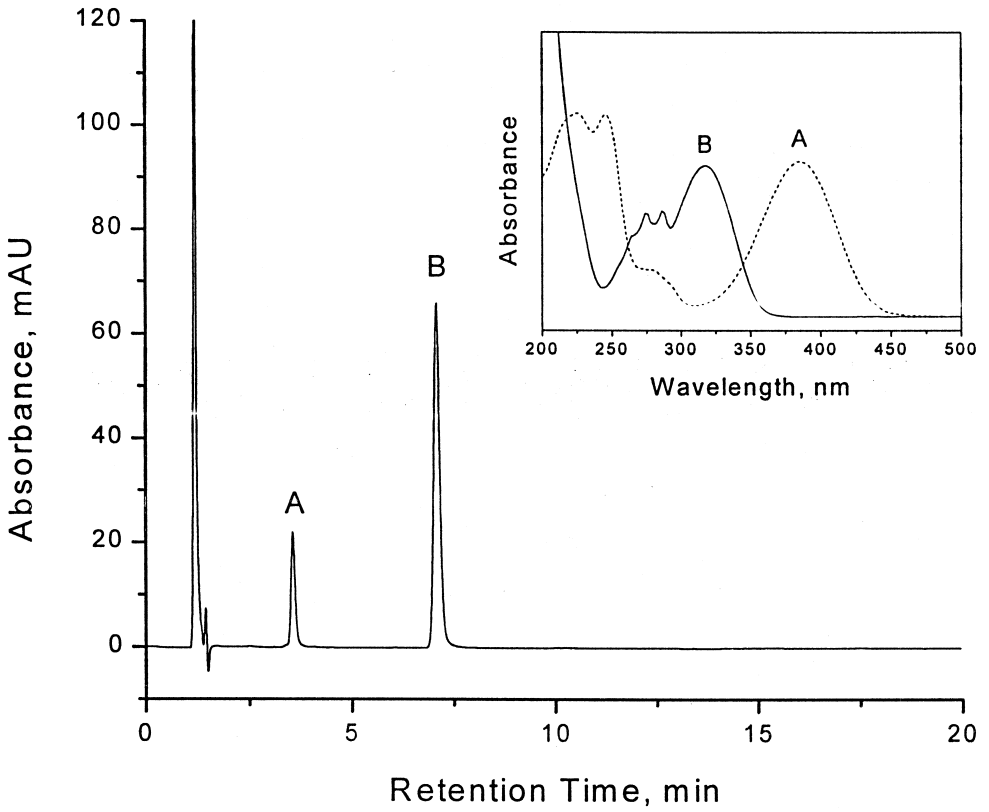

Fig. 2. Chromatogram showing separation of ABD-homocysteine (A) from ABD-F (B) and their UV spectra.

3.2. Choice of chiral selector

Chiral separation using CD is achieved through formation of an inclusion complex. The size of the nonpolar cavity of CD is critical for formation of the inclusion complex. To determine whether α-, β- or γ-CD forms inclusion complex most effectively with ABD-homocysteine, delay in migration time upon addition of CD was monitored while carrying out CE at pH 2.25. At low pH, ABD-homocysteine carries positive charge and will have electrophoretic mobility toward the negative electrode in addition to the electroosmotic flow (EOF) in the same direction. When neutral CD is added to the buffer, migration of ABD-homocysteine will be slowed down if it forms an inclusion complex with CD, which is neutral and migrates with the EOF.

In the absence of CD, the migration time of ABD-homocysteine was 22 min under the experimental conditions. When 20 m*M* α-CD was added, no change in the migration time was observed. Therefore, it appeared that ABD-homocysteine does not interact significantly with α-CD. When 20 m*M* β-CD was added, the migration time was increased to 27 min suggesting that ABD-homocysteine forms an inclusion complex with β-CD. However, no chiral separation was observed. Interestingly, the migration time was 26 min with 20 m*M* γ-CD and chiral separation was observed with a resolution of 0.80. A plausible explanation is that ABD-homocysteine interacts a little less strongly with γ-CD, yet the two enantiomers of ABD-homocysteine forms inclusion complex with γ-CD with slightly different free energy. Anyhow, it was clear that γ-CD is the best choice among the three CDs commercially available.

3.3. Influence of pH

Since CD is neutral and migrates with EOF, it is desirable to reduce the EOF and allow sufficient time for the analyte to interact with the chiral selector, CD. In CE low EOF is obtained at low pH. On the other hand, homocysteine is expected to carry positive charge around pH 2 and have substantial electrophoretic mobility toward the negative electrode, because the p*K* values for carboxyl and amino groups in homocysteine are 2.22 and 10.86, respectively [17], and the ABD moiety has an additional amino group. As a result, the analyte will migrate faster than the EOF in normal polarity, where EOF is from the positive to the negative electrode. Therefore, one needs to find optimal pH under a generally acidic condition. When the applied voltage was 25 kV and the γ-CD concentration 30 m*M*, the highest resolution of 1.06 was observed at pH 2.5. However, the migration time was too long (56 min) due to the low electrophoretic mobility of ABD-homocysteine. Migration time was decreased to 29 min at pH 2.0; however, resolution was sacrificed (R_s=0.93). We feel that a reasonable compromise in migration time and resolution is achieved at pH 2.25.

3.4. Optimum γ-CD concentration

Higher concentration of chiral selector is expected to improve chiral selectivity. Guttman et al. showed that chiral selectivity (relative retention) of dansylated leucine is increased from 1.05 to 1.15 as the concentration of γ-CD is increased from 10 to 100 m*M* in high-performance capillary gel electrophoresis [18]. However, chiral selectors are expensive and it is desirable to use lowest concentration compatible with a reasonably good chiral separation.

At pH 2.25 and 25 kV applied voltage D,L-ABD-homocysteine were barely resolved (R_s=0.68) with 10 m*M* γ-CD. Resolution improved as the γ-CD concentration was increased to 30 and 50 m*M*. Baseline separation was obtained with 80 m*M* γ-CD. However, the improvement in resolution between 50 m*M* (R_s=1.26) and 80 m*M* (R_s=1.29) was minimal, and we chose 50 m*M* as optimum CD concentration. Dependence of resolution on pH and the γ-CD concentration is summarized in Table 1.

3.5. Effect of applied voltage

Since higher resolution should be achieved when more time is available for diffusion-controlled interaction between the analyte and the chiral selector, use of lower voltage, namely 20 kV instead of 25 kV, was expected to improve resolution [19], Actually, migration time was increased and baseline separation was obtained at 20 kV using 50 m*M* γ-CD. Fig. 3 shows optimized chiral separation conditions, namely, pH 2.25, 50 m*M* γ-CD concentration, and 20 kV

Table 1
Dependence of migration time and resolution on pH and γ-CD concentration

	Migration time (min)	Resolution
30 m*M* γ-CD:		
pH		
2.00	28.4	0.93
2.25	35.2	1.01
2.50	55.3	1.06
pH 2.25:		
γ-CD (m*M*)		
10	31.1	0.68
30	36.1	1.08
50	41.4	1.26
80	49.4	1.29

applied voltage. Fig. 3 also shows that ABD-homocysteine migrates faster in the absence of γ-CD due to the combined effect of EOF and electrophoretic mobility. When neutral γ-CD is added, it slows down ABD-homocysteine. Clearly, both D- and L-ABD-homocysteine interact significantly with γ-CD. Yet, a small difference in interaction energy causes one enantiomer to migrate faster than the other.

3.6. Order of migration

When the mixture of D- and L-ABD-homocysteine was spiked with L-ABD-homocysteine, obtained by reducing L-homocystine with DTT and derivatizing the resulting L-homocysteine, the signal intensity of the earlier peak was enhanced as shown in Fig. 4. Clearly, D-ABD-homocysteine interacts more strongly with γ-CD and migrates more slowly than L-ABD-homocysteine. The stronger interaction between D-ABD-homocysteine and γ-CD was confirmed by chiral HPLC. D-ABD-Homocysteine was retained longer than L-ABD-homocysteine from a silica gel column derivatized with γ-CD [20].

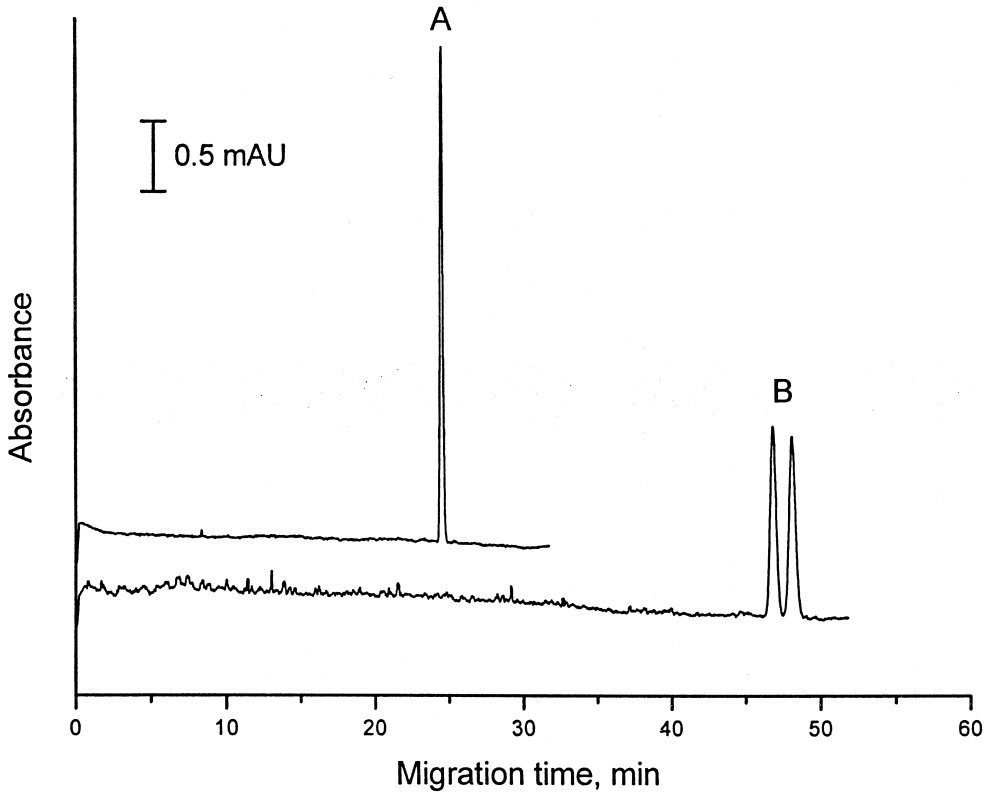

Fig. 3. Chiral separation of D,L-ABD-homocysteine (A) without γ-CD, (B) 50 m*M* γ-CD. Capillary: fused-silica, 57 cm×50 μm I.D. UV detection at 220 nm. Running buffer: 50 m*M* sodium phosphate, pH 2.25. Applied voltage, 20 kV; current 30 μA for (A) and 25 μA for (B).

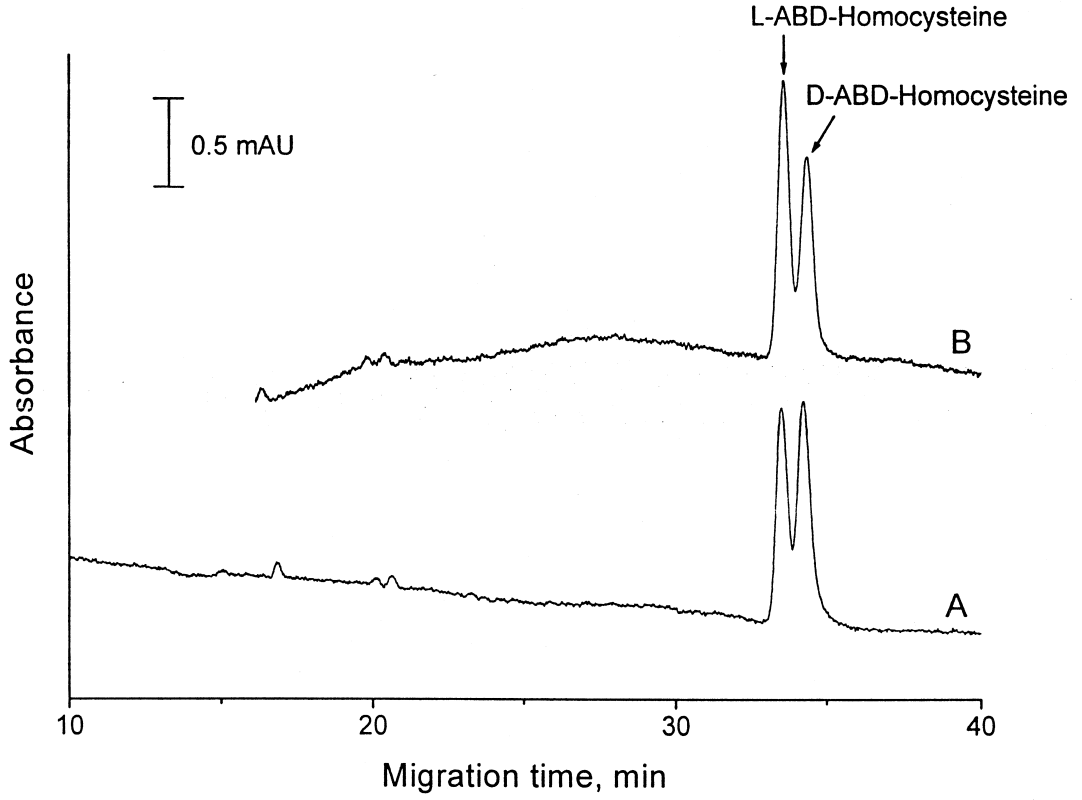

Fig. 4. Enhancement of the earlier peak in a spiking experiment using L-ABD-homocysteine obtained from L-homocystine by DTT reduction followed by ABD-F derivatization. CE conditions as in Fig. 3 except 20 m*M* γ-CD and 25 kV applied voltage.

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References

[1] G.N. Welch, J. Loscalzo, New Engl. J. Med. 338 (1998) 1042.
[2] P.M. Ridker, J.E. Manson, J.E. Buring, J. Shih, M. Matias, C.H. Hennekens, J. Am. Med. Assoc. 281 (19) (1999) 1817.
[3] A. Araki, Y. Sako, J. Chromatogr. 422 (1987) 43.
[4] I. Fermo, C. Arcelloni, G. Mazzola, A. D'Angelo, R. Paroni, J. Chromatogr. B 719 (1998) 31.
[5] S.H. Kang, J.-W. Kim, D.S. Chung, J. Pharm. Biomed. Anal. 15 (1997) 1435.
[6] R. Gotti, R. Pomponio, V. Andrisano, V. Cavrini, J. Chromatogr. A 844 (1999) 361.
[7] G. Vecchione, M. Margaglione, E. Grandone, D. Colaizzo, G. Cappucci, I. Fermo, A. D'Angelo, G. Di Minno, Electrophoresis 20 (1999) 569.
[8] E. Causse, N. Siri, H. Bellet, S. Champagne, C. Bayle, P. Valdiguie, R. Salvayre, F. Couderc, Clin. Chem. 45 (1999) 412.
[9] V. Cavrini, R. Gotti, V. Andrisano, R. Gatti, Chromatographia 42 (1996) 515.
[10] J. Russell, D.L. Rabenstein, Anal. Biochem. 242 (1996) 136.
[11] R.A. Winters, J. Zukowski, N. Ercal, R.H. Mathews, D.R. Spitz, Anal. Biochem. 227 (1995) 14.
[12] D. Tsikas, J. Sandmann, M. Ikic, J. Fauler, D.O. Stichtenoth, J.C. Frolich, J. Chromatogr. B 708 (1998) 55.
[13] A. Hidayat, D.B. Hibbert, P.W. Alexander, J. Chromatogr. B 693 (1997) 139.
[14] G. Favaro, M. Fiorani, Anal. Chim. Acta 332 (1996) 249.

[15] T. Toyo'oka, K. Imai, Anal. Chem. 56 (1984) 2461.
[16] S.H. Kang, Ph.D. Thesis, Seoul National University, Seoul, 1998.
[17] S. Budavari (Ed.), The Merck Index, 11th ed., Merck, Rahway, NJ, 1989, p. 748.
[18] A. Guttman, A. Paulus, A.S. Cohen, N. Grinberg, B.L. Karger, J. Chromatogr. 448 (1988) 41.
[19] M.W.F. Nielen, Anal. Chem. 65 (1993) 885.
[20] I.-J. Kim, S.-J. Park. H.-J. Kim, unpublished results.