ELSEVIER

Journal of Chromatography B, 738 (2000) 387–394

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Reversed-phase liquid chromatographic column switching for the determination of *N*-methylcarbamates and some of their main metabolites in urine

P. Parrilla Vázquez, J.L. Martínez Vidal*, J. Martínez Fernández

Department of Analytical Chemistry, University of Almería, 04120 Almería, Spain

Received 14 July 1999; received in revised form 24 November 1999; accepted 24 November 1999

Abstract

A column switching system for the determination of some polar pesticides and their main metabolites, such as aldicarb, aldicarb sulphoxide, aldicarb sulphone, carbofuran and 3-hydroxicarbofuran, in human urine has been developed. The limits of detection were between 0.3 and 1 μg/l. We used a simple solid-phase extraction with graphite carbon and a RPLC–LC analysis with UV detection yielding average recoveries between 84 and 110% ($N=5$) with RSD between 4 and 8%.

Keywords: *N*-Methylcarbamates

1. Introduction

The increasing knowledge of the effects of pesticides on the state of public health and the environment results in an ever growing need for fast, sensitive and reliable analytical techniques for the biological monitoring of pesticides.

Conventional HPLC techniques are not suitable for trace analysis of pesticide in complicated matrices such as biological fluids and should include other additional sample pre-treatment steps. The sample treatment in these methods is tedious and may be accomplished by multiple extraction steps and protein precipitation [1].

HPLC column switching seems to be of growing interest for the analysis of medicine in biological samples [2–6] but analysis of pesticides residues based on LC–LC in biological samples is minor [7]. The relevant aspects of column switching optimization for the determination of moderately polar pesticides have been reviewed [8].

The main advantage of coupled chromatographic techniques is the enhancement of both selectivity and sensitivity combined with a high potential for automation. Sensitivity is enhanced by the use of large-volume injection techniques combined with peak compression. The enhancement of selectivity is quite obvious if one uses two separations with different selectivity instead of one. Normally we start to optimise the column switching method with columns based on similar functionalities because it is easier and in this way we avoid problems with the compatibility of solvents or changes in the retention times of the compounds. If the obtained selectivity is satisfactory it is not necessary to use columns with

*Corresponding author. Tel.: +34-950-215-024; fax: +34-950-215-070.

0378-4347/00/$ – see front matter
PII: S0378-4347(99)00560-5

different selectivity. Moreover, the use of on-line techniques reduces the amount of manual operations in the analytical procedure [8,9].

The aim of this study was to develop a method using a column switching technique for the determination of aldicarb and its major metabolites (aldicarb sulphoxide and aldicarb sulphone) and carbofuran with its metabolite 3-hydroxicarbofuran in human urine samples for the biomonitoring of agricultural workers.

Gas chromatography (GC) is one commonly used technique for the determination of carbamates [10]. Direct GLC causes most *N*-methylcarbamates to be decomposed into their respective phenols to different extents. However, GC analysis for carbamate phenols (possible hydrolysis products of carbofuran) is not a widely accepted practice because the determination of phenols is hindered by the low sensitivity of flame ionization detectors for these compounds; nevertheless, on derivatization to their ethers [11] or acetates [12], these substances can be determined. Moreover, many derivatizing reagents are toxic, carcinogenic or explosive. GC–MS has been used for the determination of aldicarb in serum [13]. Alternative techniques used for the determination of *N*-methylcarbamates include spectrophotometry [14], TLC [15] and HPLC [16,17]. McGarvey [18] reviewed the literature concerning all aspects of determination of *N*-methylcarbamate residues in water, plants, and air by HPLC, including extraction, clean-up, chromatographic separation and detection.

Aldicarb and carbofuran are added to the soil for nematode control and for insect or mites control as a result of its efficient translocation (systemic activity) by the plant. The primary oxidative degradates for aldicarb are aldicarb sulphoxide and aldicarb sulphone and for carbofuran is 3-hydroxycarbofuran. The metabolites are extremely polar [19]. Information on the analytical methods for these very polar metabolites is limited in comparison with aldicarb and carbofuran. Unfortunately, each of the quoted analytes displayed one distinctly unfavourable characteristic, e.g., very low retention on C_{18}-bonded silica (aldicarb sulphoxide and aldicarb sulphone), an extremely non-selective detection wavelength or small molecular extinction coefficient (see Table 1). To achieve that, RPLC–LC–UV had to be preceeded by off-line solid-phase extraction (SPE).

2. Experimental

2.1. Materials, reagents and solvents

Aldicarb and carbofuran (content >99%) were obtained from Riedel-de Haën (Seelze, Germany). Aldicarb sulphoxide, aldicarb sulphone and 3-hydroxycarbofuran were obtained from Dr. S. Ehrenstorfer (Promochem, Wesel, Germany). Acetonitrile (HPLC-grade) from Merck (Darmstadt, Germany), and HPLC-grade water was obtained by purifying demineralized water in a Milli-Q system (Millipore, Bedford, MA, USA).

A mixture of $CH_3CN–H_2O$ (5:95, v/v) was used as the first mobile phase (M-1) while the second one (M-2) consisted of an $CH_3CN–H_2O$ gradient (see Table 2). Analytes were monitored at 210 nm.

Disposable 3-ml SPE cartridges containing 500 mg of graphite carbon obtained from Supelco (Bellefonte, USA). Cartridges were pre-conditioned sequentially with 10 ml of ethyl acetate, 15 ml of CH_3CN and 10 ml of Milli-Q water.

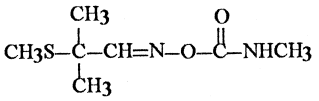
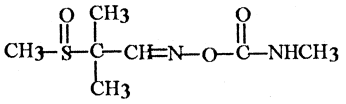
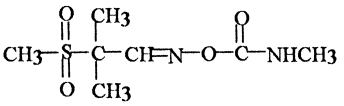
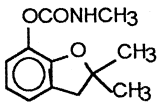
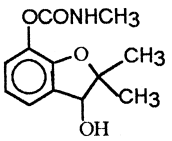
2.2. Preparation of standard solutions

Stock standard solutions of analytes were prepared by dissolving accurately weighed amounts of each pesticide in acetonitrile (500 μg/ml). Working standard solutions were made by diluting aliquots of the stock standard solutions to the appropriate concentrations in water. Fresh solutions were prepared daily. Aliquots of these working standard solutions were used to prepare calibration standards or to spike urine samples. The diluted solutions were kept in a refrigerator at 4°C.

2.3. Handling and storage of biological fluids

Urine samples were collected from a worker occupationally exposed to these pesticides. In addition, urine samples from ten people without exposure to these compounds were used as controls. All samples were collected in polyethylene bottles and

Table 1
Structural formulae and some characteristics of the studied compounds.

Compound	Formulae	VP[a] (mPa)	Sol. H_2O (g l^{-1})	λ_{max} (nm)	$\epsilon_{200\ nm}$ (l mol^{-1} m^{-1})
Aldicarb		13.0	6	200	19 000
Aldicarb-sulphoxide		–	330	200	15 500
Aldicarb-sulphone		12.0	10	200	16 700
Carbofuran		2.7	0.7	200	38 700
3-OH-Carbofuran		–	–	200	47 000

[a] Vapour pressure.

frozen at −20°C until analysis. They were brought to ambient temperature for 1 h before analysis.

2.4. Sample preparation procedure

A 5-ml volume of centrifuged human urine (20 min at 1600 *g*) diluted with 5 ml of water, mixed on a vortex mixer for 60 s was applied to a preconditioned 500 mg graphite carbon cartridge and dried by passing air for 10 min. After elution with 5 ml of acetonitrile the eluate was evaporated to dryness under a gentle stream of nitrogen and redissolved in 2 ml of LC-grade water. An aliquot of 1 ml was finally injected in the LC–LC system.

Table 2
Mobile phase gradient (M-2) used for the determination of aldicarb, carbofuran and their metabolites

Time (min)	Acetonitrile (%)	HPLC water (%)
0	15	85
6	100	0
9	100	0
14	15	85

2.5. Equipment

The system consists of an isocratic Model 510 LC pump (P-1), a gradient Model 600 LC pump (P-2) from Waters, a Rheodyne six-port injection valve (Model 7725), a Type 7000 high pressure column switching valve (V) from Rheodyne (Berkeley, CA, USA) and a Model 486 variable wavelength UV–Vis detector (D) from Waters set at 210 nm.

A 30×4.6 mm I.D. first separation column (C-1) packed with 5 μm Spherisorb ODS-2 from Waters and a 100×3 mm I.D. second separation column (C-2) packed with 5 μm Hypersil Shandon Green Env. C_{18} column.

Recording of chromatograms and quantitative measurements of peak areas were performed with a Baseline 810 (Waters).

2.6. LC–LC conditions

A schematic diagram of dual-column system used in the present study is shown in Fig. 1. The analytical process was as follows: a 1 ml aliquot of urine extract was injected into the sample loop and was transferred to the pre-separation column at a flow-rate of 1 ml/min (status B). In 2.0 min from the injection, the switching valve was turned to the alternate position, so that the pre-separation column and main separation column were connected together (status A).

The mobile phase used in the pre-separation column (pump 2) carried analytes from the pre-separation column to the main column was M-1. After a duration of 5.7 min, the switching valve was turned back to the initial position to disconnect the pre-separation column and the main separation column, so as to re-equilibrate the former with the mobile phase in the pre-separation (status B). Analytes were eluted from the main column with the mobile phase used for the main separation (pump 2) (M-2). (See Table 2). The time required for one analytical run was approximately 14 min.

A guard column was mounted before the analytical column in order to protect it from interfering endogenous constituents in urine.

3. Results and discussion

3.1. Switching parameters

The chromatographic system for the separation of the analytes was chosen in order to get optimum separation with high selectivity in a short analysis time.

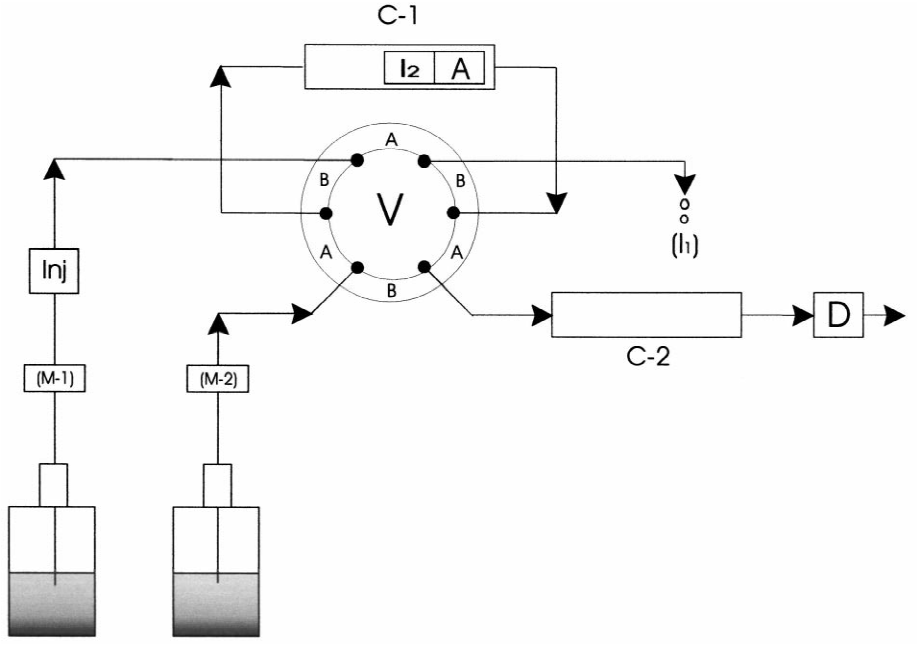


Fig. 1. Schematic presentation of separation procedure involved in coupled-column RPLC. Inj=Injector; C-1, C-2=first and second separation column, respectively; M-1, M-2=mobile phases; I1, I2=interferences; A=target analytes; V=high-pressure valve; D=detector.

The C-1 column has to be selected in order to improve the clean up performance of the system and, thus, to develop a successful RPLC column switching operation for compounds in the polarity range of aldicarb sulphoxide and aldicarb sulphone. Previous works [2,20] showed that the use of short columns packed with 5 μm C_{18} improved significantly the separation between interferents and analytes. A wavelength of 210 nm was chosen for detecting these compounds. Lower wavelengths allow a higher sensitivity but the matrix urine samples caused troubles because of the co-extracted interferences which elute as a broad hump in the chromatogram.

Firstly, retention on a C_{18} column was studied using C-2. A variety of mobile phases were tested in order to find out the optimum chromatographic system for the analysis of these compounds. The mobile phases, were binary mixtures of an aqueous solution with acetonitrile in several ratios. Capacity factors (K') for different mobile phases, with an acetonitrile content ranging from 0 to 100% were obtained. A buffer to control the pH of the mobile phase was not necessary for these analytes because satisfactory peaks were obtained and the validation of the method was good, this results in a simpler mobile phase. The results show that problems occur with aldicarb sulphoxide and aldicarb sulphone as they show insufficient retention even in a completely aqueous mobile phase and this results in an early breakthrough and excessive band broadening on transfer to the analytical column.

The investigations with different loops were performed in order to optimize the signal-to-noise ratio and thus to improve the detection limit. Different sample volumes were essayed (from 20 to 2000 μl) injecting standard mixtures of the analytes on C-1. A sample volume of 1 ml was selected as a compromise between sensitivity and speed of analysis. On injecting 2000 μl the peaks show some band broadening.

A gradient elution mode was selected for the analytical separation (Table 2). Increasing the percentage of organic modifier in the mobile phase for the second column afforded peak compression and hence increased sensitivity. Fig. 2 shows a chromatogram of an standard solution using the selected experimental conditions and a transfer volume of 3.7 ml.

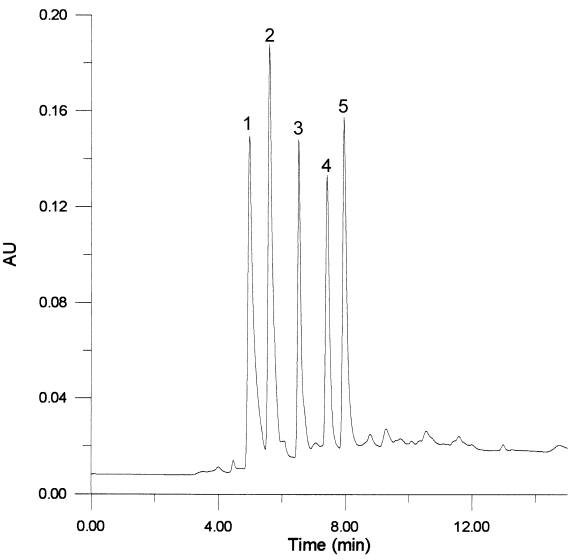


Fig. 2. LC–LC–UV chromatogram of a standard solution containing 1 μg/ml of aldicarb sulphoxide and aldicarb sulphone, 0.5 μg/ml of 3-hydroxicarbofuran, aldicarb and carbofuran in LC-grade water registered at 210 nm. Peak number: (1) aldicarb sulphoxide, (2) aldicarb sulphone, (3) 3-hydroxycarbofuran, (4) aldicarb, (5) carbofuran.

3.2. Recovery and clean-up

According to previous extraction studies for these compounds it is not necessary to hydrolyse the sample [21]. Passing the urine through graphite carbon cartridges it is possible to recover analytes or to clean-up impurities from the sample [15,22]. The clean-up efficiency was tested on uncontamined human urine samples.

When analyzing a sample using SPE and only C-2 a completely masked chromatogram was obtained. The potential of the LC–LC system is clearly illustrated in Fig. 3 which compares the chromatogram corresponding to a human blank urine control sample (b) with and (a) without column switching. The chromatograms clearly show the efficiency of the clean-up when using column switching. Chromatogram (a), obtained without column switching, is completely masked by a large interfering peak from the extract. Chromatogram (c) was obtained from the same sample after column switching and SPE with disposable graphite carbon cartridge. It is evident that the background is reduced. Similar results were

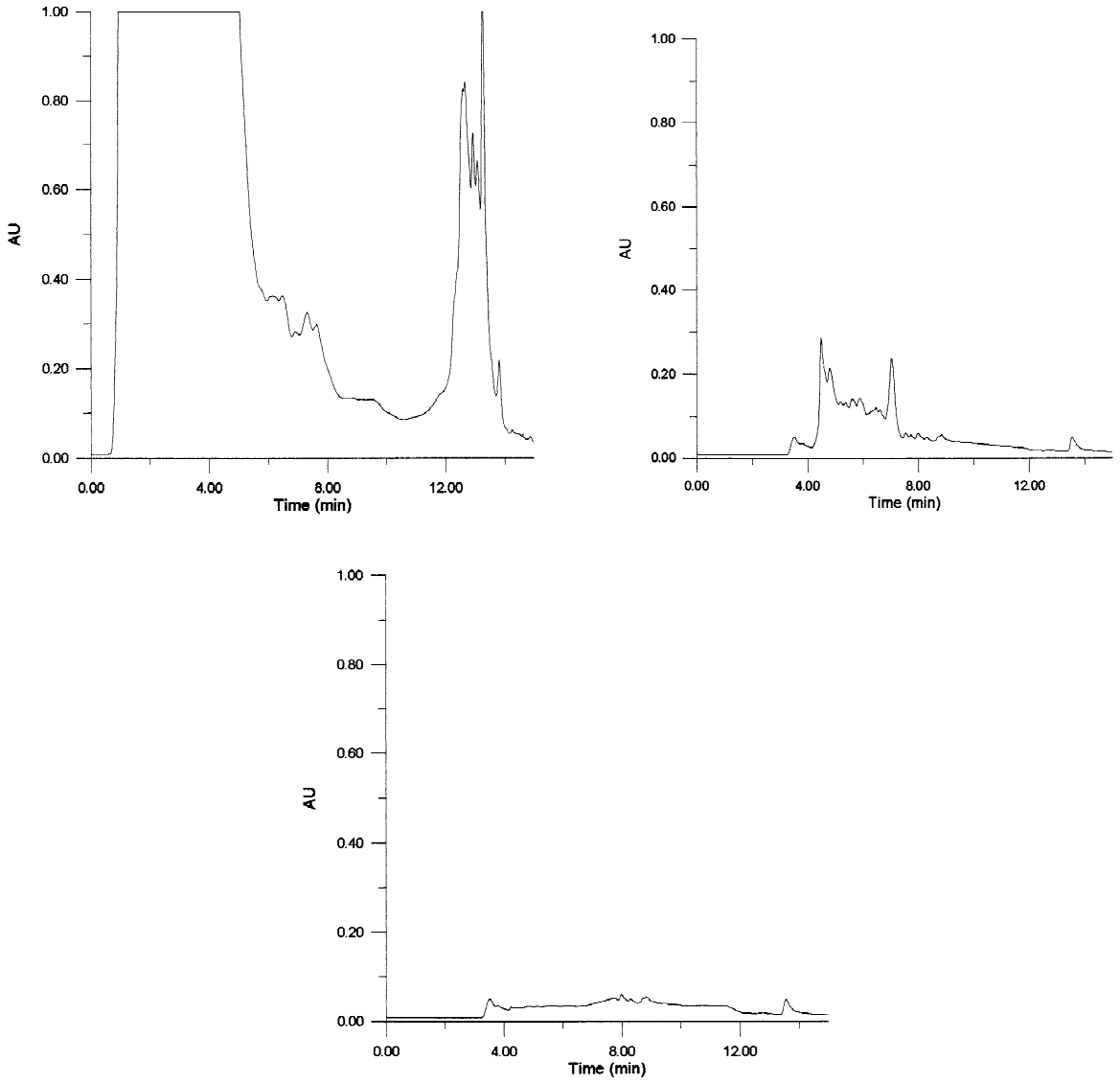


Fig. 3. Chromatograms of 1000 μl of blank urine sample, injections registered at 210 nm. Chromatogram (a) without and (b) with column switching. Chromatogram (c) with column switching and SPE with graphite carbon cartridge.

found with other different blank urine control samples.

The limits of detection were lower than 1 μg/l because the final extract contains very few interfering peaks, as shown in Fig. 3.

Quantitation of peaks was performed by an external standard method, using measurement of peak areas. Calibration graphs (nine points, $N=3$) were obtained for different concentrations of analytes (5–100 μg/l). The regression coefficients were >0.998 in all cases.

Recovery studies were performed on urine samples spiked with 0.01 and 0.1 μg/ml of each compound ($N=5$). The extracts were analyzed as previously described. Average recoveries were between 84 and 110% (Table 3). The relative standard

Table 3
Mean recoveries (R), relative standard deviation (RSD, $N=5$), detection and quantitation limits (LOD and LOQ, respectively) using SPE with graphite carbon cartridges

Compound	R (RSD)%[a]	R (RSD)%[b]	LOD (μg/l)	LOQ (μg/l)
Aldicarb	91(5)	105(6)	0.3	1
Aldicarb sulphoxide	97(5)	95(7)	1	3
Aldicarb sulphone	84(7)	88(4)	1	3
Carbofuran	110(6)	90(5)	0.3	1
3-OH-Carbofuran	102(8)	89(6)	0.5	1.7

[a] Spiking levels at 10 μg/l.
[b] Spiking levels at 100 μg/l.

deviation (RSD) were in the range 4–8%. Table 3 summarizes both detection and quantitation limits, calculated as signal-to-noise ratios of 3 and 10, respectively ($N=5$). The noise was defined as the signal of blank sample at the same time of analytes.

Fig. 4 shows the chromatogram obtained at 210 nm from an urine sample spiked with 5 μg/l of each compound and analyzed with the proposed procedure. An interesting feature of the present procedure is that only a small fraction of the interfering material reaches the second column, C-2, and subsequently the detector. With regard to the robustness of the method, both C_{18} columns maintained their performance during the time of experiments and readjustments of column-switching conditions (clean-up and transfer time) were not necessary, even 3 months later.

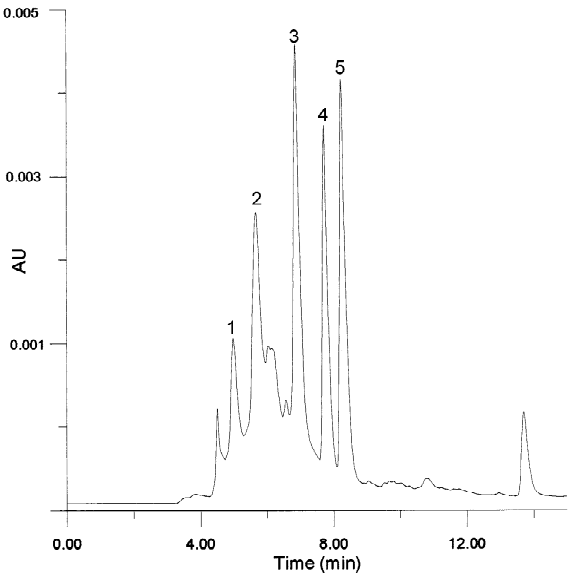


Fig. 4. LC–LC–UV chromatogram of a urine sample at 5 μg/l of each compound and analyzed with the proposed method. Wavelength 210 nm. Peak number=1 aldicarb sulphoxide, 2 aldicarb sulphone, 3 3 hydroxycarbofuran, 4 aldicarb, 5 carbofuran.

3.3. Human studies

Real samples of a pest control operator were analyzed after application in the field of Temik 10G (aldicarb 10% GR) applied directly under the ground at a rate 40 kg/ha and Cekufuran 20 LA (carbofuran 20% LA) at a rate of 4 l/ha, during 2 h. The operator wore protective equipment. Urine samples were collected over 36 h after application, starting with the first void after commencing work activities on the day of using the pesticides and we follow collecting all urine in this interval of time. Residues of these compounds were not detected.

4. Conclusions

This work has demonstrated the suitability of a simple LC–LC–UV system for the separation and identification of the aldicarb, aldicarb sulphoxide, aldicarb sulphone, carbofuran and 3-hydroxicarbofuran in human urine.

Since highly sensitive analytical methods are required for the identification and quantification of pesticides in biological samples, a minimum off-line sample manipulation is involved. Using solid-phase extraction, sensitivity was increased, and interfering products of the biological material were removed, providing acceptable recoveries of the compounds.

Acknowledgements

This study has been financially supported by the European Union Standard Measurement and Testing Programme, contract number SMT4-CT96-2048 and by the CICYT Project AMB97-1194-CE.

References

[1] P. Carbonnelle, S. Boukortt, D. Lison, J.P. Buchet, Analyst 121 (1996) 663.
[2] R. Wyss, F. Bucheli, B. Hess, J. Chromatogr. A 729 (1996) 315.
[3] E. Woolf, T. Au, H. Haddix, B. Matuszewski, J. Chromatogr. A 692 (1995) 45.
[4] S. Bompadre, L. Ferrante, M. De Martinis, L. Leone, J. Chromatogr. A 812 (1998) 249.
[5] G. Horvai, A. Hrabéczy-Páll, V. Horváth, I. Klebovich, Mikrochim. Acta 113 (1994) 171.
[6] E.J. Woolf, T. Au, B. Matuszewski, Chromatographia 39 (1/2) (1994) 79.
[7] R. Wintersteiger, B. Ofner, H. Juan, M. Windisch, J. Chromatogr. A 660 (1994) 205.
[8] E.A. Hogendoorn, C.E. Goewie, P. Van Zoonen, Fresenius J. Anal. Chem. 339 (1991) 348.
[9] P. Van Zoonen, The Science of the Total Environment 132 (1993) 105.
[10] E. Ballesteros, M. Gallego, M. Valcárcel, J. Chromatogr. 633 (1993) 169.
[11] L. Ogierman, J. Assoc. Off. Anal. Chem. 65 (1982) 1452.
[12] R.T. Coutts, E.E. Hargesheimer, F.M. Pasutto, J. Chromatogr. 195 (1980) 105.
[13] M. Lifshitz, E. Shahak, A. Bolotin, S. Sofer, J. Toxicol. Clin. Toxicol. 35 (1997) 25.
[14] M.C. Quintero, M. Silva, D. Pérez-Bendito, Analyst 114 (1989) 497.
[15] H. De la Vigne, D. Janchen, J. Planar Chromatogr. Mod. TLC 3 (1990) 6.
[16] A. Di Corcia, M. Marchetti, Anal. Chem. 63 (1991) 580.
[17] W. Blad, Fresenius J. Anal. Chem. 339 (1991) 340.
[18] B. McGarvey, J. Chromatogr. 642 (1993) 89.
[19] C.L. Munster, R.W. Skaggs, V.R. Pemmireddy, Am. Soc. Agric. Engrs. 39 (1) (1996) 55.
[20] L. Torchio, F. Lombardi, M. Visconti, E. Doyle, J. Chromatogr. A 666 (1995) 169.
[21] F.D. Harper, C.P. Weisskopf, G.P. Cobb, Anal. Chem. 70 (1998) 3329.
[22] A. Di Corcia, S. Marchese, R. Samperi, J. Chromatogr. 642 (1993) 163.