Journal of Chromatography, 366 (1986) 135–144
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 748

PARALLEL COLUMN ION EXCHANGE FOR POST-SEPARATION pH MODIFICATION IN LIQUID CHROMATOGRAPHY

APPLICATION TO BARBITURATES AND MINIATURIZATION

H. JANSEN, C. J. M. VERMUNT, U. A. Th. BRINKMAN and R. W. FREI*
Department of Analytical Chemistry, Free University, De Boelelaan 1083, 1081 HV Amsterdam (The Netherlands)
(Received April 25th, 1986)

SUMMARY

A new approach to the UV detection of barbiturates in high-performance liquid chromatography is demonstrated. The analytical system comprises an anion-exchange column inserted parallel to the injection valve and analytical column. One part of the acetate-containing mobile phase flows through the injection valve and analytical column to achieve the separation, the other part flows through the anion-exchange column where the acetate ions cause the release of hydroxide ions from this column. Finally, the alkaline stream from the anion-exchange column is recombined with the analytical column effluent. This results in an alkaline medium which can be favourable for many detection processes. A series of barbiturates, which show enhanced UV detectability at 254 nm in alkaline solution, was chosen to demonstrate the potential of such a method. Applications of this principle to the analysis of urine and plasma samples are described. In the system only one pump is needed for the separation and the post-column pH modification. A critical comparison between conventional scale and narrow-bore systems is made.

INTRODUCTION

The use of a strongly alkaline detection medium can be advantageous for the detection. For example, some pesticides, such as the non-fluorescent benzotriazinone compounds and pyrazine compounds can be hydrolyzed with base to fluorescent derivatives[1]. Several anilines show fluorescence only in alkaline solution[2] and barbiturates exhibit *ca.* 20–50 fold higher UV absorption at 254 nm in alkaline as compared to non-alkaline (pH < *ca.* 7) solution[3]. However, the use of an alkaline mobile phase is restricted to non-silica-based packings, *i.e.*, ion-exchange resins and copolymers such as PRP-1 which have an alkali-resistant styrene–divinylbenzene backbone. Moreover, columns packed with these materials often exhibit low efficiency. Further, alkaline mobile phases may cause problems in the pumping system or may simply not be suitable for a separation process. When the mobile phase is not alkaline the

0021-9673/86/$03.50

high pH required for the detection can be achieved by mixing the column effluent with a suitable buffer solution with the consequent need for an additional pump. Recently, our group evaluated the simultaneous use of an anion-exchange column for both separation and pH modification in which case the additional pump can be omitted[4]. Although the system operated well, a disadvantage was the need for relatively frequent regeneration.

The present paper describes a method for post-column pH modification that utilizes an anion-exchange column mounted parallel to the injection valve and the C_{18}-bonded silica analytical column. The anion-exchange resin is converted into the hydroxide form prior to use. Upstream from the injection valve, the mobile phase is split. One part flows through the analytical column while in the other stream hydroxide ions on the anion-exchange resin are released by acetate ions present in the mobile phase. The resulting alkaline stream is recombined with the stream from the analytical column, giving a suitable detection medium. An important advantage of the present system is that only one pump is needed for both the separation and the post-column pH modification. In addition, it is not necessary to pump a corrosive alkaline solution. Since the analytes do not pass through the ion-exchange column this column does not contribute to band broadening and, hence, a low-cost large particle resin can be used. The anion-exchange column can be large, hence it can be used for a long period before regeneration is needed.

The new approach is demonstrated for UV detection of barbiturates, a class of drugs widely prescribed as hypnotics and sedatives. In a non-alkaline medium (pH $<$ *ca.* 7), barbiturates absorb only in the low UV region, *i.e.*, below about 230 nm. This results in considerable interferences when analyzing urine and plasma samples. One way to overcome this problem is via sample pretreatment[5]. Selective on-line sample clean-up with precolumns was recently investigated by our group[6]. Another possibility is to detect the barbiturates at 254 nm in their anionic forms which requires an alkaline medium[3] (see above), hence low-cost fixed-wavelength (254 nm) detectors can be used and the selectivity is improved.

Both a conventional scale (3.0 mm I.D. analytical column) and a narrow-bore (1.0 mm I.D. analytical column) high-performance liquid chromatographic (HPLC) system are described and a critical comparison between the two is made.

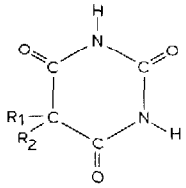
EXPERIMENTAL

Chemicals

HPLC-grade water produced by a Milli-Q system (Millipore, Bedford, MA, U.S.A.) was used throughout this work. The eluent was prepared by mixing equal volumes of HPLC-quality methanol (J. T. Baker, Deventer, The Netherlands) and aqueous 44 m*M* acetic acid, adjusted to pH 7.0 with sodium hydroxide. This pH adjustment is needed to avoid the acetate buffer action. Stock solutions of each of the barbiturates used, *i.e.*, barbital (BA), butobarbital (BB), amobarbital (AB) and secobarbital (SB) (*cf.*, Table I) were made by dissolving the compounds at a level of *ca.* 2 mg/ml in methanol. The solutions were stored in a refrigerator at 4°C and were diluted in water to the desired concentration. The regeneration solution for the anion-exchange column was prepared by mixing equal volumes of methanol (Baker) and aqueous 1 *M* sodium hydroxide.

TABLE I

STRUCTURES OF THE BARBITURATES USED



Compound	*Abbreviation*	R_1	R_2	*Supplier*
Barbital	BA	Ethyl	Ethyl	Brocacef*
Butobarbital	BB	Ethyl	1-Methylpropyl	Brocacef
Amobarbital	AB	Ethyl	3-Methylbutyl	OPG**
Secobarbital	SB	Allyl	1-Methylbutyl	OPG

* Maarssen, The Netherlands.
** Utrecht, The Netherlands.

Apparatus

The experimental set-up is shown in Fig. 1. The conventional scale system was built from a double head reciprocating high-pressure pump (Spectroflow 400, Kratos, Ramsey, NJ, U.S.A.), an injection valve with a 10-μl sample loop and a laboratory built mixing T-piece that is a conventional scale version of the micro T-piece described before[7]. The narrow-bore system consisted of a laboratory-built syringe pump, capable of pulse-free pumping in the 25–500 μl/min range at a maximum pressure of 250 bar, a laboratory-built injection valve[8] with an internal loop of 0.57 μl and a laboratory-built micro mixing T-piece[7]. The splitting T-piece was obtained from Valco (Houston, TX, U.S.A.). Two flow restrictions were used to control the splitting ratio. As restrictions, chromatographic columns or capillaries of small inner diameters were used. The UV detector (SF 757, Kratos) was equipped either with a 12-μl (8-mm optical path length) flow cell for the conventional scale system or with a 0.5-μl (1-mm optical pathlength) cell for the narrow-bore system. In the conventional scale system, the analytical column (100 mm × 3 mm I.D.) was laboratory-packed with 5-μm Spherisorb ODS-1 (Phase-Sep, Queensferry, U.K.), whereas in the narrow-bore system a 100 mm × 1 mm I.D. glass-lined column was laboratory-packed with 5-μm Hypersil ODS (Shandon, Runcorn, U.K.).

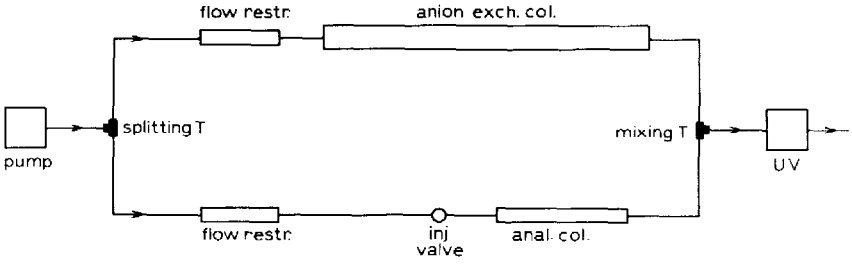


Fig. 1. Schematic diagram of the experimental set-up.

The anion-exchange column (250 mm × 4.6 mm I.D.) was dry packed with a low-cost large particle strong anion-exchange resin (Amberlite CG-400; BDH, Poole, U.K.). In the narrow-bore system, all connections between the injection valve and detector were of the shortest possible lengths of 0.12 mm I.D. stainless-steel tubing. All other connections and the connections in the conventional scale system were made from 0.25 mm I.D. stainless-steel tubing. All chromatograms were registered by a strip-chart recorder (BD 40; Kipp & Zonen, Delft, The Netherlands). An high-pressure pump (Orlita, Giessen, F.R.G.) was used for pumping the regeneration solution.

Start-up procedure

Prior to use the anion-exchange column was flushed off-line with 25 ml of methanol–1 *M* aqueous sodium hydroxide (50:50) in order to convert the resin into the hydroxide form. Next, the column was flushed with a few ml of mobile phase followed by insertion of the column in the analytical system. After use, the pump was flushed with water to remove the highly alkaline solution. The splitting ratio was determined by measuring the dead volume of the system with the parallel anion-exchange column and, next, without the ion-exchange column and at a known flow-rate through the analytical column in order to calculate the latter's volume.

For further chromatographic conditions see the legends to the figures.

RESULTS AND DISCUSSION

Conventional scale system

A chromatogram of a test mixture of four barbiturates in the system C_{18} bonded silica with methanol–aqueous acetic acid pH 7.0 as the mobile phase is shown in Fig. 2. Baseline separation of the four solutes is obtained in less than 7 min.

Relevant data concerning the conventional scale system have been summarized in Table II. For these measurements, 25–45 ng amounts of each of the barbiturates were injected. The within-one-day repeatabilities of both retention time and peak height measurements were good. Obviously, the splitting ratio is not subject to large fluctuations. There was no measurable difference in band broadening when the anion-exchange column was removed and the analytical column was directly connected with the detector, which is not surprising since a low dead volume T-piece is the only additional contribution. With the 10-μl injection volume and the 0.3–0.9 ng detection limits, the detection limits in concentration units ranged from 30 ppb* for barbital to 90 ppb for secobarbital.

Application to urine samples. The method was used for the determination of barbiturates in spiked urine. Urine from healthy male individuals was filtered over a Millipore filter (pore width 0.2 μm) to remove microparticulate matter prior to use. No further pretreatment was carried out. In Fig. 3 the chromatogram of urine spiked at the 2-ppm level with BB, AB and SB is given. The dotted line indicates the blank signal. Determination down to the *ca.* 1-ppm level is possible before chemical interferences start to intervene. The clean-up procedure with precolumns as proposed by

* The American billion (10^9) is meant.

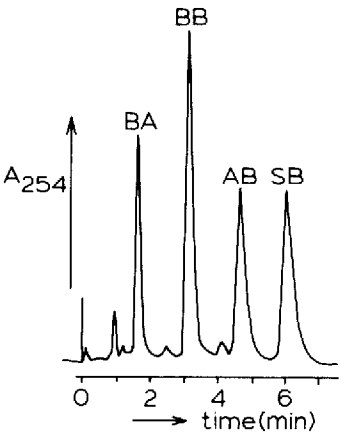


Fig. 2. Chromatogram of a test mixture of barbiturates. BA = Barbital; BB = butobarbital; AB = amobarbital; SB = secobarbital. Conditions: flow-rate of mobile phase [methanol–44 m*M* acetic acid pH 7 (50:50)] 0.6 ml/min; 0.46 ml/min through the analytical column (100 mm × 3.0 mm I.D., packed with 5-μm Spherisorb ODS-1) and 0.14 ml/min through the anion-exchange column (250 mm × 4.6 mm I.D., packed with Amberlite CG-400, OH^-); injection volume 10 μl; UV detection, 254 nm; cell volume 12 μl; optical pathlength 8 mm.

De Jong *et al.*[6] might well be beneficial to improve this situation. For all three analytes, the recovery was higher than 95% and the repeatability of retention time measurements was better than 2.5% relative S.D. (n = 6). Further, there were no trend effects. These facts indicate that, also when analyzing urine, there are no problems in maintaining a constant splitting ratio.

Fig. 3 also shows the chromatogram of a urine blank without using the anion-exchange column, *i.e.*, the analytical column outlet was directly connected with the detector set to 220 nm. The flow-rate through the analytical column was main-

TABLE II

ANALYTICAL DATA CONCERNING THE CONVENTIONAL SCALE SYSTEM

Conditions. Flow-rate of mobile phase [methanol–44 m*M* acetic acid pH 7 (50:50)], 0.6 ml/min; 0.46 ml/min through the analytical column (100 mm × 3.0 mm I.D., packed with 5-μm Spherisorb ODS-1) and 0.14 ml/min through the anion-exchange column (250 mm × 4.6 mm I.D., packed with Amberlite CG-400 in the hydroxide form); injection volume, 10 μl; UV detection at 254 nm; cell volume 12 μl; optical pathlength 8 mm. t_R = Retention time.

Compound	t_R *(min)*	*Repeatability (% relative S.D., n = 7) of*		σ_t *(s)*	*Detection limit (ng)**
		t_R	*Peak height*		
BA	1.60	1.7	4.1	3.8	0.3
BB	3.12	1.3	3.1	5.3	0.5
AB	4.62	1.5	1.5	6.7	0.8
SB	6.04	1.3	1.4	8.3	0.9

* Signal-to-noise ratio S/N = 3:1.

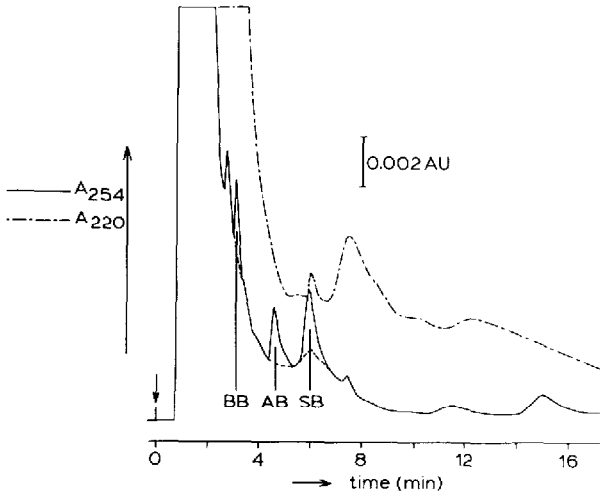


Fig. 3. ———, Chromatogram of an urine sample spiked with 2.0 ppm BB, 1.9 ppm AB and 2.0 ppm SB; ---------, blank signal. Conditions as in Fig. 2. — · — · —, Chromatogram of a blank urine sample obtained without using the anion-exchange column (neutral detection medium). Flow-rate 0.46 ml/min. UV detection at 220 nm. All other conditions as in Fig. 2.

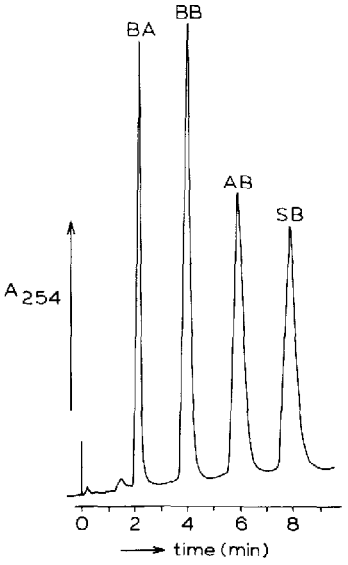


Fig. 4. Chromatogram of a test mixture of barbiturates. Conditions: flow-rate of mobile phase [methanol–44 m*M* acetic acid, pH 7 (50:50)] 93 μl/min; 62 μl/min through the analytical column (100 mm × 1.0 mm I.D., packed with 5-μm Hypersil ODS) and 31 μl/min through the anion-exchange column (250 mm × 4.6 mm I.D., packed with Amberlite CG-400, OH^-); injection volume 0.57 μl; UV detection at 254 nm; cell volume 0.5 μl; optical pathlength 1 mm.

tained at 0.46 ml/min. From the chromatogram, it is seen that the large interference peak at the beginning has increased and that the background absorbance is higher throughout the chromatogram. The responses of the neutral barbiturates at 220 nm were found to be roughly equal to those at 254 nm observed in the split system with pH modification.

Regeneration of the anion-exchange column. Since acetate ions displace hydroxide ions from the anion-exchange column this column will be exhausted as a source for hydroxide ions under our experimental conditions after approximately 17 h. During this time, 140 ml of alkaline solution were delivered; the pH remained essentially constant until it suddenly decreased to the pH of the mobile phase. Regeneration was carried out as described under Experimentals for the first conversion into the hydroxide form of an unused column.

Narrow-bore system

In the narrow-bore system the same anion-exchange column dimensions as in the conventional scale system were used. The chromatogram obtained upon injection of a test mixture in this system was similar to the one obtained in the conventional scale system (Fig. 4). Again, baseline separation was achieved in about 8 min.

Table III lists some relevant analytical data. The retention times were somewhat larger than in the conventional scale system, in spite of the *ca.* 20% higher linear velocity through the column. This is due to the use of a C_{18} material supplied by another manufacturer. As a result, the values for band broadening in time units (σ_t) are somewhat higher as compared to the conventional scale system. However, it can be concluded that they are of the same order of magnitude in both systems. Again, we found good repeatabilities for both retention time and peak height measurements. For these measurements, 50–90 ng amounts of the four barbiturates tested were injected.

The absolute detection limit was about two-fold higher than with the conventional scale system for all four barbiturates tested. Linearity of response was found

TABLE III

ANALYTICAL DATA CONCERNING THE NARROW-BORE SYSTEM

Conditions. Flow-rate of mobile phase [methanol–44 m*M* acetic acid pH 7 (50:50)], 93 μl/min; 62 μl/min through the analytical column (100 mm × 1.0 mm I.D., packed with 5-μm Hypersil ODS) and 31 μl/min through the anion-exchange column (250 mm × 4.6 mm I.D., packed with Amberlite CG-400 in the hydroxide form), injection volume, 0.57 μl; UV detection at 254 nm; cell volume 0.5 μl; optical pathlength 1 mm.

Compound	t_R *(min)*	*Repeatability (% relative S.D., n = 7) of*		σ_t *(s)*	*Detection limit (ng)**
		t_R	*Peak height*		
BA	2.06	2.0	1.6	4.5	0.5
BB	3.88	1.5	1.3	6.6	0.8
AB	5.94	0.8	2.2	9.6	1.1
SB	7.76	1.0	1.5	11.0	1.4

* S/N = 3:1.

up to at least an injected amount of 50 ng of barbital and 90 ng of the three other barbiturates, these being the most concentrated sample solutions analyzed. Since the flow-rate through the ion-exchange column is lower than in the conventional scale system, regeneration is now needed only after several days.

Application to urine samples. Urine was again used as test sample, utilizing the same procedure as before. The chromatogram obtained after injection of 0.57 μl of urine spiked at the *ca.* 3-ppm level with BB, AB and SB, and the blank signal (dotted line), are given in Fig. 5. This concentration level must be seen as the lowest at which determination is possible bearing in mind the electronic noise. This differs from the situation in the conventional scale system where the presence of interferences limited detection to *ca.* 1 ppm in urine. The signal-to-noise ratios are worse in the narrow-bore system since the analytes are detected in a cell with a relatively short optical pathlength. The repeatability of peak-height measurement in urine, spiked at the 7–8 ppm level, was found to be *ca.* 5% rel. S.D. (n = 8).

Application to plasma samples. Plasma samples were pretreated by mixing 1 ml plasma with 1 ml aqueous 25% trichloroacetic acid and subsequent centrifugation for *ca.* 15 min in a desk-top centrifuge (2800 rpm, 1300 *g*). 0.57-μl Aliquots of the clear supernatant were analyzed in the split system. The resultant chromatogram for a plasma sample spiked at the 17–19 ppm level with BB, AB and SB is given in Fig. 6. This shows that the analysis of plasma is feasible down to the 6–10 ppm level, even without any on-line precolumn trace-enrichment procedure. With suitable precolumns permitting direct injection of plasma samples[9], this level could well be decreased by at least one order of magnitude. Recoveries were found to be 85 ± 2.5% for BB, 73 ± 3.0% for AB and 60 ± 3.5% for SB (n = 6). They did not vary with the barbiturate concentration over the 6–30 ppm range.

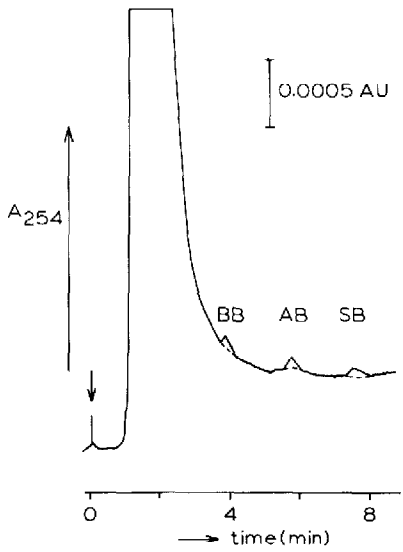


Fig. 5. Chromatogram of an urine sample spiked with 3.1 ppm BB, 3.1 ppm AB and 2.8 ppm SB. ---------, Blank signal. Conditions as in Fig. 4.

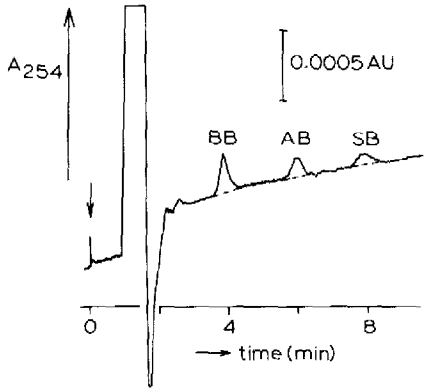


Fig. 6. Chromatogram of a plasma sample spiked with 18.8 ppm BB, 18.8 ppm AB and 17.4 ppm SB. --------, Blank signal. Sample pretreatment with 25% trichloroacetic acid (*cf.*, text). Conditions as in Fig. 4.

CONCLUSIONS

The detection principle described with a parallel anion-exchange column as a source of hydroxide ions gave fully satisfactory results with increased selectivity of UV detection for some barbiturates. It has the advantage that only one high-quality HPLC pump is needed for both the separation and the post-column pH modification. Another important point is the fact that the ion-exchange column does not contribute to band broadening since the analytes do not pass through it. This is in contrast to the situation in which an ion-exchange column is mounted downstream from the analytical column. The parallel coupling therefore allows the use of large columns (less frequent regeneration required) packed with low-cost large particle materials. The same advantages hold if other types of replenishable reactor columns are used in parallel.

The comparative study of the conventional scale and the narrow-bore system shows that band broadening in time units, σ_t, is of the same order of magnitude in both cases while band broadening in volume units (σ_v) is, of course, much lower in the latter case. In both systems we obtained reproducible analytical data and no problems were encountered concerning the maintenance of a constant splitting ratio.

In the conventional scale system the anion-exchange column can be used for 17 h before regeneration is needed. The reduced volumetric flow-rate in the narrow-bore system has the advantage that regeneration is needed only after several days.

The main disadvantage of the narrow-bore system is the less sensitive UV detection. In absolute amounts, the detection limits in both systems are roughly the same. This is not surprising since, if equal amounts are injected in the conventional scale and in the narrow-bore system, the peaks will be less diluted in the latter. However, they were detected using a detector with a shorter optical pathlength leading to roughly equal signal-to-noise ratios. The noise was slightly less in the narrow-bore system as a result of the completely pulse-free pumping system. Since the injection volume was considerably smaller in the narrow-bore system the detection limit in concentration units is worse. Similar conclusions on the concentration sensitivity were given by Van den Berg *et al.*[10].

Future work will concentrate on further applications of this split-flow principle to various types of post-column reagent addition from a parallel column. The use of pH modification can be worthwhile for fluorescence enhancement of various systems via cation or anion exchangers. Also the addition of an oxidator or reductor via a parallel column seems feasible. In peroxyoxalate chemiluminescence detection systems the addition of reagents from a parallel column is currently being studied by our group[11].

ACKNOWLEDGEMENTS

This work was supported by the Dutch Foundation for Technical Sciences, grant No. VCH 33.0436. The barbiturates were a gift from Mr. P. de Goede (Free University Academic Hospital Dispensary). The Onze Lieve Vrouwe Gasthuis in Amsterdam is acknowledged for providing the plasma samples. Kratos is thanked for the loan of HPLC equipment.

REFERENCES

1 R. T. Krause, *J. Chromatogr.*, 255 (1983) 497.
2 J. W. Bridges and R. T. Williams, *Biochem. J.*, 107 (1968) 225.
3 C. R. Clark and J. L. Chan, *Anal. Chem.*, 50 (1978) 635.
4 H. Jansen, E. G. van der Velde, U. A. Th. Brinkman, R. W. Frei and H. Veening, *Anal. Chem.*, 58 (1986) 1380.
5 R. N. Gupta, *J. Chromatogr.*, 340 (1985) 139.
6 J. de Jong, M. W. F. Nielen, R. W. Frei and U. A. Th. Brinkman, *J. Chromatogr.*, 381 (1986) 431.
7 H. Jansen, U. A. Th. Brinkman and R. W. Frei, *Chromatographia*, 20 (1985) 453.
8 J. A. Apffel, U. A. Th. Brinkman and R. W. Frei, *Chromatographia*, 17 (1983) 125.
9 M. W. F. Nielen, R. C. A. Koordes, R. W. Frei and U. A. Th. Brinkman, *J. Chromatogr.*, 330 (1985) 113.
10 J. H. M. van den Berg, H. W. M. Horsels and R. J. M. Groenen, *Chromatographia*, 18 (1984) 574.
11 J. R. Poulsen, J. W. Birks, P. van Zoonen, C. Gooijer, N. H. Velthorst and R. W. Frei, submitted for publication.