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Journal of Chromatography A, 898 (2000) 185–191

JOURNAL OF CHROMATOGRAPHY A

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Simplified determining procedure for routine residue monitoring of sulphamethazine and sulphadimethoxine in milk

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Received 30 May 2000; received in revised form 1 August 2000; accepted 8 August 2000

Abstract

A simplified determining/identifying method for residual sulphamethazine (SMZ) and sulphadimethoxine (SDM) in milk by using a high-performance liquid chromatography (HPLC) with a photo-diode array detector was presented. Both sulphonamides in cow's milk samples were extracted by only stirring with ethanol followed by an Ultrafree®–MC/Biomax as a centrifugal ultra-filtration unit. For determination/identification of SMZ and SDM, a Mightysil® RP-18 GP *Aqua* column and a mobile phase of 25% (v/v) ethanol solution (in water) with a photo-diode array detector was used. Average recoveries from spiked SMZ and SDM (10–1000 ng/ml each drug) were ⩾83% with the relative standard deviations between 1.4 and 3.7%. The limit of quantitation (LOQ) were calculated to be 5 ng/ml for SMZ and 10 ng/ml for SDM, respectively. The values were below the MRL/tolerance (SMZ, 25 ng/ml; SDM, 10 ng/ml). The total time and solvent required for the analysis of one sample were <35 min and <2 ml of only ethanol, respectively. No toxic solvents were used. The developed procedure was harmless to the human and environment.

Keywords: Sulphamethazine; Sulphadimethoxine

1. Introduction

Sulphamethazine (SMZ) and sulphadimethoxine (SDM) are regularly used in food-producing animals for therapeutic, prophylactic, or growth-promoting purposes. Improper use of these sulphonamides in lactating dairy cows is of great concern, in particular because the sulphonamide residues are turning up in milk, an important component in the diets consumed by almost young and growing children and most adults every day. Indeed, evidence has implicated SMZ as a possible carcinogen [1], which has magnified risk concerns.

To prevent any health problems with consumers, the maximum residue limit (MRL) or the tolerance in milk have been established by European Union (EU), the Joint Expert Committee for Food Additives (JECFA, Codex Alimentarium Commission in FAO/WHO), the Japanese Ministry of Health and Welfare, or, the Food and Drug Administration (FDA): EU, SMZ and SDM 100 ng/ml MRL [2]; JECFA [3,4] and Japan, SMZ 25 ng/ml MRL; FDA, SDM 10 ng/ml tolerance [1,5].

Analytical methods for routine residue monitoring should be accurate, simple, economical in time and cost to permit monitoring of large numbers of

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0021-9673/00/$ – see front matter
PII: S0021-9673(00)00843-8

samples, and capable of detecting the residues below tolerance/MPL, thus harmless to the environment. Discharging the toxic waste of organic solvents is a severe problem on a world scale. From the viewpoint of the toxicity of solvents and environmental affects, the method should avoid the use of toxic solvents and reagents.

Previous papers have described various methods for determination of SMZ or SDM in milk using high-performance liquid chromatography (HPLC) [6–14]. The sample preparation procedure, in recent years, has been increased use of solid-phase extraction (SPE) columns [6,8,10,11,13,15] or matrix solid-phase dispersion (MSPD) [7], because of their easy use, and reduction of analysis time. However, there are two problems with these methods: (1) recoveries and reproducibilities are somewhat/sometimes low; (2) they require the use of some toxic solvents, like chloroform, dichloromethane, acetonitrile and methanol (Merck Catalogue, 2000/2001), as the extraction solvents and/or the HPLC mobile phase, which is harmful to the environment. At present, a better method than the above is necessary.

The present paper describes a simple HPLC determination procedure for routine residue monitoring of SMZ and SDM in milk, which is harmless to both humans and the environment. The HPLC photo-diode array detector chosen allowed the separation and identification of the target compounds by their retention times and spectra.

2. Experimental

2.1. Materials and reagents

Paper-packed cow's milk served as a sample, and was stored in a refrigerator until analysis. Ethanol and distilled water (HPLC grade) were obtained from Wako Pure Chemicals (Osaka, Japan). Sulphamethazine (SMZ) and sulphadimethoxine (SDM) standards were also obtained from Wako. Stock standard solutions of SMZ and SDM, respectively, were prepared using distilled water. Working mixed standard solutions of SMZ and SDM were prepared by diluting the stock solutions with distilled water. These solutions can be kept in a refrigerator for up to 1 month.

2.2. Apparatus

The following apparatus was used in the sample preparation: a Vortex mixer (Model MT-51, Yamato Science, Tokyo, Japan); a micro-centrifuge, Biofuge® fresco (Kendo Lab. Products, Hanau, Germany).

As centrifugal ultra-filtration units, three membrane types of the Ultrafree®–MC series (nominal molecular mass limit (NMWL)=5000, sample size ≤0.5 ml) were purchased from Millipore (Bedford, MA, USA): Ultrafree–MC/Biomax (Biomax™ high-flux polysulphone ultra-filtration membrane); –MC/PL (regenerated cellulose ultra-filtration membrane); –MC/PT (polysulphone ultra-filtration membrane).

Four types of silica-based reversed-phase C_{18} columns (particle size 5 μm) (250×4.6 mm I.D.) with their guard columns (5×4.6 mm I.D.) for HPLC analysis were purchased from Merck (Darmstadt, Germany) (LiChrospher® 100 RP-18 and LiChrosorb® 100 RP-18) and Kanto Chemicals (Tokyo, Japan) (Mightysil® RP-18 GP and Mightysil® RP-18 GP *Aqua*).

HPLC analysis of the target compound was conducted using a Jasco HPLC (Model PU-980 pump and DG-980-50 degasser) (Jasco, Tokyo, Japan), equipped with an SPD-M10A_{VP} diode array detector (Shimadzu, Kyoto, Japan) interfaced with a Fujitsu FMV-5133D7 personal computer (Fujitsu, Tokyo, Japan). The separation was performed on a Mightysil RP-18 GP *Aqua* column with a guard column using a mixture of 25% (v/v) ethanol solution (in water) as the mobile phase at a flow-rate of 0.8 ml/min at ambient temperature. The injection volume was 50 μl.

2.3. Procedure

A 0.5-ml sample was placed into a micro-centrifuge tube together with 0.5 ml of 50% (v/v) ethanol solution (in water). The tube capped was tried on an ultrasonic cleaner for 30 s. After 30 s, the tube was stirred for 30 s and centrifuged at 10 000 *g* for 5 min. A 0.4-ml sample of the supernatant liquid was put into an Ultrafree–MC/Biomax and centrifuged at 2000 *g* for 5 min. The ultra-filtrate was injected into the HPLC system.

2.4. Application of SMZ and SDM to the Ultrafree–MCs

The recoveries of SMZ and SDM from the Ultrafree–MCs were examined. The milk extract processed with 50% (v/v) ethanol solution was centrifuged as shown above. The supernatant was fortified with a mixed standard solution and mixed. A 0.4-ml sample of the extract containing 400 ng of SMZ and SDM, respectively, was applied to the Ultrafree–MCs. Ultra-filtered solutions were determined by the HPLC.

2.5. Recovery test

The recoveries of SMZ and SDM from blank milk samples spiked at 10, 50, 100, 500 and 1000 ng/ml were determined. These fortification concentrations were prepared by adding 20 μl of five mixed standard solutions of SMZ and SDM (250, 1250, 2500, 12 500 and 25 000 ng/ml, respectively) to separated 1-ml portions of the sample. Fortified samples were allowed to stand at 4°C for 1 h after sulphonamide standards addition and then mixed prior to workup.

In the test, coefficients of variation (C.V.s) determined for each spiked concentration were then averaged, which resulted in a mean±the relative standard deviation (RSD) This was defined as inter-assay variability. Intra-assay variability was defined as the C.V. for the mean of five replicates of the same sample and represents the variability associated with the analytical procedure used.

3. Results and discussion

3.1. Sample preparation

The present method could be rapidly determined SMZ and SDM in milk using HPLC without complex extraction and clean-up procedures; moreover, no use of toxic solvents and reagents was also achieved.

The extraction was performed in an ultrasonic cleaner with 50% ethanol solution (in water) followed by stirring with a vortex mixer. Use an ultrasonic cleaner as the extraction procedure gave fine recoveries of SMZ and SDM (Table 1).

Table 1
Comparison on recoveries of sulphamethazine (SMZ) and sulphadimethoxine (SDM) from Ultrafree®–MCs[a]

Membrane type	Recovery (%)[b]	
	SMZ	SDM
Ultrafree–MC/Biomax	94 (1.7)	92 (1.3)
–MC/PL	93 (1.9)	90 (1.2)
–MC/PT	81 (2.8)	83 (2.1)

[a] The extract from milk was fortified with a mixed standard solution of SMZ and SDM and applied to the ultra-filter unit.
[b] Data are averages ($n=8$). Values in parentheses are coefficients of variation.

Using three types of centrifugal ultra-filtration units, Ultrafree–MCs, the present study was tested and compared to the recovery of SMZ and SDM from the units. As shown in Table 1, an Ultrafree–MC/Biomax gave the best recoveries and precision for both target compounds.

This ultra-filtration unit was able to deproteinize the extracted solution (a smaller sample, ≤0.5 ml) easily, in a short period (around 5 min), only with centrifuging. Ultrafree–MC/Biomax eliminates many steps and problems associated with classical clean-up techniques, e.g., reduces recoveries, and consequently increases clean-up yields. The procedure was presented here for rapid and efficient purification of SMZ and SDM resulting high recovery and reproducibility (Fig. 1).

3.2. HPLC operating conditions

Some researchers have previously reported acceptable determination of sulphonamides (including SMZ and SDM) by HPLC using a reversed-phase (RP) (silica-based C_{18}) column and a mixture of acetonitrile–methanol and buffer solution as the mobile phase [6,8,12,13].

Acetonitrile and methanol are usually used in the mobile phase for the RP-HPLC analyses of various compounds. According to the Swiss toxicity classification [16], these organic solvents are handled as toxic solvents (e.g., acetonitrile: poison class, very strong toxin; LD_{50} oral, 200 mg/kg). Even methanol is a toxin. In contrast, the influence of the ethanol

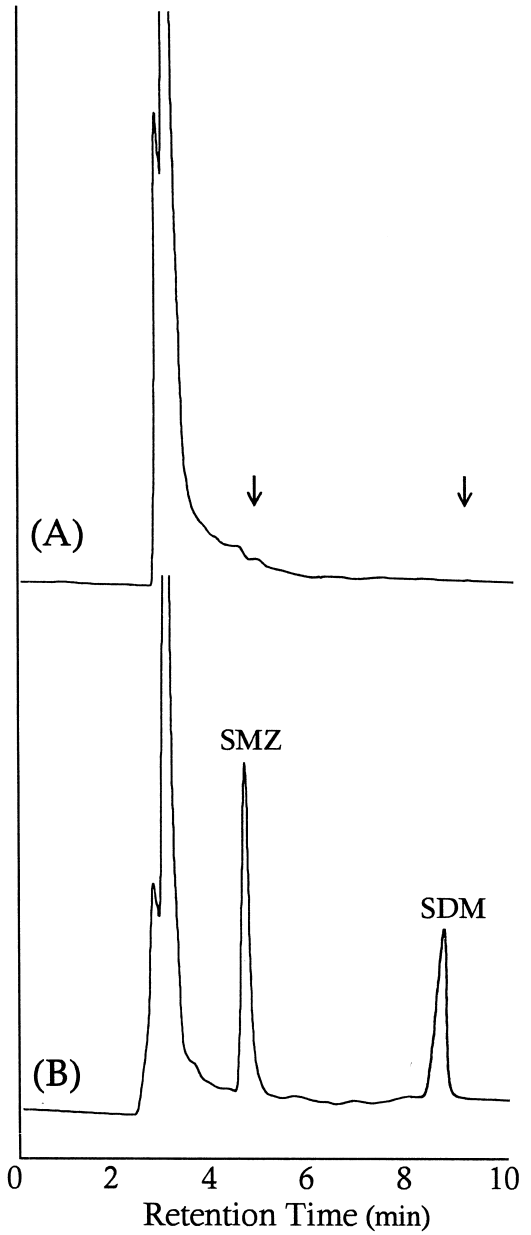

Fig. 1. HPLC chromatograms obtained from milk samples (photo-diode array detector set at 266 nm). (A) Blank milk sample; (B) spiked (100 ng/ml of SMZ and SDM, respectively) milk sample. Peaks: sulphamethazine (SMZ) (retention time, 4.7 min); sulphadimethoxine (SDM) (8.8 min). Arrows indicate the retention times of SMZ and SDM.

used in this study on the environment and humans is negligible (poison class, not subject to toxicity).

Since the chromatographic characteristics of RP columns can be different because of variations in the residual silanol shielding or carbon content [17,18], four types of C_{18} columns, LiChrospher 100 RP-18, LiChrosorb 100 RP-18, Mightysil RP-18GP and Mightysil RP-18GP *Aqua*, with a mixture of ethanol and water as the mobile phase, were tested and compared with regard to the separation: SMZ and SDM, and their from interfering peaks; the their sharp peaks obtained upon injection of equal amounts. Mobile phases with ethanol concentrations between 15–30% were tested.

The observed capacity factors and peak forms for SMZ and SDM with 30% (v/v) ethanol solution (in water) as a mobile phase are presented in Table 2. As expected, the capacity factors on the C_{18} columns of SMZ and SDM are mainly governed by the carbon content of the columns. The capacity factors of SMZ and SDM were the highest on Mightysil RP-18GP, followed by LiChrospher 100 RP-18, LiChrosorb 100 RP-18 and Mightysil RP-18GP *Aqua*. On the LiChrospher 100 RP-18 and Mightysil RP-18GP, both target compound peaks were easy to detect as significant broadening peaks (Table 2). Resolution of 100% purity between SMZ and SDM on the LiChrosorb 100 RP-18, which gave the lowest capacity factor for SDM, is not possible. From data shown in Table 2, it is likely that the pore volume in the column is a critical parameter with regard to the retention of SMZ and SDM and their peak forms. The LiChrospher 100 RP-18, LiChrosorb 100 RP-18 and Mightysil RP-18GP columns were difficult to separate between SMZ, and the interference of the resulting milk extract. The best chromatogram with complete separation of target compounds and clear/short retention times was obtained by using a Mightysil RP-18GP *Aqua* column and an isocratic mobile phase of 25% ethanol (in water) solution. This method made it unnecessary to use the gradient system to improve the separation.

SMZ and SDM spectra were measured using a photo-diode array detector and a common maximum absorbance was chosen: 266 nm was selected for SMZ and SDM monitoring wavelength. The retention times of SMZ and SDM were 4.7 and 8.8 min,

Table 2
Properties of the C_{18} column[a] materials examined and chromatographic sulphonamide peaks obtained with a mobile phase of 30% (v/v) ethanol (in water)

Designation	Property		Capacity factor		Peak form[b]	
	Pore volume (ml/g)	Carbon content (%)	SMZ[c]	SDM	SMZ	SDM
LiChrosher 100 RP-18	1.25	21.5	0.73	4.00	−	−
LiChrosorb 100 RP-18	1.0	16.0	0.59	0.71	±	±
Mightysil RP-18 GP[d]	1.1	21.4	2.52	5.40	−	−
Mightysil RP-18 GP *Aqua*[d]	0.9	15	0.51	1.50	+	+

[a] Particle size 5 μm (250×4.6 mm I.D.).
[b] +, remarkably sharp; ±, comparatively sharp; −, broadening.
[c] SMZ, sulphamethazine; SDM, sulphadimethoxine.
[d] End-capped.

respectively. The solvent (only ethanol) consumption per sample was estimated to be <2 ml.

Fig. 1 shows examples of typical HPLC traces of standards of a blank and a spiked (100 ng/ml each drug) milk sample obtained under the established procedure. The resulting extracts were free from interfering compounds for detection and identification in all HPLC traces. This finding indicates that satisfactory purification could be archived by the present method.

With the proposed procedures, shorter analysis time and use of less organic solvent (no toxicity solvents and reagents) were achieved. Analytical time and solvent consumption were <35 min per sample and <2 ml of ethanol per sample, respectively.

3.3. Calibration

The calibration graphs that generated by plotting peak areas against amount were linear over the range 0.1 – 20 ng for SMZ and 0.2 – 20 ng for SDM and passed though the origin (slopes: 341 for SMZ; 204 for SDM). The correlation coefficients, 0.999 for SMZ and 0.998 for SDM, were highly significant statistically ($P<0.01$). The minimum detectable amounts of SMZ and SDM were 0.1 and 0.2 ng, respectively. The precision of the HPLC procedure was obtained from relative standard deviation of areas calculated for 10 replicate injections of 1 ng of each target compound. The values were 1.0% for SMZ and 1.3% for SDM.

3.4. Recoveries and identification

Table 3 summarizes the average recoveries from milk samples at five different spiking levels (10, 50,

Table 3
Recoveries of sulphamethazine (SMZ) and sulphadimethoxine (SDM) from milk[a]

Spiked (ng/ml)	Recovery (%) (mean±RSD, $n=5$)	
	SMZ	SDM
10	86±2.6	84±2.8
50	85±2.5	83±3.3
100	87±1.4	85±3.7
500	92±1.3	91±2.0
1000	89±2.0	85±1.6
Total ($n=25$)	88±1.9	86±2.7
Correlation coefficient[b] ($n=5$)	0.998±0.001	0.997±0.002
Inter-assay variability (%±RSD)	2.2±0.7	3.1±1.0
Intra-assay variability (%, $n=5$)	1.4	2.0
LOD (ng/ml)	3	5
LOQ (ng/ml)	5	10

[a] n=number of replicates; RSD, relative standard deviation; LOD, limit of detection; LOQ, limit of quantitation.
[b] Standard spiking graph.

100, 500 and 1000 ng/ml), correlation coefficients of 'standard spiking graphs', inter- and intra-assay variabilities for SMZ and SDM.

The average recoveries from milk samples at five different spiking levels (10, 50, 100, 500 and 1000 ng/ml), correlation coefficients of standard curves, inter- and intra-assay variabilities for SMZ and SDM. The average recoveries were greater than 83% with the RSD between 1.4 and 3.7%. Intra-assay variability is quite low for both sulphonamides. These findings were satisfactory for strict residue analysis.

The 'standard spiking graphs' for SMZ and SDM were generated by plotting peak areas of fortified sample extracts ranged 10–1000 ng/ml. The graph was constructed from five points and each point represented the mean of the five injections. The resulting correlation coefficients, 0.998 for SMZ and 0.997 for SDM, were highly significant statistically ($P<0.01$). For the two sulphonamides, respectively, the standard spiking graph and its pure standard (aqueous) graph was able to pool statistically, indicating that slope of the standard graph is similar to that of pure standard. The calibration can be performed with the simplest procedure using pure standards.

To properly characterize the practical residue monitoring, the limit of detection (LOD) and limit of quantitation (LOQ) for target compounds were calculated in accordance with the CCMAS 1993 (Codex Committee for Methods Analyses and Sampling). Based on the peak areas in HPLC chromatograms, LOD is defined as the average background plus three times the standard deviations (SD). LOQ is defined as the average background plus 10 times the SD. Four different blank milk samples were analyzed in duplicate. The LODs were 3 ng/ml for SMZ and 5 ng/ml for SDM. The LOQ were 5 ng/ml for SMZ and 10 ng/ml for SDM (Table 3). These values were below the MRLs/tolerance (SMZ, 25 ng/ml; SDM, 10 ng/ml).

The aforementioned findings, i.e., high recovery and low variability, together with the low LOD and LOQ, indicate that the present method may be precise and accurate.

In HPLC analysis for residual drug monitoring, a photo-diode array gives spectral information and is an easy way for the confirmation of the drug. HPLC combined with the diode array system proved to be able to detect a wide range of molecules and ensure identification of target compound. The retention time and spectrum provide strong evidence of its identity. SMZ and SDM can be identified in the milk sample with their retention times and absorption spectra. The spectrum of SMZ obtained from sample is practically identical with that of the standard. Fig. 2A gives a spectrum of the SMZ peak of milk sample obtained with the photo-diode array detector. Similar results were obtained from SDM (Fig. 2B). The present sample preparation allowed a reliable confirmation.

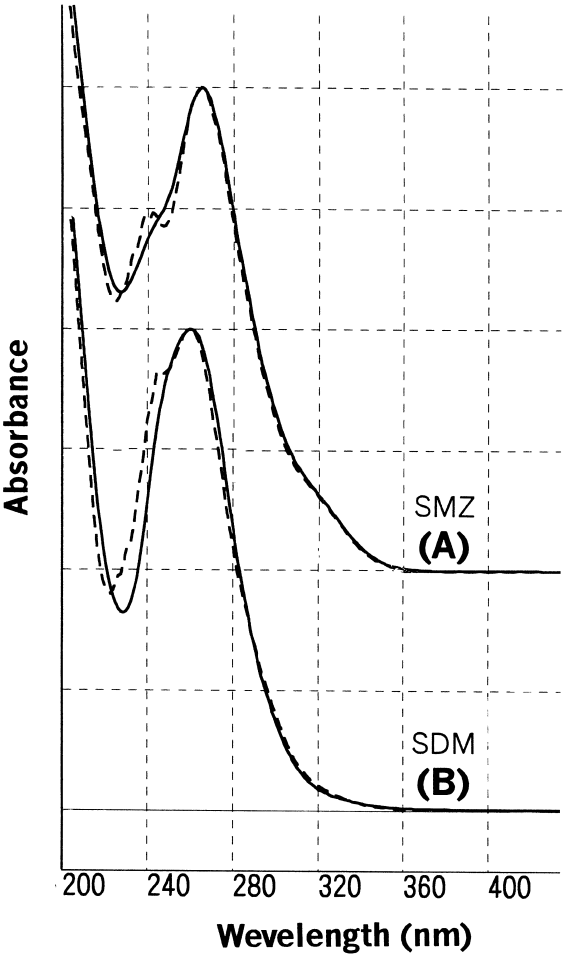

Fig. 2. Normal absorption spectra of peaks at 4.7 min (A) for SMZ and 8.8 min (B) for SDM, respectively, in chromatograms (Fig. 1). Standards (solid line); spiked milk sample (dashed line).

3.5. Monitoring residue in marketing animal tissues

Thirty different samples of marketing milk that were circulated in Osaka City were analyzed by using the present method. No SMZ and SDM were detected. There were no interfering peaks in the resulting chromatograms.

In conclusion, a simple and rapid method for determining SMZ and SDM in milk using HPLC was developed. The main characteristics of the proposed procedure are summarized as follows: shorter analysis time (total $<$35 min per sample); high precision (RSD$\leqslant$3.7% in the recovery test); harmless to the environment (total solvent consumption $<$2 ml of ethanol per sample). This procedure may, therefore, be useful for the routine residue monitoring of SMZ and SDM in milk.

References

[1] Feb. Regist., 53 FR 9492, March 23 1988, NCTR Technical Report Experiment Number 418, March 1988, NCTR, Jefferson, AR 72079.
[2] Commission of the European Communities, Establishment by the European Community of maximum residue limits (MRLs) for residues of veterinary medical products in foodstuffs of animal origin, The rules governing medical products in the European Community VI, October 1991, ECSC-EEC-EAEC, Brussels.
[3] WHO Technical Report Series No. 799, 36th Report of the Joint FAO/WHO Expert Committee on Food Additives, WHO, Switzerland, 1990.
[4] FAO Food and Nutrition Papers 41/4, 1991, Residues of some veterinary drugs in animal and foods, Monographs prepared by the Thirty-eighth Meeting of the Joint FAO/WHO Expert Committee on Food Additives, January, Rome.
[5] FDA/CVM: General principles for evaluating the safety of compounds used in food producing animals, FDA guideline.
[6] V.K. Agarwal, J. Liq. Chromatogr. 13 (1990) 3531.
[7] A.R. Long, C.R. Short, S.A. Barker, J. Chromatogr. 502 (1990) 87.
[8] A. Hoffmeister, G. Suhren, W. Heeschen, Milchwissenschaft 46 (1991) 770.
[9] J.M. Diserens, C. Renaud-Bezot, M.C. Savoy-Perroud, Deutshe Lebensmittel-Roundschau 87 (1991) 205.
[10] V.K. Agarwal, J. Chromatogr. 624 (1992) 441.
[11] V.K. Agarwal, J. Liq. Chromatogr. 16 (1993) 3793.
[12] J. Laloux, J.M. Romnee, C. Marin, D. Vanwijnsberghe, Milchwissenschaft 51 (1996) 517.
[13] D. Decolin, P. Leroy, A. Nicolas, P. Archimbault, J. Chromatogr. Sci. 35 (1997) 557.
[14] N. Furusawa, Fresenius J. Anal. Chem. 364 (1999) 270.
[15] N. Furusawa, Chromatographia 49 (1999) 369.
[16] The Merck Catalogue 2000/2001, Merck KgaA, Darmstadt, Germany, 2000.
[17] R.E. Majors, J. Chromatogr. Sci. 18 (1980) 393.
[18] M.M.L. Aerts, W.M.J. Beek, J. Chromatogr. 435 (1988) 97.