Journal of Chromatography, 457 (1988) 299–307
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 20 925

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF ALIPHATIC ALDEHYDES BY MEANS OF POST-COLUMN EXTRACTION WITH FLUOROMETRIC DETECTION

HITOSHI KOIZUMI* and YOSHIHITO SUZUKI
Department of Basic Engineering, Faculty of Engineering, Yamanashi University, Takeda 4, Kofu, Yamanashi 400 (Japan)
(First received April 29th, 1988; revised manuscript received August 3rd, 1988)

SUMMARY

7-Hydrazino-4-nitrobenzo-2,1,3-oxadiazole (NBD hydrazine) was synthesized from a reaction of NBD chloride with hydrazine. NBD hydrazones were formed from aldehydes and ketones but only the aldehyde derivatives were fluorescent in an antiprotonic solvent. An on-line post-column extraction system suitable for fluorometric detection of aldehyde-NBD hydrazones in reversed-phase high-performance liquid chromatography is presented. The system consists of a segmentor, a PTFE extraction coil and a PTFE membrane phase separator. The determination of aldehydes in whisky is described.

INTRODUCTION

In many foods, aliphatic aldehydes occur which smell of oxidative rancidity[1] over prolonged storage. Since long chain aldehydes are produced as degradation products of many kinds of polymers and the raw materials for polymer syntheses, it is required to determine trace amounts of aldehydes in order to evaluate the extent of deterioration. The presence of carbonyl compounds in the environment arises also from industrial pollution and from automobile exhausts. Carbonyl compounds, aldehydes in particular, are analyzed in order to estimate environmental pollutions in air and waste water. Since, in many cases, all kinds of carbonyl compounds exist in samples, it is necessary to separate a homologous series and determine them.

Several derivatization reactions have been described for liquid chromatographic (LC) determination of trace amounts of carbonyl compounds. Derivatization with 2,4-dinitrophenyl (DNP) hydrazine is usually employed for the determination of carbonyl compounds in automobile exhaust gases[2,3] and in rain-water[4] since increased sensitivity is achieved by the enhanced molar absorptivity of the derivatized compounds with respect to the starting carbonyl compounds. Recently, dabsylhydrazine was synthesized from the reaction of dabsyl chloride with hydrazine. This newly developed chromophoric reagent has been demonstrated to be very promising for the determination of aliphatic aldehydes with resonance Raman detection[5] and for that

0021-9673/88/$03.50

of monosaccharides[6]. For highly sensitive detection, dansylhydrazine[7] as a fluorescent reagent has been employed. Hydrazine type reagents react readily with carbonyl compounds. Derivatizations by the Hatzsch reaction for fluorometric detection have been reported: reaction with ethyl acetoacetate[8], acetylacetone[9] and cyclohexane-1,3-dione[10,11]. These derivatization reactions are specific with respect to aldehyde. In particular, the reaction product of methanal with ethyl acetoacetate in the presence of ammonium acetate has a very intensive fluorescence.

In this work, 7-hydrazino-4-nitrobenzo-2,1,3-oxadiazole (NBD hydrazine) was synthesized from the reaction of NBD-Cl with hydrazine. Both aldehydes and ketones were treated with NBD hydrazine[12] and converted into hydrazones, but only the aldehyde derivatives was fluorescent. The aldehyde derivatives give a light green fluorescense, only when present in an anti-protonic solvent such as carbon tetrachloride, chloroform and ethyl acetate, not in a protonic solvent. As described above, NBD hydrazones would promote a selective fluorometric detection in high-performance liquid chromatography (HPLC). However, when NBD hydrazones are separated in reversed-phase HPLC, fluorometric detection cannot be applied because of the lack of fluorescence in aqueous solvents.

As in conventional HPLC, detection systems can be improved by making use of on-line post-column detection techniques. The use of on-line liquid–liquid extraction techniques for flow injection analysis[13,14] and in post-column systems for HPLC[15–18] has been reported. The extraction is performed by adding an immiscible organic solvent such as chloroform or benzene to the aqueous LC effluent, thereby producing a stream of extremely small aqueous and organic solvent segments. A approach to phase separation has been employed by exploiting semipermeable membranes. In general, the design of the phase separator consists of a manifold through which a segmented flow comes into contact with a hydrophobic membrane. The aqueous solvent cannot pass through the membrane, while the organic one can.

In the present paper, we report the use of a membrane phase separator suitable for selective fluorometric detection of NBD hydrazones in a reversed-phase HPLC system with post-column extraction.

EXPERIMENTAL

Chemicals

The aliphatic aldehydes methanal, ethanal, propanal, butanal, pentanal, 2-methylbutanal and aliphatic ketones acetone and 2-butanone were used as obtained without further purification. NBD-Cl and hydrazine monohydrate were obtained from Tokyo Kasei (Tokyo, Japan). Potassium dihydrogenphosphate and sodium dihydrogenphosphate were obtained from Kanto (Tokyo, Japan). All solvents were HPLC grade. Acetonitrile, chloroform and carbon tetrachloride were obtained from Nakarai (Kyoto, Japan). The distilled water was purified in-house using a Milli-Q II system (Nippon Millipore, Tokyo). Purified water was used in all experiments.

Apparatus

Fig. 1 shows a schematic diagram of the post-column extraction chromatographic system. The mobile phase was delivered with a Tri Rotar V pump (JASCO, Tokyo, Japan) and samples were injected by a Rheodyne Model 7125 equipped with a

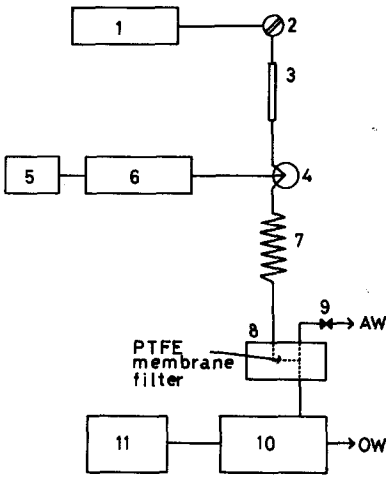

Fig. 1. Schematic diagram of the post-column extraction system: 1 = mobile phase pump; 2 = loop injector; 3 = analytical column (Develosil C_8, 5 μm, 150 mm × 4.6 mm I.D.); 4 = segmentor; 5 = degasser; 6 = segmenting liquid pump; 7 = extraction coil (1 m × 0.5 mm I.D. PTFE); 8 = membrane phase separator; 9 = needle valve; 10 = fluorescence detector; 11 = recorder; AW = aqueous waste; OW = organic waste.

50-μl loop. The column was packed with 5-μm Develosil C_8 (Nomura, Japan) using a slurry packing technique. A JASCO FP-110 spectrofluorometer equipped with a xenon lamp (output 75 W) was used. The detector, equipped a 17-μl flow cell, was operated with the excitation monochromator set at 470 nm and the emission monochromator set at 530 nm. The chromatograms were recorded with a SIC chromatocorder 11 (System Instrument, Tokyo, Japan). For the post-column extraction system, a JASCO Tri Rotar V pump which delivers flows in the range 0.3–1.0 ml/min, previously degassed on-line with a Shodex degas (Showa Denko, Tokyo). The column effluent and the segmenting liquid were mixed with a mixing joint (PTFE: Chromato Research, Sagamihara, Japan) in which three 1/16 in. O.D. PTFE tubings were jointed at angles of 60°. The extraction coil consisted of 100 cm × 0.5 mm I.D. PTFE tubing.

Several types of phase separator have ben reported[13,16–21]. The design of the phase separator used in this work[14] was a modification of that of Apffel *et al.*[16] who designed a miniaturized post-column extraction system. It consisted of two stainless-steel blocks, each of which has a semicylindrical groove of volume approximately 10 μl (2 mm width × 10 mm length × 0.5 mm depth) drilled into it. The membranes were Fluoropore type FG PTFE filters (Nippon Millipore) which have 0.2-μm pores and were bonded to a PTFE net facing the segmented liquid inlet. With a needle valve (Type BMCV; Gasukuro Kogyo, Tokyo) connected to the aqueous outlet, and applying back pressure, the amount of organic phase passing through the membrane filter can be regulated. In this work, the flow through the membrane filter was adjusted to 80% by a needle valve.

Synthesis of NBD hydrazine

Hydrazine monohydrate (0.3 ml) was dissolved in a mixture of 5 ml tetrahydrofuran and 5 ml of disodium hydrogenphosphate (1/15 *M*). NBD-Cl (400 mg, 2

m*M*) dissolved in 50 ml acetonitrile was added dropwise with stirring to the hydrazine solution at 40°C for 30 min, then for 60 min and finally cooled to room temperature. The reaction solution was filtered, and to the filtrate was added enough acetonitrile to give 100 ml. This reagent solution contains NBD hydrazine and has been stored for 2 weeks in the dark at −20°C without loss of potency.

Preparation of standard carbonyl-NBD hydrazone

A 1.0-ml volume of an aqueous solution containing 45 ppm of each carbonyl compound was mixed with 1.0 ml of NBD hydrazine solution and one drop of hydrochloric acid. The mixture was heated at 40°C for 20 min, and then cooled to room temperature. The carbonyl compounds were converted into NBD hydrazones. Aliquots (5μl) of the reaction mixture were injected directly for HPLC.

Determination procedure for HPLC

A 1-ml volume of an aqueous solution of aliphatic aldehyde containing 10 μg of 2-methylbutanal as an internal standard was placed in a 5-ml vial, followed by 1 ml NBD hydrazine solution and one drop of hydrochloric acid. The vial was sealed with a PTFE-lined screwcap and placed in a water-bath. The yields of the derivatization were examined at reaction temperatures of 40, 50 and 60 °C and reaction times of 10, 20 and 30 min. A 10-μl volume of reaction mixture was injected for HPLC. No difference in peak height or peak area obtained from chromatograms were detected under the above reaction conditions. The derivatization reaction at 40°C was complete after 10 min. Since a very small amount of NBD hydrazine was extracted into an organic solvent and fluoresced, fluorometric detection of NBD hydrazones was subject to interference by the excess of reagent. So the vial contents were mixed with 20 μl of 2-butanone in order to destroy the excess of NBD hydrazine. The vial was returned to the water-bath (40°C) for 10 min. According to this procedure, the excess of NBD hydrazine was converted into the hydrazone of the ketone which did not fluoresce. A 10-μl aliquot of the reaction mixture was injected for HPLC with post-column extraction.

Determination of aldehydes in whisky

To a 10-ml volume of a commercial whisky were added 1 ml of 2-methylbutanal (200 μg/ml) as an internal standard (IS) and enough water to give 20 ml. A 1.0-ml aliquot of the diluted solution was derivatized with NBD hydrazine as described above. A blank experiment was also performed with water–carbonyl free ethanol (70:30, v/v) containing an internal standard. A 10-μl aliquot of the reaction mixture was injected for HPLC.

RESULTS AND DISCUSSION

HPLC separation of NBD hydrazone

Isocratic elution conditions were investigated by using acetonitrile–water mixtures with the post-column extraction system (0.6 ml/min carbon tetrachloride as an organic solvent) as shown in Fig. 1. NBD hydrazones were not separated successfully because of weak interactions with the stationary phase. So we investigated the retention behaviour of NBD hydrazones using acetonitrile–phosphate buffers (Michaelis

buffer, potassium dihydrogenphosphate–disodium hydrogenphosphate, 1/30 *M*, pH 7.0) in volume ratios ranging between 20 and 50% acetonitrile, at a flow-rate of 1.0 ml/min.

Stable segments were not produced with mobile phases containing above 50% acetonitrile in water. As a result, the retention time of each NBD hydrazone increased with decreasing acetonitrile content. However, a complete separation of methanal and ethanal was not achieved at 20% acetonitrile. On the other hand, when the pH value of the phosphate buffer solution was varied in the range between 6.4 and 7.7, the retention time decreased with increasing pH, and was constant above pH 7.0. Good separation of NBD hydrazones was obtained by using acetonitrile–phosphate buffer pH 7.0 (22:5:77.5, v/v) on a Develosil C_8 column.

Effect of extraction coil

In order to examine the extraction efficiency, the length and inside diameter of the extraction coils were studied with the post-column extraction system. The helical coiling of the tubings (coiling diameter 20 mm) was used in order to reduce band broadening by introducing secondary flow phenomena. The measurements of the total band broadening of the HPLC system with post-column extraction were based on peak widths at half height and at 10% peak height.

At a mobile phase flow-rate of 1.0 ml/min a flow-rate of extraction solvent of 0.6 ml/min the maximum peak heights and minimum band broadenings were obtained when the tubing was 100 cm × 0.5 mm I.D. as shown in Table I. This tubing was therefore routinely used. It has a volume of approximately 196 μl and yields an extraction time of 7.4 s.

Effect of extraction solvent

For extractions in flowing systems[22], the detection sensitivity increases when the ratio of the flow-rate of the organic (extractant) phase to that of the aqueous phase decreases. Also band broadening increases with decreasing flow-rate of the organic solvent at a constant flow-rate of LC effluent.

In the present system, the flow-rate of carbon tetrachloride as the extraction solvent was varied from 0.3 to 1.0 ml/min, while the LC effluent flow-rate was kept constant at 1.0 ml/min. Eighty percent of the extraction solvent at each flow-rate was

TABLE I

EFFECT OF EXTRACTION COIL ON DETECTION

Develosil C_8 column: acetonitrile–phosphate buffer (22.5:77.5) mobile phase; flow-rate 1.0 ml/min; extraction solvent, carbon tetrachloride 0.6 ml/min; sample, propanal-NBD hydrazone. Number of experiments: 5.

Coil dimension (cm × mm I.D.)	*Peak height*	*Half width*	*Width at 10% peak height*
50 × 0.8	79 ± 0.5	7.0 ± 0.0	14.9 ± 0.1
100 × 0.8	78 ± 0.8	7.0 ± 0.1	15.0 ± 0.1
50 × 0.5	77 ± 0.6	7.5 ± 0.0	14.5 ± 0.0
100 × 0.5	85 ± 0.8	6.6 ± 0.0	14.0 ± 0.0

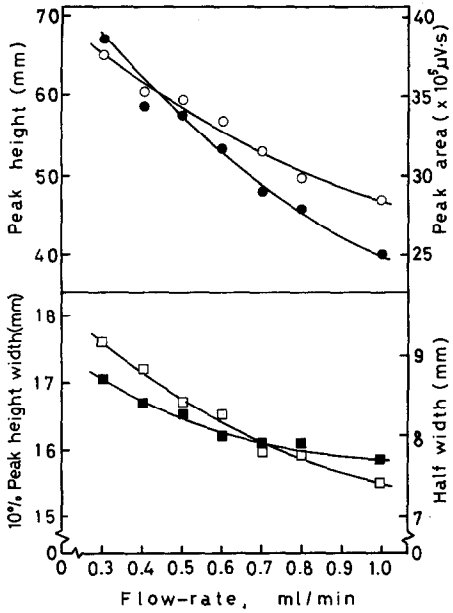

Fig. 2. Effect of flow-rate on extraction efficiency. Extraction solvent; carbon tetrachloride. Sample: propanal-NBD hydrazone. ○ = Peak height; ● = peak area; □ = width at 10% peak height; ■ = half width. Number of experiments: 5. Average values plotted, R.S.D. within 3.2%.

led through the detector by adjustment of the back pressure regulator. The extraction efficiency was investigated by measuring not only the peak height and peak area for detection sensitivity but also the width at 10% peak height and the half width for band broadening as a function of the flow-rate of the extraction solvent. The results are shown in Fig. 2.

As shown in Fig. 2, band broadening at 10% peak height increases clearly with decreasing flow-rate of the organic solvent. On the other hand, both the peak height and peak area decrease because the concentration of NBD hydrazone in the extraction solvent will be lower. This indicates that the lower the flow-rate of extraction solvent, the higher is the detection sensitivity. In practice, however, unstable segments were frequently produced in the extraction coil at a flow-rate of 0.4 ml/min, often followed by a breakthrough of the aqueous phase to the fluorometric detector. In view of these results, the preferred flow-rate of the extraction solvent 0.5 ml/min, seems to be a good compromise.

One further contrast between the use of carbon tetrachloride and chloroform as extraction solvent, was examined. In the case of carbon tetrachloride, the extraction efficiency increased for NBD hydrazones of aliphatic aldehydes with longer alkyl chains. With chloroform, the extraction efficiency of aliphatic aldehydes having short alkyl chain were higher, and the detection sensitivity as a whole was about three times better than that with carbon tetrachloride. The extraction efficiency of NBD hydrazones was influenced by the extraction solvent, therefore we have examined the extraction efficiency by varying the contents of each extraction solvent (chloroform–carbon tetrachloride). Fig. 3 shows the percentage of the relative peak area of each

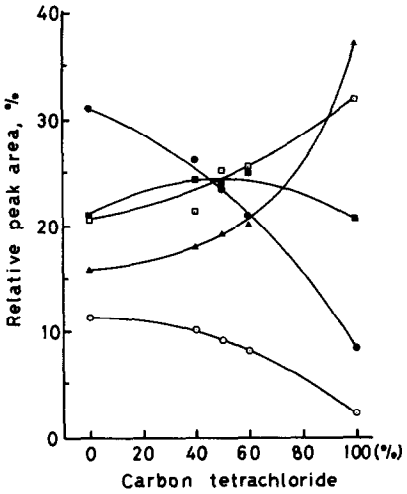

Fig. 3. Effect of the extraction solvent composition (carbon tetrachloride–chloroform) on the peak area. ○ = Methanal; ● = ethanal; ■ = propanal; □ = butanal; ▲ = pentanal.

NBD hydrazone as a function of the carbon tetrachloride contents in chloroform.

The results show that the extraction efficiency of propanal-NBD hydrazone was little affected by the composition of the extraction solvent, but those of methanal and ethanal decreased with increasing carbon tetrachloride content and those of butanal and pentanal increased. Consequently, chloroform–carbon tetrachloride (50:50, v/v) was used as the extraction solvent in order to reduce the differencies between the extraction efficiency of each NBD hydrazone. A typical chromatogram obtained under the optimum conditions is shown in Fig. 4.

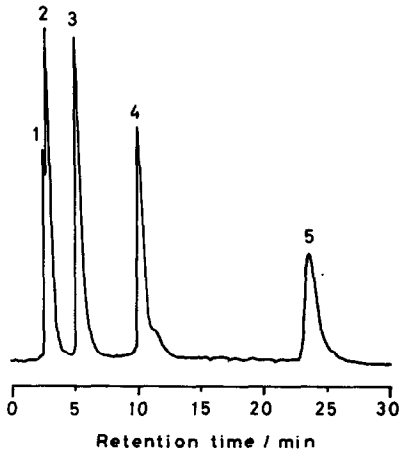

Fig. 4. Typical chromatogram of NBD hydrazones obtained under optimum conditions. Column: Develosil C_8 (5 μm, 150 mm × 4.6 mm I.D.). Mobile phase: acetonitrile–phosphate buffer pH 7.0 (22.5:77.5, v/v). Flow-rate: 1.0 ml/min. Extraction solvent: carbon tetrachloride–chloroform (50:50, v/v), 0.5 ml/min. Fluorescence detector; excitation 470 nm; emission 530 nm. Peaks: 1 = methanal; 2 = ethanal; 3 = propanal; 4 = butanal; 5 = pentanal.

TABLE II

CALIBRATION GRAPHS AND RELATIVE SENSITIVITY OF ALIPHATIC ALDEHYDES WITH POST-COLUMN EXTRACTION DETECTION

Develosil C_8 column; acetonitrile–phosphate buffer (22.5:77.5); flow-rate 1.0 ml/min; extraction solvent, carbon tetrachloride–chloroform (50:50), flow-rate 0.5 ml/min; extraction coil, 100 cm × 0.5 mm I.D. a = Slope, b = intercept, r^2 = regression coefficient.

Aldehyde	*Area*				*Height*			
	Calibration graph			*Relative sensitivity*	*Calibration graph*			*Relative sensitivity*
	a	*b*	r^2		*a*	*b*	r^2	
Ethanal	2.18	0.11	0.997	1.00	4.64	0.08	0.998	1.00
Propanal	3.02	0.05	0.999	1.38	7.88	0.07	0.999	1.70
Butanal	4.03	0.20	0.998	1.85	7.41	0.06	0.999	1.60
Pentanal	3.73	0.14	0.999	1.71	3.38	0.05	0.999	0.73

Calibration graph and reproducibility for aldehydes

Mixtures containing various amounts of four aliphatic aldehydes except methanal were determined with the procedure described for HPLC. Table II shows the calibration graphs obtained by plotting the ratios of the peak areas of NBD hydrazones to that of the internal standard against the known concentration of aldehydes. Linear relationships were found with the peak area and/or peak height ratio in the concentration ranges of 1.7–55 ppm (0.038–1.25 μmol/ml) for ethanal, 2.5–82 ppm (0.044–1.41 μmol/ml) for propanal, 2.8–90 ppm (0.039–1.25 μmol/ml) for butanal and 3.0–97 ppm (0.035–1.13 μmol/ml) for pentanal. The varying relative sensitivity shown in Table II evidenced by the different slopes of the regression lines reflects the varying extraction efficiency of NBD hydrazones with the post-column extraction HPLC system. However, the variation of responce was less than 1.85-fold by the peak area method, 1.70-fold by the peak height method.

The within-run precision of the method was measured by processing aliquots of reaction mixture through the procedure during a single day. The relative standard

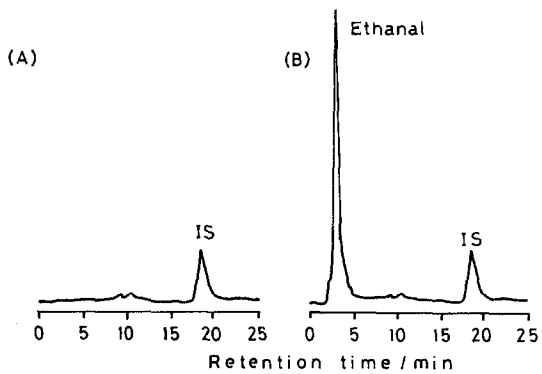

Fig. 5. Chromatagram of NBD hydrazones obtained from (A) a blank experiment and (B) a whisky. Chromatographic conditions as in Fig. 4.

deviation (R.S.D.) of the peak area ratio obtained by means of the internal standard procedure for five analyses of ethanal was 2.3% at 20 ppm (0.45 μmol/ml) and 5.4% at 4.5 ppm (0.10 μmol/ml).

Application

The present method was applied to the determination of aliphatic aldehydes in a commercial whisky. Chromatograms obtained from a blank experiment and a whisky are shown in Fig. 5. Ethanal was determined to be 90.6 ppm (2.06 μmol/ml) from the calibration graph using the peak area method. Other aldehydes were not detected in whisky.

In the present study NBD hydrazones were formed from carbonyl compounds but only the aldehyde derivatives were fluorescent in the anti-protonic solvent. A selective fluorometric detection of only aldehyde derivatives was achieved by means of a post-column extraction system which consists of a segmentor, an extraction coil and a membrane phase separator.

ACKNOWLEDGEMENT

This work was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education of Japan.

REFERENCES

1 E. Puputti and P. Lehtonen, *J. Chromatogr.*, 353 (1986) 163.
2 J. B. DeAndrade and A. H. Mignel, *Int. J. Environ. Anal. Chem.*, 21 (1985) 229.
3 M. Possanzini, P. Ciccioli, V. DiPalo and R. Draisci, *Chromatographia*, 23 (1987) 829.
4 M. Matsumoto, Y. Nishikawa, K. Murano and T. Fukuyama, *Bunseki Kagaku*, 36 (1987) 179.
5 H. Koizumi, H. Koshiyama and Y. Suzuki, *Bunseki Kagaku*, 34 (1985) 376.
6 Jen-Kun Lin and Shan-Shou Wu, *Anal. Chem.*, 59 (1987) 1320.
7 R. W. Frei, J. F. Flawrence, J. Hope and R. M. Cassidy, *J. Chromatogr. Sci.*, 12 (1974) 40.
8 Y. Suzuki and K. Tani, *Bunseki Kagaku*, 29 (1980) 849.
9 Y. Suzuki and K. Tani, *Bunseki Kagaku*, 34 (1985) 55.
10 Y. Suzuki, *Bunseki Kagaku*, 34 (1985) 314.
11 K. Yoshino, T. Mastuura, M. Sano, S. Saito and I. Tomita, *Chem. Pharm. Bull.*, 34 (1986) 1694.
12 D. R. Knapp, *Handbook of Analytical Derivatization Reactions*, Wiley, New York, 1979.
13 S. Motomizu, Y. Hazaki, M. Oshima and K. Toei, *Anal. Sci.*, 3 (1987) 265.
14 H. Koizumi and Y. Suzuki, *Anal. Sci.*, 4 (1988) 537.
15 J. F. Lawrence, U. A. Th. Brinkman and R. W. Frei, *J. Chromatogr.*, 171 (1979) 73.
16 J. A. Apffel, U. A. Th. Brinkman and R. W. Frei, *Chromatographia*, 18 (1984) 5.
17 F. A. Maris, M. Nijenhuis, R. W. Frei, G. J. de Jong and U. A. Th. Brinkman, *Chromatographia*, 22 (1986) 235.
18 J. W. Hellgeth and L. T. Taylor, *Anal. Chem.*, 59 (1987) 295.
19 J. Kawase, *Anal. Chem.*, 52 (1980) 2124.
20 T. Imasaka, T. Harada and N. Ishibashi, *Anal. Chim. Acta*, 129 (1981) 195.
21 K. Ogata, K. Taguchi and T. Imanari, *Anal. Chem.*, 54 (1982) 2127.
22 A. H. M. T. Scholten, U. A. Th. Brinkman and R. W. Frei, *Anal. Chem.*, 54 (1982) 1932.