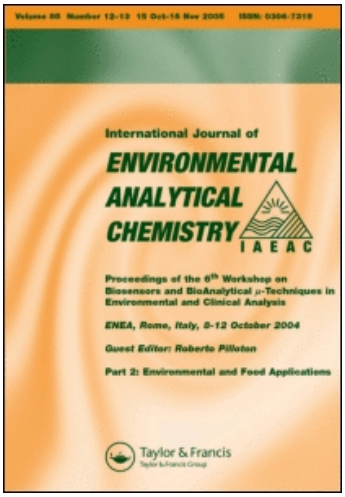

International Journal of Environmental Analytical Chemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713640455

Simultaneous Determination of Urinary Metabolites of Toluene, Xylene, Styrene and Ethyl Benzene by Solid-Phase Extraction Technique and High-Performance Liquid Chromatographic/Photo Diode Array Detection

I. F. Mao[a]; M. L. Chen[a]; E. W. Lo[a]
[a] Institute of Public Health, School of Medicine, National Yang-Ming University, Taipei, Taiwan

To cite this Article Mao, I. F. , Chen, M. L. and Lo, E. W.(1996) 'Simultaneous Determination of Urinary Metabolites of Toluene, Xylene, Styrene and Ethyl Benzene by Solid-Phase Extraction Technique and High-Performance Liquid Chromatographic/Photo Diode Array Detection', International Journal of Environmental Analytical Chemistry, 64: 1, 1 — 9
To link to this Article: DOI: 10.1080/03067319608028330
URL: http://dx.doi.org/10.1080/03067319608028330

Intern. J. Environ. Anal. Chem., Vol. 64, pp. 1–9
Reprints available directly from the publisher
Photocopying permitted by license only

SIMULTANEOUS DETERMINATION OF URINARY METABOLITES OF TOLUENE, XYLENE, STYRENE AND ETHYL BENZENE BY SOLID-PHASE EXTRACTION TECHNIQUE AND HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC/PHOTO DIODE ARRAY DETECTION

I. F. MAO, M. L. CHEN* and E. W. LO

Institute of Public Health, School of Medicine, National Yang-Ming University, Shih-Pai, Taipei, Taiwan

(Received, 15 August 1995; in final form, 1 November 1995)

To determine the level of urinary hippuric acid, methylhippuric acid, mandelic acid, and phenylglyoxylic acid, a method by solid-phase extraction (SPE) pretreatment and reversed-phase high performance liquid chromatography with photo diode array detection has been developed in this study. Six milliliter of methanol is the only solvent used in the SPE procedures. After extraction, the urine sample is injected onto a 5 μm RP18-LiChrospher 100 column (150 mm × 4 mm I.D.). The mobile phase is a mixture of acetonitrile (15 parts) and phosphate buffer (85 parts) containing tetrabutyl ammonium hydrogen sulfate (8.5 mM). The entire procedure is performed in 40 min. The method is simple, rapid and highly specific, and applicable in field study of toluene, xylene, styrene and ethyl benzene exposure.

KEY WORDS: Hippuric acid, methylhippuric acid, mandelic acid, phenylglyoxylic acid, solid phase extraction, photo diode array detection.

INTRODUCTION

Toluene, xylene, styrene and ethyl benzene are widely used in industry, and they have the characteristics of temporal and spatial variation of concentrations in the workplace[1]. These chemicals can be inhaled and/or absorbed via the skin, and since the metabolic pathways of them have been proposed by many toxicokinetic studies[2–5], determining the urinary metabolites of these organic solvents has been reported for monitoring the worker's exposure[6–9]. This kind of biological monitoring provides a reliable evaluation of the occupational exposure of the individual worker exposing to these chemicals[10–11].

Toluene and xylene are generally used as organic solvents[12]. Xylene exists in three isomers: ortho-, meta- and para-xylene. Commercial xylene, referred to as xylol, consists of a mixture of xylene with ethyl benzene[13]. Styrene has found extensive application in the synthetic resin industry. Most of the toluene and o-, m- and p- xylene inhaled by

* Corresponding author.

workers are metabolized and excreted in urine as hippuric acid (HA) and o-, m- and p-methylhippuric acid (MHA), respectively. Ethyl benzene and styrene are metabolized to the same metabolites, they are mandelic acid (MA) and phenylglyoxylic acid (PhGA). US/ACGIH (1985) documented the biological exposure indices (BEI) on urinary metabolites of toluene as hippuric acid (2.5 g/g creatinine), xylene as methylhippuric acid (1.5 g/g creatinine), styrene as mandelic acid (0.8 g/g creatinine) and phenylglyoxylic acid (240 mg/g creatinine) and ethyl benzene as mandelic acid (1.5 g/g creatinine)[14].

Urine specimen can be obtained in a noninvasive manner and available in sufficient volume at workplace. But a cleanup procedure is required[15]. Liquid-liquid extraction has found extensive application as a pretreatment technique. However, solid-phase extraction (SPE) has recently become a convenient, inexpensive, and time-saving alternative to liquid-liquid extraction. In addition, SPE methods significantly reduce the volume of organic solvents required for a typical sample preparation[16].

Because of the concurrent use of toluene, xylene, ethyl benzene and styrene in factory, urinary metabolites have to be measured simultaneously for workers' exposure. Liquid chromatography is a conventional method and previous investigations used a single wavelength of UV detector for measuring these metabolites[6,17–19,20]. The disadvantage of using a single wavelength is less sensitive to some analytes during compromising with simultaneous determination of all interested analytes because the maximum molar absorption of these four metabolites are not the same. To simultaneously determine the urinary hippuric acid, methylhippuric acid, mandelic acid and phenylglyoxylic acid, a new method, including the SPE pretreatment of urine samples and an isocractic HPLC with photo-diode array detection is developed in this study. Additionally, the method is employed to evaluate workers exposed predominantly to toluene, xylene, and ethyl benzene.

EXPERIMENTAL

Reagents

Hippuric acid, mandelic acid, phenylglyoxylic acid, phosphate buffer (pH 6.6 at 25°C) were purchased from Sigma Chemical (St. Louis, MO, USA). 3- methylhippuric acid, methanol, sodium hydroxide, hydrochloric acid, acetonitrile, tetrabutylammonium hydrogen sulfate were obtained from E. Merck (Darmstadt, Germany). Solvent and all other chemicals were of the reagent grade. Milli Q (Nihon Millipore, Yonezawa, Japan) deionized water was used for all aqueous solutions.

Apparatus

A vacuum manifold for twelve columns (J & W Scientific, Folsom, CA, USA) was used for manipulating the eluting rate of sample during solid-phase extraction. Analysis was performed by a Hitachi (Tokyo, Japan) high-performance liquid chromatography. The Hitachi system consists of a model L-6200 intelligent pump, a valve injector fitted with a 25- μL loop, a model L-3000 photo diode array detector, and a model L-6100 interface for linking the UV detector and a model D-6000 data management software. The software was executed through a computer (Copam computer, Taiwan) for on-line

recording the absorption spectra. A Anna model 8520 (Italy) pH meter was used for pH values measurement.

Urine collection

The shift-end urine samples of workers in a paint manufacturing plant were collected for the metabolite analyses; sixteen workers participated in this study. The urine samples were collected in glass containers and chill transported to the laboratory within the same day, then stored at –20°C and analyzed within one week.

Chromatographic conditions

A reversed-phase ion pair HPLC system was developed; a 150 mm × 4 mm I.D., 5-μm, RP18-LiChrospher 100 column (E. Merck, Darmstadt, Germany) was used. The mobile phase was a mixture of acetonitrile (15 parts) and pH = 6.6, 34 mM phosphate buffer (85 parts) containing 8.5 mM tetrabutyl ammonium hydrogen sulfate. The flow rate was 0.8 ml/min.

Solid-phase extraction

Frozen urine samples were thawed and made homogeneous by gentle stirring. The pH values of urine samples (1 ml) were adjusted to 7.5 with 0.5 N sodium hydroxide solution and applied onto the top of a strong anion exchanger column, the SAX 730079 cartridge (40 μm particle size, 500 mg, Macherey-Nagel Co., Germany), which had been preconditioned with 6.0 ml of methanol and 6.0 ml of water. Washing with 5 ml of water followed the sample applying and then the adsorbed compounds on the cartridge were eluted with 1 ml of 4.0 N sodium hydroxide solution. The washing and elution were proceed under the vacuum manifold. After elution, the extract was adjusted to pH = 6.6 with a 10 N or 2 N hydrogen chloride solution and filtered through a 0.2 μm nylon filter (25 mm in diameter, Sun Brokers Co., USA). The filtrate was collected and 25 μl of this solution was injected into LC injector.

Preparation of standard curve

The standard curves were prepared by dissolving hippuric acid, methylhippuric acid, mandelic acid, and phenylglyoxylic acid with methanol-water (1:1, v/v). After series dilution, working solutions including the described compounds in optimal concentration ranges were used for worker's urinary metabolites measurement. Standard curves were constructed by plotting the peak area against the concentration of the analyte.

Recovery

For the calculation of the recovery, urine sample was homogeneously mixed and divided into two portions. These two portions were treated with identical procedures except for the addition of a known amount of the four metabolites in one portion. After SPE

treatment and chromatographic measurement, the recovery was calculated by comparing the concentrations obtained and the added known amount of the four metabolites.

Precision

Within-day, precision for each metabolite was determined by analyzing urine samples at several spiked concentrations for triple tests, Between-day, precision was evaluated by assessing urine samples at different spiked concentrations for each metabolite on three separate occasions. The coefficient of variation for each metabolite was calculated from the standard deviation of the arithmetic mean of the peak area at each concentration.

Detection limit

The limit of detection was determined by the concentration presented with a signal-to-noise ratio of 3.

The measurement of urine specimen was expressed as mg/g creatinine. The creatinine was measured by Jaffe's method[21].

RESULTS AND DISCUSSION

The typical UV spectra 200–400 nm at pH 6.6 for the metabolites are determined. The light absorption at the wavelength of 235 nm was chosen for the quantitative analyses of hippuric acid, methylhippuric acid and mandelic acid. Alternatively light absorption at 254 nm was used for phenylglyoxylic acid in this study. Obviously, the maximum molar absorptivities of the four metabolites are different. However, an identical single wavelength, such as 225 nm[6,17–19] or 257 nm[20] was used to measure the four compounds in previous studies.

The photo diode array detector provides an additional advantage through means of multi-signal detection to indicate the characteristic of peak-area ratio when the confirmation of peak identity and peak homogeneity is required. Since the absorbance ratio for pure compound is constant, a coeluting impurity peak subsequently leads to the deviation from these peak-area ratios.

The chromatograms of a standard solution of the four metabolites and urine with and without SPE pretreatment are presented in Figure 1. Those results indicate that these analytes are separated in good peak shape (Figue 1-a). One urine specimen, filtered through a 0.22 μm nylon-66 filter only, seems overloading the LC column and indicates complicated background peaks in chromatogram (Figure 1-b). Besides, the overloading and complex matrixes caused the shift of retention times. After SPE pretreatment of the other urine specimen, most of these peaks are excluded (Figure 1-c), the retention times are quite identical with the standard solution. A little shift of retention time from Figure 1-a may result from the variation of pH values between standard solutions and SPE treated samples. The SPE pretreatment favors the maintenance and performance of LC column.

Because C18 phase of SPE cartridge was not satisfactory to the simultaneous extraction of four analytes in a pilot study, a strong anion exchanger phase column was used. To improve the selectivity and recovery of the column, sorbent conditioning, sample treatment, wash procedure and sample elution of the SPE procedures were

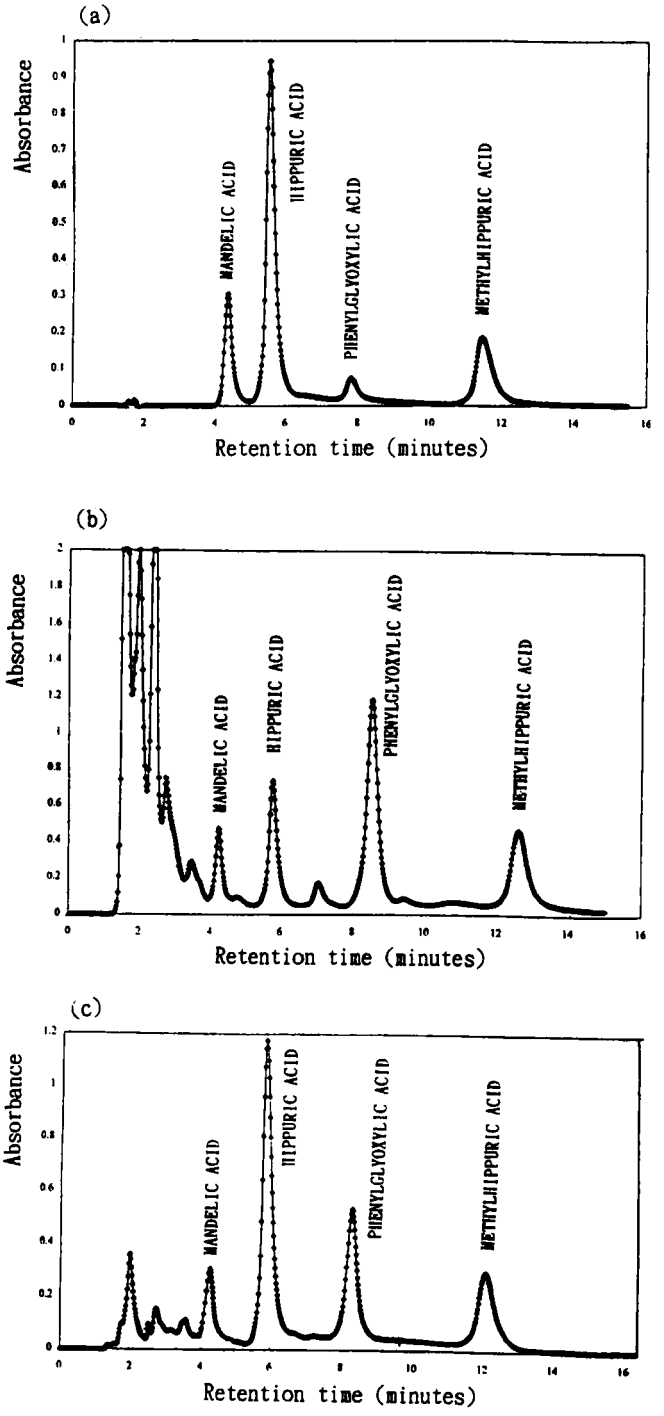

Figure 1 Chromatogram of (a) a standard solution with a mixture of the four metabolites tested, (b) a urine sample filtered through a 0.22 μm filter and (c) a urine sample pretreated with solid-phase extraction.

repeatedly tested, especially the elution step. The strong adsorption behavior of four metabolites on the column led to the application of solutions with high pH value to elute the adsorbents. Thereafter, neutralization and filtration are deemed necessary for the eluent to compromise with the chromatographic condition.

Several mixtures of mobile phases were tested in the present experiment. The ion-pair method proves to be a better separation for simultaneous analysis of the four metabolites than any other mixed solutions of methanol or acetonitrile. The isomer of o-methylhippuric acid (The peak has a retention time of 7.5 min in Figure 1-b) is well separated from m- and p-methylhippuric acid (The peak has a retention time of 12.7 min in Figure 1-b). However, the latter two isomers are eluted at the same retention time. Since m-xylene is the predominant component in isomer mixture and thus the m-methylhippuric acid is a major metabolite of worker, only m-methylhippuric acid was evaluated for xylene exposed workers in this study.

Standard curves for all of these metabolites in methanol-water solution are linear with the coefficients of correlation greater than 0.9994. The ranges from 4 to 3000 mg/L for hippuric acid. The concentrations of meta-methylhippuric acid ranges from 8 to 2000 mg/L; those of mandelic acid and phenylglyoxylic acid ranges from 25 to 10000 mg/l and 6 to 1000 mg/l, respectively. These linear dynamic ranges are very wide and all of the minimum quantitative concentrations are far below the biological exposure indices recommended by US/ACGIH[11]. A known concentration of each metabolite was added to three urine samples for determining the quantization ranges of the urinary metabolites. Analysis was performed after pretreatment by SPE, the results show in Table 1. The overall recovery rate is 82.21 ± 1.03% for hippuric acid, the quantization range is from 12.5 ng/L to 2000 mg/L; 88.15 ± 5.50% for methylhippuric acid, the range is from 12.5 mg/L to 500 mg/L; 84.79 ± mandelic acid, the range is from 25 mg/L to 800 mg/L; 97.78 ± 4.74% for phenylglyoxylic acid, the range is from 12.5 mg/L to 500 mg/L. The quantitative ranges in this study are determined by the concentration of workers urinary metabolites; the recovery and precision are also established in these ranges.

Comparing the recovery rates of the study method with liquid-liquid extraction[22], the latter possessed recovery rates from 95.67% to 108% for these four metabolites. The superior solvent strength inherent in liquid phase results in the higher recovery rate. However, the precision of this developed method is satisfactory. The pooled within-day coefficients of variation by triple analysis of series dilution for hippuric acid, m-methylhippuric acid, mandelic acid, and phenylglyoxylic acid are 5.47%, 6.39%, 6.48% and 6.50%, respectively. The pooled between-day C.V. are 3.97%, 5.54%, 5.00% and 5.68%, respectively for the analysts (Table 2). The precision, comparable with that of liquid-liquid extraction, ranged from 3.00% to 7.23%[22]. In addition to the elimination of using solvent, the solid phase extraction procedure takes only twenty minutes but the liquid-liquid extraction needs more than forty minutes. Since the reliable precision of this method, it provides an obvious advantage over the conventional liquid-liquid extraction method.

The lower limits of detection were determined by analyzing each analyst with decreasing concentrations of the compound tested. The detection limits are 2.45 mg/L, 2.26 mg/L, 7.79 mg/L and 2.63 mg/L for hippuric acid, m-methylhippuric acid, mandelic acid, and phenylglyoxylic acid, respectively.

The concentrations of metabolites in urine of the sixteen exposed workers are summarized in Table 3. Excluded the samples below he quantitative dynamic range, all of other samples are calculated into the geometric mean concentration of the group. The geometric mean level of 16 workers' urinary hippuric acid is 354 mg/g Cr. at the

Table 1 Recoveries of added HA, m-MHA, MA and PhGA from unexposed urine.

Metabolites	*Concentration (mg/L)*	*Recovery (%)*
HA	12.5	84.13 ± 5.25*
	25	82.93 ± 1.99
	50	83.41 ± 7.28
	100	81.30 ± 1.68
	200	80.68 ± 3.96
	500	82.07 ± 3.20
	1000	81.57 ± 3.45
	2000	83.57 ± 1.85
mean ± SD		82.21 ± 1.03
m-MHA	12.5	87.57 ± 3.47
	25	96.50 ± 3.57
	50	91.56 ± 4.02
	100	88.48 ± 1.69
	200	81.13 ± 3.12
	500	83.67 ± 2.00
mean ± SD		88.15 ± 5.50
MA	25	84.37 ± 5.55
	50	83.00 ± 2.04
	100	83.80 ± 2.98
	200	91.54 ± 4.15
	400	85.39 ± 1.59
	800	80.65 ± 2.27
mean ± SD		84.79 ± 3.67
PhGA	12.5	105.77 ± 5.05
	25	100.90 ± 4.63
	50	96.19 ± 1.40
	100	93.06 ± 0.82
	200	94.30 ± 1.08
	500	96.47 ± 1.86
mean ± SD		97.78 ± 4.74

* Mean values and SD of three determinations with urine samples

end of shift, with the highest concentration of 1590 mg/g Cr.. The geometric mean of the workers' urinary methylhippuric acid is 95 mg/g Cr.; the highest concentration is 380 mg/g Cr.. Eight workers' urinary mandelic acid levels are below 25 mg/L; others are 355 mg/g in geometric mean concentration. For phenylglyoxylic acid, the geometric mean concentration is 56 mg/g, excluding the samples below 12.5 mg/L. None of the exposed workers has urinary metabolite exceeding the BEI recommended by US/ACGIH.

Liquid-liquid extraction or centrifuging is a traditional method in urine pretreatment. The development of filtering material and sorbent and demand for curtailing the organic solvent use make the cleanup techniques have had dramatic changes. Ultra filtration and solid-phase extraction have become preferable alternatives. Coupled with high-performance liquid chromatography, solid-phase extraction provides a complete cleanup sample for feeding to the LC injector if the extraction procedure is well-established. Advantages of using SPE for pretreatment are: (1) the overloading of LC column will not occur; (2) the column can be easily maintained without overusing of

Table 2 Within- and between-day precisions of HA, m-MHA, MA and PhGA.

Concentration (mg/L)	*Within-day (n = 3) C.V. (%)*	*Between-day (n = 9) C.V. (%)*
HA		
12.5	4.58	3.48
50	4.89	3.40
100	5.23	3.09
200	6.40	5.00
1000	5.90	4.78
2000	5.79	4.06
mean ± SD	5.47 ± 0.68	3.97 ± 0.78
m-MHA		
12.5	6.67	6.05
50	5.62	5.29
100	6.54	4.99
200	5.92	4.41
500	7.19	6.95
mean ± SD	6.39 ± 0.62	5.54 ± 0.99
MA		
25	5.73	5.17
200	5.27	4.69
400	8.13	5.06
800	6.79	5.07
mean ± SD	6.48 ± 1.27	5.00 ± 0.21
PhGA		
12.5	5.64	4.66
25	5.06	7.20
50	6.75	3.12
100	7.64	6.44
500	7.42	6.97
mean ± SD	6.50 ± 1.12	5.68 ± 1.74

Table 3 Levels of urinary HA, MHA, MA, PhGA in exposed workers (unit: mg/g Cr.).

Metabolites	*Sample no.*	*Concentration*	
		*GM**	*GSD***
HA	16	354	262
MHA	16	95	94
MA	8	355	159
PhGA	8	56	95

* GM means geometric mean.
** GSD means geometric standard deviation.

solvent; and (3) the column can be used in extended life even in a complicated sample, such as urine. By selecting the column phase, packing material, and extraction solvent, solid-phase extraction method has been developed for monitoring polar and non-polar compounds in various samples[23–25].

The solid-phase extraction method is performed in 20 minutes and only 6 ml of methanol are used, excluding aqueous solution. The entire procedure (including extraction and chromatographic run) can be achieved in 40 minutes, which makes our method more feasible than previous methods for the urinary metabolite determination.

In conclusion, we have developed a simple, precise and accurate method for the simultaneous measurement of toluene, xylene, styrene, and ethyl benzene metabolites in urine. The high sensitivity and wide dynamic range of linearity make it available for routine evaluation of workers' exposure in the field study.

Acknowledgement

This study was supported by a grant (No. NSC83-0421-D-010-049Z) from the National Science Council, Republic of China.

References

1. L. Parmeggiani, *Encyclopedia of Occupational Health and Safety*, (ILO, 1983), 3rd ed., p. 1583, p. 2114, p. 2184, p. 2335.
2. L. D. Pagnotto and L. M. Liberman, *Am. Ind. Hyg. Assoc. J.*, March-April, 129–134 (1967).
3. L. Drummond, J. Caldwell and H. K. Wilsom, *Xenobiotica*, **19**, 199–207 (1989).
4. L. Drummond, J. Caldwell and H. K. Wilsom, *Xenobiotica*, **20**, 159–168 (1990).
5. G. A. Snowronski, R. M. Turkall and A. R. Kadry, *Environ. Research*, **51**, 182–193 (1990).
6. M. Ogata and T. Taguchi, *Int. Arch. Occup. Environ. Health*, **58**, 121–129 (1986).
7. T. Kawai, K. Mizunuma and T. Yasugi, *Int. Arch. Occup. Environ. Health*, **63**, 69–75 (1991).
8. O. Inoue, K. Seiji and T. Kawai, *Int. Arch. Occup. Environ. Health*, **64**, 533–539 (1993).
9. M. Y. Huang, C. Jin and Y. T. Liu, *Occup. Environ. Med.*, **51**, 42–46 (1994).
10. L. Parmeggiani, *Encyclopedia of occupational health and safety*, (ILO, 1983), 3rd ed., p. 272.
11. US/ACGIH: Threshold limit values and biological exposure indices for 1993–1994. (American Conference of Governmental Industrial Hygienists, 1994).
12. A. B. William, *Recognition of health hazards in industry. A review of materials processes*, (John Wiley & Sons, 1981), p. 102, p. 208.
13. A. Hamilton and H. L. Hardy, *Industrial toxicology*. Revised by A. J. Finkel, (John Wright • PSG Inc., 1983), 4th ed., p. 252.
14. US/NIOSH, Documentation of biological exposure indices, (US/NIOSH, 1988), BEI-11, BEI-29, BEI-35, BEI-47.
15. R. S. S. Murthy, J. Holzbecher and D. E. Ryan, *Anal. Chem.*, **6**, 113 (1982).
16. J. Hartleb, S. Eue and A. Kemper, *J. Chromatog.*, **622**, 161–171 (1993).
17. G. Poggi, M. Giusiani and U. Palagi, *Int. Arch. Occup. Environ. Health*, **50**, 25–31 (1982).
18. M. Ogata and T. Taguchi, *Int. Arch. Occup. Environ. Health*, **59**, 263–272 (1987).
19. M. Ogata and T. Taguchi, *Int. Arch. Occup. Environ. Health*, **61**, 131–140 (1988).
20. O. Inoue, K. Seiji and T. Suzuki *et al.*, *Bull. Environ. Contam. Toxicol.*, **47**, 204–210 (1991).
21. D. Heinegard and G. Tiderstrom, *Clin. Chim. Acta*, **43**, 305 (1973).
22. J. Angerer and K. H. Schaller, title in proofs (VCH, Germany, 1988), 52–66.
23. A. DiCorcia and M. Marchetti, *Anal. Chem.*, **63**, 580 final page (1991).
24. F. Andreolini, *Anal. Chem.*, **59**, 1720 final page (1987).
25. F. P. Bigley and R. L. Grob, *J. Chromatogr.*, **360**, 407–416 (1985).