Journal of Chromatography, 620 (1993) 47–53
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 7059

Automated column-switching high-performance liquid chromatography for the determination of 2-thiothiazolidine-4-carboxylic acid in urine

P. Simon* and T. Nicot

Institut National de Recherche et de Sécurité, Avenue de Bourgogne, 54501 Vandoeuvre (France)

(First received May 24th, 1993; revised manuscript received July 20th, 1993)

ABSTRACT

2-Thiothiazolidine-4-carboxylic acid (TTCA) is the accepted urinary indicator of occupational exposure to carbon disulfide. An extractionless automated column-switching procedure for the determination of this compound in urine was developed. The biological fluid is directly injected into the chromatographic system after dilution. A clean-up procedure is performed in an anion-exchange pre-column, with aqueous formic acid as eluent, followed by a transfer to a cyan-amino or amino analytical column in order to carry out analysis of TTCA under isocratic elution conditions, with a mixture of acetonitrile, water and formic acid as eluent, combined with ultraviolet detection. The detection limit of the method in urine is estimated to be 0.1 mg/l.

INTRODUCTION

Exposure to carbon disulfide (CS_2), a neurotoxic compound, occurs principally in the viscose industry. Biological monitoring for exposure to this solvent is performed by measuring the concentration of 2-thiothiazolidine-4-carboxylic acid (TTCA) in the urine of exposed workers. A biological exposure index (BEI) of 5 mg of TTCA per g of creatinine and BAT (Biologischer Arbeitsstofftoleranzwert) of 8 mg/l are recommended [1,2]. TTCA can also be used for the biological monitoring of workers exposed to fungicides such as captan [3,4] or disulfiram [5]. These compounds are partly metabolized to form thiophosgene or CS_2, which, after formation of a glutathione conjugate followed by an enzymatic degradation and ring-closure reaction, are excreted in urine as TTCA.

* Corresponding author.

Only a few methods are available for the measurement of TTCA in urine [6–9], and they are based on either capillary gas chromatography with thermoionic detection [6] or liquid chromatography with ultraviolet detection [7–9] as analytical techniques. Furthermore, they all require a time-consuming liquid–liquid extraction step, including the selective method of Thienpont *et al.* [9], which additionally uses affinity chromatography on organomercurial agarose gel for the isolation of TTCA. The column-switching technique, which has found many applications in clinical laboratories, such as therapeutic drug monitoring, is very attractive for sample clean-up and routine analysis in liquid chromatography.

The method described in this work omits the liquid–liquid extraction step and allows the direct injection of urine after a simple dilution by using column switching as a clean-up procedure. The purification step is performed on an anion-exchange column, and, after transfer of the fraction

0378-4347/93/$06.00 © 1993 Elsevier Science Publishers B.V. All rights reserved

of interest, the analysis is carried out in isocratic mode on a cyan-amino or amino column combined with UV detection at 275 nm.

EXPERIMENTAL

Chemicals and reagents

All chemicals were of analytical reagent grade. Formic acid and perchloric acid were obtained from Merck (Darmstadt, Germany). Water was purified by passage through a Milli-Q treatment system (Millipore, Bedford, MA, USA). Acetonitrile and methanol of chromatographic grade were obtained from Varian (San Fernando, CA, USA). TTCA was at first synthesized and purified according to the method of Rosier *et al.* [10], then obtained from Aldrich (Steinheim, Germany).

Apparatus

The HPLC system consisted of two chromatographic pumps, a Model 590 from Waters Associates (Milford, MA, USA) and a Model 5000 from Varian, two automated switching valves (Model 7000, Rheodyne, Berkeley, CA, USA), a Model 805 autosampler from Varian, associated with a Model 7126 automatic sample injector (Rheodyne) equipped with a 10-μl sample loop, and a Model SPD-6A ultraviolet detector (Shimadzu, Kyoto, Japan) set at 275 nm, all of which were controlled by a Waters chromatographic pump.

Columns

The anion-exchange column was a 5 cm × 0.32 cm I.D. stainless-steel tube packed with 10-μm Partisil SAX (Whatman, Fairfield, NJ, USA) or 5-μm Spherisorb SAX (Phase Separations, Deeside, Clwyd, UK) connected to a 3 cm × 0.21 cm I.D., 37–53 μm pellicular ODS (Whatman) guard column. The analytical column was a 15 cm × 0.32 cm I.D. stainless-steel tube packed with 5-μm Partisil cyan-amino (Whatman) or 5-μm Nucleosil NH_2 (Macherey-Nagel, Düren, Germany). With the exception of the guard column, which is dry packed, all columns were made in the laboratory and slurry-packed at $4 \cdot 10^7$ Pa using a mixture of 95% ethanol–2-propanol–toluene (1:1:1, v/v/v) as slurry solvent and methanol followed by water as displacement liquid.

Other silica-bonded phase anion exchangers were tested. These included: a 5-μm Chromegabond RP-SAX (ES Industries, Berlin, NJ, USA), a 10-μm Vydac AN (Vydac, Hesparia, CA, USA), a 5-μm Rosil AN (ResearchSeparations Laboratories, Eke, Belgium) and a 5-μm Nucleosil SB (Macherey-Nagel, Düren, Germany).

Chromatographic analysis

The mobile phases used in this study were aqueous formic acid (0.2 *M*) for the anionic–exchange column and a mixture of water–acetonitrile–formic acid (75–65:24–34/1, v/v/v) for the analytical columns, the strength depending on the experimental conditions. The flow-rate for both columns was 0.6 ml/min.

Spiked urine pretreatment

Blank human urine samples spiked with known amounts of TTCA (16 mg/l, 4 mg/l and 0.8 mg/l) were diluted 1:40, 1:10 and 1:5 with eluent and an aliquot of 10 μl was injected into the HPLC system. When urine samples were not being analysed, they were kept frozen at −20°C.

Analysis of TTCA

For a given urine sample, the retention time was compared with that of an external standard, and the method of peak-height measurement was used for quantitative assessment. The analysis of each sample was completed within about 20 min.

Urine preclean-up procedure on C_{18} cartridges

In order to protect the HPLC columns and increase their lifetime, the urine samples were purified before their injection into the HPLC system by a clean-up on C_{18} cartridges: 0.5 ml of the urinary sample were passed through a Bond Elut cartridge filled with 0.1 g of C_{18} sorbent previously conditioned with 1 ml of methanol, 1 ml of distilled water and 1 ml of ammonium acetate or monopotassium phosphate $2 \cdot 10^{-2}$ *M*. After recovery of the eluate in a gauged flask, *ca.* 0.5 ml of the previous buffer solution was passed

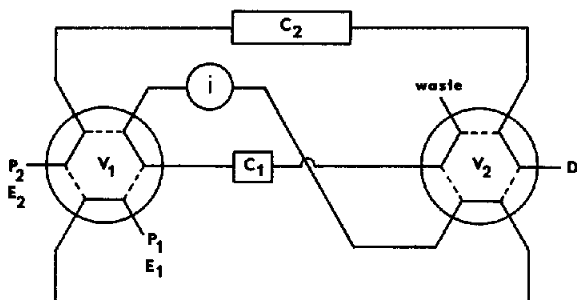

Fig. 1. Schematic diagram of the column-switching system. V_1, V_2 = Switching valves; P_1, P_2 = HPLC pumps; E_1, E_2 = eluents; i = injector; C_1 = purification column; C_2 = analytical column; D = detector.

through the cartridge, mixed with the previous eluate and adjusted to 2.5 ml or 10 ml with water or buffer solution.

TABLE I

TTCA CAPACITY FACTOR (k') ON DIFFERENT ION-EXCHANGE BONDED COLUMNS

Injection of 10 μl of a TTCA standard (0.4 mg/l in eluent). Eluent: 0.2 *M* aqueous formic acid; flow-rate: 0.6 ml/min; columns: 5 cm × 0.32 cm I.D.; t_0 was determined by injection of deionized water or acetonitrile.

Packing material	k'
Chromegabond RP-SAX	5.9
Vydac AN	7.8
Partisil SAX	9
Spherisorb SAX	11.1
Rosil AN	16.8
Nucleosil SB	52.1

RESULTS AND DISCUSSION

Liquid–liquid extraction of TTCA from urine is a rather restrictive and non-selective technique. The use of column switching for sample urine clean-up in order to reduce the handling of biological material seems attractive. This technique is not new, and Ramsteiner [11] has described the main principles in a review. The direct transfer technique was employed here, and consists of discarding the uninteresting parts of a sample initially eluted on a primary column in order to select the fraction of interest and to transfer it onto the secondary column with a minimum of overlapping interferences.

A schematic diagram of the switching system is shown in Fig. 1. Initially, after the injection of sample into an anion exchange column (C_1), purification was performed with eluent E_1 and pump P_1 by dispatching the bulk of the undesired matrix to waste over approximately 6 min. Following this, valve 1 (V_1) was switched, and the analyte fraction of interest was transferred from C_1 onto the analytical column (C_2) with eluent E_1 and pump P_1 for *ca.* 2 min. After the complete elution of analyte from the primary column, valve 2 (V_2) was rotated and TTCA was eluted and separated on column C_2 with eluent E_2 and pump P_2. The valves and connections were ordered so as to inject the sample on column C_1 or on the column C_2 without the need to switch them.

The ionizable carboxylic functional group of TTCA suggests the use of an anion-exchange phase for preliminary chromatographic fractionation since either an ion suppression or association technique must be used to detect it by reversed phase chromatography [7,8]. In preliminary experiments, the ionic force and the pH were optimized to obtain a sufficient difference in retention between the TTCA and UV-absorbing interfering compounds, such as other organic acids, while minimizing the total retention time. In this way, the number of potentially interfering components to be transferred to the secondary column with TTCA was reduced. A solution of 0.2 *M* formic acid in water was retained as mobile phase. The capacity factor of TTCA was determined for a series of columns with different anion-exchange bonded phases using 0.2 *M* formic acid as eluent. The results are summarized in Table I. Partisil SAX and Spherisorb SAX formed the best compromise, offering a minimum number of interfering components transferred in a minimum retention time.

A C_{18} bonded phase was not used in the ana-

lytical column since the separation and the sensitivity were reduced by sample dilution throughout the analysis, because the eluent strengths of the mobile phases of the two columns were too similar.

The combination of the selected strong anion-exchange phase with weak anion-exchange phases, cyan-amino, NH_2 and $N(CH_2)_3$, was tested. Highly selective chromatographic separations were obtained with the first two phases. A mixture of water–acetonitrile–formic acid (75:24:1, v/v/v) was selected as an eluent for the cyan-amino phase and 65:34:1 (v/v/v) for the amino phase. Given the low pressure obtained with the cyan-amino phase, the analyses were carried out preferably and principally in this phase. Typical chromatograms are shown in Fig. 2.

The minimum detection limit for the determination of standard TTCA was 0.02 mg/l (signal-to-noise >3), and estimated to be 0.1 mg/l for urinary TTCA.

The calibration curve for TTCA was linear over the investigated range. The regression equation was $y = 0.037 + 0.161x$, with a correlation coefficient greater than 0.999.

Different quantities of TTCA were added to urine samples. The recovery of TTCA from these spiked urine samples is shown in Table II.

The concentrations were determined by external standardization with aqueous standards and were not corrected for recovery. No internal standardization method was performed because of the difficulty in finding an appropriate internal standard by using the heart-cut column-switching technique; the internal standard requiring two conditions: to have the same retention time

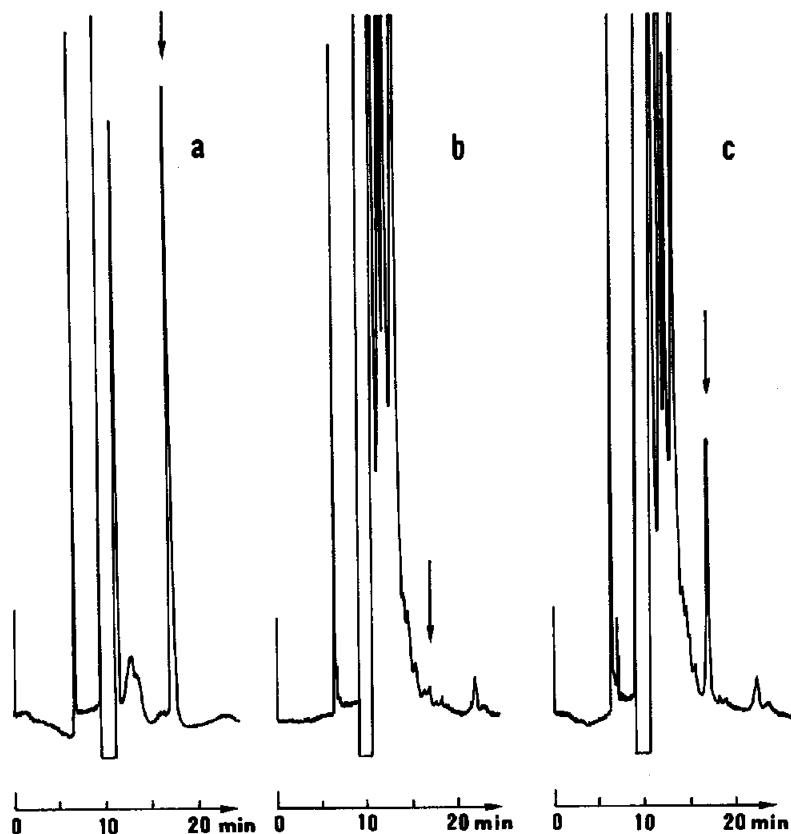

Fig. 2. Chromatograms of : (a) standard of TTCA 0.4 mg/l; (b) blank urine sample (dilution 1:5); TTCA less than 0.05 mg/l; (c) blank urine sample spiked with 0.8 mg/l TTCA (dilution 1:5). Chromatographic conditions: C_1, Partisil SAX; eluent, H_2O–HCOOH (99.2:0.8, v/v); flow-rate, 0.6 ml/min; C_2, Partisil PAC; eluent, H_2O–CH_3CN–HCOOH (75:24:1, v/v/v); flow-rate, 0.6 ml/min; switch times, T_1 = 6 min, T_2 = 8.5 min; UV, 0.005 AUFS. The arrows indicate the TTCA peak.

TABLE II

TTCA RECOVERY FROM SPIKED BLANK URINE SAMPLES

Direct injection of 10 μl of urine sample (dilution 1:5) without preclean-up procedure on C_{18} cartridges. Chromatographic conditions as in Fig. 2.

Concentration (mg/l)	Recovery (%)	Standard deviation (%)
16	95.8	1.9 (n = 5)
4	95.3	1.1 (n = 5)
0.8	96.9	4.2 (n = 5)

as TTCA on the primary column and to be completely separated from TTCA and other unknown substances on the analytical column.

The within-series precision and between-day precision were established on a sample of pooled urine (1.56 mg/l creatinine) to which TTCA was added in concentrations of 0.8 mg/l, 2.4 mg/l and 8 mg/l. The analyses of the urinary samples were performed without a preclean-up procedure on C_{18} cartridges. The average values, the standard deviations (S.D.) and the variation coefficients (C.V.) are reported in Table III.

Other tests of reproducibility were performed by changing the operators and the chromatographic columns; the relative standard deviations of the assays did not exceed 5% ($\bar{x}$ = 0.76 mg/l, n = 16), 4% ($\bar{x}$ = 2.25 mg/l, n = 16) and 2.5% ($\bar{x}$ = 7.79 mg/l, n = 16).

No interference was observed with another metabolite of CS_2, 2-oxothiazolidine-4-carboxylic acid, the oxygenated compound corresponding to TTCA. To check the specificity of the method, measurements of urinary TTCA were performed on the urine of workers unexposed to CS_2. A total of 38 urine samples were analysed and the TTCA levels were lower than the estimated urinary detection limit: $\bar{x}$ = 0.07 mg/l ; S.D. = 0.04 ($\bar{x}$ = 0.05 mg per g of urinary creatinine ; S.D. = 0.03).

The disadvantage of the method is the limited lifetime of the anion purification column, which is due to the relatively small amount of the phase contained in the column, *ca.* 0.3 g. The lifetime was checked by measuring of the TTCA standard retention time every five injections and ranged from 70 to 100 injections of diluted urine. This lifetime was doubled by the introduction of a C_{18} guard column above the anion-exchange column. The guard column was replaced every 50 injections of biological samples.

Another way of protecting the anion purification column and increasing its lifetime is to carry out an additional clean-up step of the urine sample prior to the injection. The method involves using C_{18} silica cartridges. It is simple and easy, and no loss of TTCA occurs during the procedure. The recovery of TTCA from spiked urine containing concentrations of 0.8 mg/l and 4 mg/l using solid phase clean-up alone was 99.8% (S.D. = 1.8%, n = 5) and 99.7% (S.D. =

TABLE III

WITHIN-SERIES AND BETWEEN-DAY PRECISIONS AT THREE CONCENTRATION LEVELS ON URINARY SAMPLES SPIKED WITH TTCA

Direct injection of 10 μl of urine sample (dilution 1:5) without preclean-up procedure on C_{18} cartridges. Chromatographic conditions as in Fig. 2.

Concentration of added TTCA (mg/l)	Within-series (n = 6)			Between-day (n = 6)		
	$\bar{x}$ (mg/l)	S.D. (mg/l)	C.V. (%)	$\bar{x}$ (mg/l)	S.D. (mg/l)	C.V. (%)
0.8	0.76	0.02	2.6	0.76	0.03	3.9
2.4	2.26	0.02	0.9	2.29	0.04	1.8
8	7.62	0.08	1	7.87	0.16	2

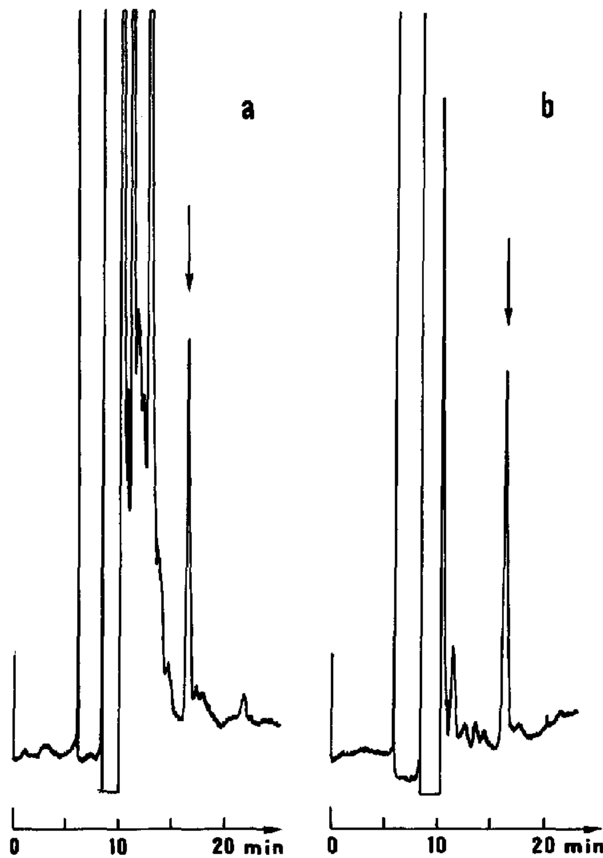

Fig. 3. Chromatograms of: (a) direct injection of a blank urine sample spiked with 0.8 mg/l TTCA without clean-up procedure prior to HPLC injection (dilution 1:5); (b) same sample with a clean-up procedure on C_{18} cartridge prior to HPLC injection (dilution 1:5). Chromatographic conditions as in Fig. 2. The arrows indicate the TTCA peak.

1.25%, n = 5), respectively. This method led to an additional manipulation of urine sample, but a useful purification is nevertheless obtained in the case of "concentrated" urine. An example of the purification obtained in this way is shown in Fig. 3. No direct analyses of spiked urine samples in an anion-exchange column alone or a cyan-amino column are shown because the TTCA peak is lost due to large, positive signal broadening.

During the assessment of the applicability of the method to various urine samples it was observed that mobile phase containing perchloric acid (0.01 M), instead of formic acid, can also be used with the two columns of the chromato-

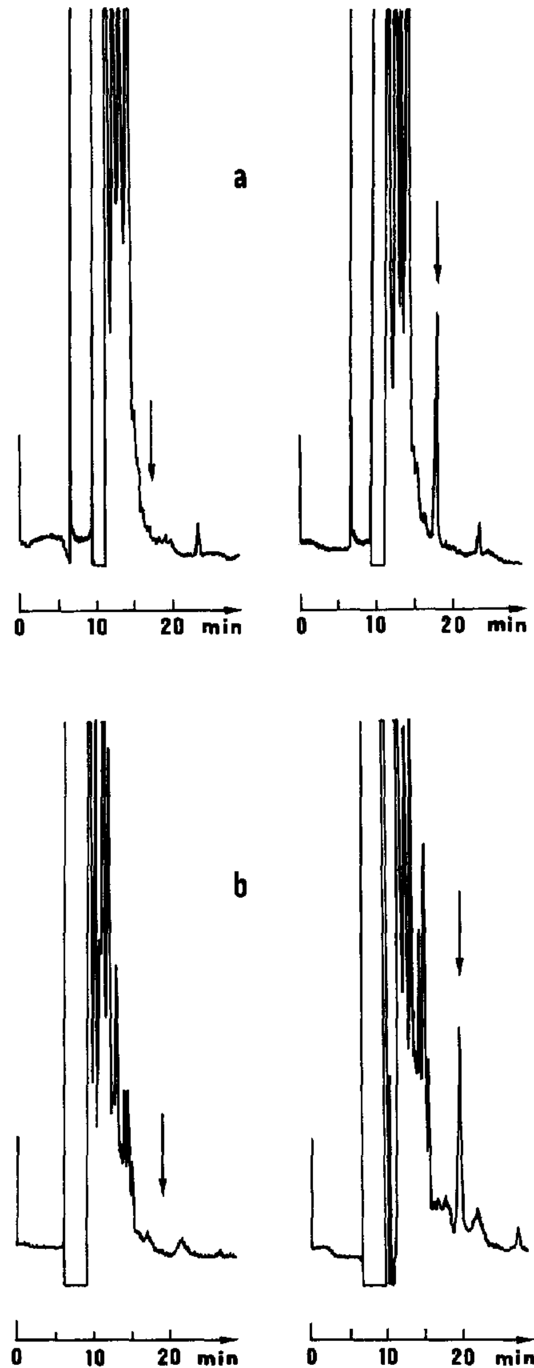

Fig. 4. Chromatograms of a blank urine sample, TTCA less than 0.05 mg/l, and an urine sample spiked with 0.8 mg/l TTCA (dilution 1/5) on: (a) Partisil PAC as analytical column (chromatographic conditions as in Figs. 2 and 3); (b) Nucleosil NH_2 as analytical column. Chromatographic conditions: C_1, as in Figs. 2 and 3; C_2, Nucleosil NH_2 ; eluent, H_2O-CH_3CN-HCOOH (65:34:1, v/v/v); flow-rate, 0.6 ml/min; switch times, T_1 = 6 min, T_2 = 8.5 min; UV, 0.005 AUFS. The arrows indicate the TTCA peak.

graphic system. This results in a change in the selectivity of the chromatographic separation, and thus the use of an NH_2 phase instead of the cyan-amino phase should not be neglected. An example of the difference in selectivity between the cyan-amino and NH_2 phases is illustrated in Fig. 4.

No reconditioning of the anion-exchange column was needed between injections as the column was always flushed with the same mobile phase. However, backflushing with a stronger eluent, which is used on the analytical column for instance, can be performed after a series of ten injections in order to elute strongly retained components.

CONCLUSION

The great advantage of the proposed column-switching method described in this work is the minimization of sample handling through omission of the tedious liquid–liquid extraction stage, which makes it particularly useful for routine analysis. The method is easy and rapid, and the detection limit, 0.1 mg/l of TTCA, is much lower than the recommended BEI (for a mean creatinine level of 1.6 g/l).

REFERENCES

1 ACGIH, Recommended carbon disulfide BEI, *Documentation of the Threshold Limit Values and Biological Exposure Indices, American Conference of Governmental and Industrial Hygienists, Cincinnati, OH, 1992.*
2 DFG, *Maximale Arbeitsplatzkonzentrationen und Biologische Arbeitsstofftoleranzwerte 1990*, VCH, Weinheim, 1990.
3 J. R. De Baum, J. B. Miaullis, J. Knarr, A. Mihailovski and J. J. Menn, *Xenobiotica*, 4 (1974) 101.
4 R. T. H. Van Welie, P. Van Duyn, E. K. Lamme, P. Jäger, B. L. M. Van Baar and N. P. E. Vermeulen, *Int. Arch. Occup. Environ. Health*, 63 (1991) 181.
5 R. Van Doorn, C. P. M. J. M. Leijdekkers, S. M. Nossent and P. T. Henderson, *Toxicol. Lett.*, 12 (1982) 59.
6 D. Mancas, M. Leanca and C. Chiriac, *J. High Resolut. Chromatogr. Chromatogr. Commun.*, 12 (1989) 828.
7 R. Van Doorn, L. P. C. Delbressine, C. P. M. J. M. Leijdekkers, P. G. Vertin and P. Henderson, *Arch. Toxicol.*, 47 (1981) 51.
8 M. Ogata and T. Taguchi, *Ind. Health*, 27 (1989) 31.
9 L. M. Thienpont, G. C. Depourcq, H. J. Nelis and A. P. De Leenheer, *Anal Chem.*, 62 (1990) 2673.
10 J. Rosier, R. Simonds, C. Van Peteghem and M. Vanhoorne, *Bull. Soc. Chim. Belg.*, 92 (1983) 397.
11 K. A. Ramsteiner, *J. Chromatogr.*, 456 (1988) 3.