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Enrichment techniques and trace analysis with microbore columns in liquid chromatography*

M. KREJČI,† K. ŠLAIS, D. KOUŘILOVÁ and M. VESPALCOVÁ

Institute of Analytical Chemistry, Czechoslovak Academy of Sciences, 611 42 Brno, Czechoslovakia

Abstract: The advantages of microbore columns for trace analysis by liquid chromatography are identified, with reference to on-column enrichment techniques performed on analytical micro-columns. The selectivity and high sensitivity of the amperometric detector are utilized in combination with a microbore column for a number of pharmaceutical and bioanalytical analyses, including phenothiazines, parabens, sulphonamides, catecholamines, tetracyclines, vitamins, amino acids and dipeptides.

Keywords: *Trace analysis; liquid chromatography; microbore columns; phenothiazines; sulphonamides; catecholamines; parabens; tetracyclines; dipeptides.*

Introduction

In order successfully to apply liquid chromatography on microbore columns [1–3] to trace analysis, it is necessary to optimize the separation system (column efficiency and selectivity), the method of detection and the sampling systems employed. Most of the significant factors of the separation system can be expressed in the relationship describing concentration of the chromatographic zone at the peak maximum [4]. The inverse quadratic dependence of the solute concentration at the peak maximum on the column diameter confers a significant advantage on micro-columns. It has been verified experimentally [3, 5] that the column diameter does not affect the height equivalent to a theoretical plate (HETP). HETP is, however, related to the square root of the particle size, d_p, a fundamental parameter influencing the diffusion path of a solute.

The maximum permissible amount of solute which can be introduced onto the column is that for which the value of the reduced HETP, h, is not affected. The value of h can be used to indicate reliably the solute amount for which the column sorption capacity is exceeded. The minimum required amount of the analyte is, on the other hand, determined by the value of the minimum detectable concentration of solute at the column outlet.

The amount of the substance introduced corresponds to the product of the sample volume injected onto the column and the total solute concentration. In analytical

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† To whom correspondence should be addressed.

practice two boundary cases can be identified. In the first case, when sample concentration is relatively large and, at the same time, sample volume is small, microbore columns will find full use, since under these circumstances sample volumes of tenths of microlitres or less can be introduced, while at the same time the concentration at the column outlet is higher in comparison with analytical columns of larger diameters. The characteristics of the detector and the sample introduction technique are most important for sensitivity. Both these factors can influence the value of h significantly and thus the value of the maximum permissible concentration.

In the second case, sample concentration is extremely small and it is necessary to introduce a large sample volume in order to obtain a detectable concentration at the column outlet. An enrichment technique must then be applied and under such conditions that the efficiency of the chromatographic column can be maintained and utilized.

Techniques and Applications

Enrichment techniques on microbore columns

The decrease in column diameter means that the sample volume which can be injected onto the column without significant loss of the efficiency also decreases. If it is specified that the volume of injected sample, V_S, should contribute to the band-broadening of the eluted zone by no more than 10%, then it follows that:

$$\sigma^2_{V_S} = V_S^2/12 \leq 0.1\ \sigma^2_{V_{0(col)}} \tag{1}$$

where $\sigma^2_{V_S}$ is the variance of the volumetric band-broadening of the solute attributable to the injected sample volume and $\sigma^2_{V_{0(col)}}$ is the variance of the volumetric band-broadening of the unsorbed solute in the column. If a 150 × 0.7 mm i.d. column packed with 6-μm particles is used, the maximum injected volume should be no more than 0.45 μl; for a 100 × 0.5 mm i.d. column, then the volume should not exceed 0.22 μl.

The adverse influence on separation efficiency caused by the injection of larger sample volumes can be overcome when the solute is injected in a non-eluting solvent [6, 7]. The advantages of this manner of using micro-columns have been shown earlier [8]. A peak-focussing effect can also be obtained by the influence of a transient change in the properties of the stationary phase [9]. This effect can be achieved, for example, by adsorption of a substance which leads to an intensive sorption of the solute during sampling. This substance can be added to the sample solution and should itself have a sufficiently low capacity factor. The enrichment effect, so far as the injected volume and band-spreading of the injected substances are concerned, is the same as in the case where the solute is injected in a non-eluting solvent.

Both injection procedures make it possible to decrease in a simple fashion the minimum detectable concentration in proportion to the increase in the injected sample volume. Thus it is possible to inject onto the micro-column a sample volume several times larger than the column dead volume, V_M, without decreasing the separation efficiency. For example, the 150 mm × 0.7 mm i.d. column (V_M = 43 μl) was used with a sampling valve provided with an outer loop of up to 1 ml in volume, i.e. 23-times the column dead volume [8]. The 5 m × 0.5 mm i.d. loop could be filled and washed with a conventional injection syringe.

When the sample is injected in a non-eluting solvent the retention volume, V_R, increases with the injected volume, V_S:

$$V_R = V_R^0 + V_S \quad (2)$$

where V_R^0 is the limiting retention volume (corresponding to the sample volume approaching zero). The contribution of the sampling procedure to the chromatographic zone broadening is independent of V_S [6]. The upper limit of the sample volume which can be introduced is determined by the ratio of the elution forces of the mobile phase and of the non-eluting solvent and by the column sorption capacity.

An example of the injection of chlorophenols in a non-eluting solvent (water) is shown in Fig. 1. For comparison of the variances of the peak-volumes in which the individual components are eluted, chromatograms are shown demonstrating injected sample volumes of 0.2 μl and 1 ml, respectively. The band-width of the components is virtually identical in both instances. The minimum detectable concentration is, of course, lower in the case of the aqueous injection (larger sample volume) by practically three orders of magnitude. For these chlorophenols the minimum detectable concentration is of the order of 10–100 ng/l (4-chlorophenol, 20 ng l^{-1}; 2,4,6-trichlorophenol, 80 ng l^{-1}); similar sensitivity is obtained, for instance, with polynuclear aromatic hydrocarbons (1,2-benzpyrene, 60 ng l^{-1}; perylene, 40 ng l^{-1}).

An example of the trace-enrichment technique, involving an admixture (with the

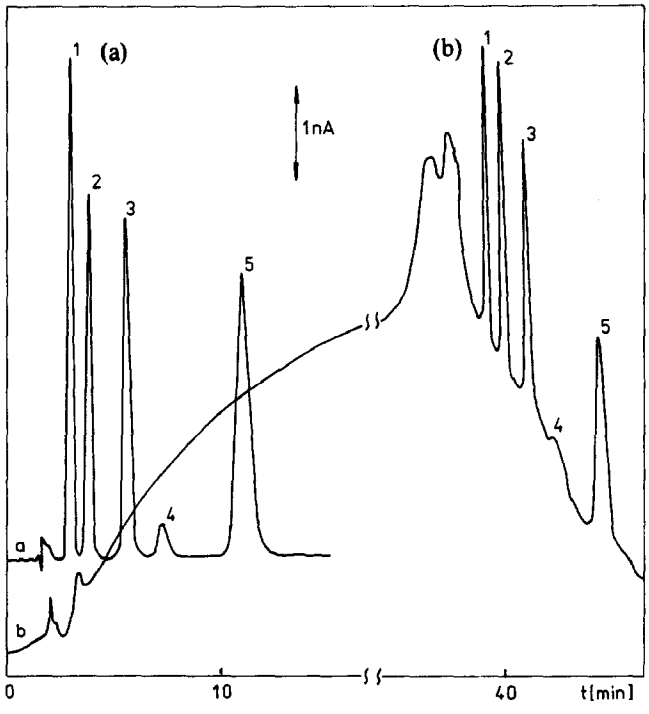

Figure 1
Chromatograms of a mixture of chlorinated phenols under conventional and non-eluting solvent conditions. (a) 0.2 μl of the mixture in mobile phase; and (b) 1 ml of the mixture in (non-eluting) aqueous solution. Key: 1, 4-chlorophenol, (a) 4.5 mg l^{-1}, (b) 1.6 μg l^{-1}; 2, 2,4-dichlorophenol, (a) 6 mg l^{-1}, (b) 4 μg l^{-1}; 3, 2,4,6-trichlorophenol, (a) 9 mg l^{-1}, (b) 6 μg l^{-1}; 4, tetrachlorophenol, (a) 3.5 mg l^{-1}, (b) 1.6 μg l^{-1}; 5, pentachlorophenol, (a) 34 mg l^{-1}, (b) 15 μg l^{-1}; concentrations are given in the injected solution. Capillary glass column (CGC) 150 × 0.7 mm i.d., packed with 7-μm LiChrosorb RP-18; mobile phase: acetonitrile–water (60:40, v/v) with 0.1 M $NaClO_4$ and 0.001 M $HClO_4$; linear velocity (a) 1.7 mm s^{-1}, (b) 1.4 mm s^{-1}. Detector: amperometric, model EMD-10, Pt electrode (Laboratory Instruments, Prague, Czechoslovakia).

injected sample of catecholamines) of an active substance capable of modifying the characteristics of the chromatographic system (stationary and mobile phases) within a defined time interval, is shown in Fig. 2.

The role of detector properties in trace analysis with microbore columns

In connection with micro-columns concentration-sensitive detectors are preferred. Because of the difficulty in designing a functioning detection cell with a sufficiently small volume, refractometric and permittivity detectors have not been exploited in practical applications. A conductometric detector with a small enough cell has been developed [10]. Spectrophotometric [11, 12] and amperometric [3, 13–16] detectors have been most frequently used and for many practical purposes their selectivity and sensitivity are satisfactory. In connection with micro-columns, the miniaturization of detector cells based on these two principles should be evaluated.

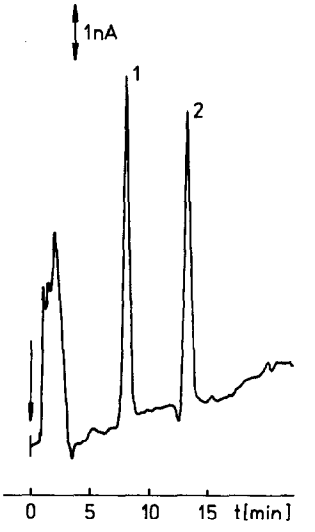

Figure 2
Trace-enrichment separation of catecholamines by peak-focussing with a co-eluting additive. Column: CGC 150 × 0.7 mm i.d., packed with 10-μm Separon Si-C_{18}; mobile phase: 10^{-1} M $NaClO_4$ + 10^{-3} M $HClO_4$ + 10^{-3} M disodium ethylenediamine tetra-acetate (EDTA) in water; flow-rate, 0.8 μl s^{-1}; amperometric detection. Sample injected: 0.1 ml of a solution of 4.10^{-7} M noradrenaline + 4.10^{-7} M adrenaline + 2.10^{-2} M $C_{10}H_7SO_3Na$. Key: 1, noradrenaline; 2, adrenaline.

In order to limit the distortion of the zone profile at the column outlet due to the contribution of the detector cell volume to 10%, for example, it is necessary to control the ratio between the standard deviation of the zone profile caused by the column, $\sigma_{V(\mathrm{col})}$, and that caused by the cell volume, $\sigma_{V(\mathrm{det})}$:

$$k_1\sigma_{V(\mathrm{col})} > \sigma_{V(\mathrm{det})}, \tag{3}$$

where k_1 is a constant determined by the level of distortion acceptable; for a limit of 10%, $k = 0.3$.

For $\sigma_{V(\mathrm{col})}$ the following relationship applies:

$$\sigma_{V(\mathrm{col})} = \frac{V_{\mathrm{M}}(1 + k')}{\sqrt{N}} . \tag{4}$$

The expression for $\sigma_{V(\mathrm{det})}$ becomes:

$$\sigma_{V\mathrm{det}} = \frac{V_{\mathrm{det}}}{\sqrt{12}} . \tag{5}$$

Here V_M is the column dead volume, k' the capacity ratio of the solute, N the theoretical plate number of the column, and V_{det} the detector cell volume. Combining relationships (3)–(5) gives the expression:

$$V_{det} \leq k_1 V_M (1 + k') \left[\frac{12}{N}\right]^{1/2}. \tag{6}$$

It follows that the detector cell volume should be diminished in proportion to the decrease in the column volume in order to limit the relative distortion of the chromatographic zone to a specified maximum.

The spectrophotometric detector will now be considered. At present, these detectors are designed in such a way that the total light flux leaving the measuring cell impinges on the light-sensitive surface of an optoelectronic sensing element, for which the resulting electric signal is proportional to the light flux, Φ, passing through the cell [17]. By use of the Beer–Lambert law, a relationship between the change in light flux, $\delta\Phi$, and the change in solute concentration, δc, can be derived:

$$-\delta\Phi = I_0 a V_{det} \cdot \delta c, \tag{7}$$

where I_0 is the incident light intensity, a is the molar absorptivity of the solute and V_{det} the product of the length and cross-section of the detector cell. The noise of the photometric detector is mainly determined by the optoelectronic sensing element. Consequently, a decrease in detector cell volume will lead to less change in the light flux, decreased detector response and a reduced signal-to-noise ratio.

With amperometric detectors, the relationship between the limiting diffusion current of the solute, I_{lim}, and solute concentration can be described in simplified terms by the following relationship (derived from [18, 19]):

$$I_{lim} = k_2 z F c (D\, u_{det} \cdot A l)^{1/2}, \tag{8}$$

where z is the number of electrons involved, F the Faraday, D the diffusion coefficient of the solute, u_{det} the linear velocity of the mobile phase in the detector cell, A the electrode surface and l a characteristic cell dimension, related to the channel width for the thin-layer electrode and to the electrode diameter for the wall-jet electrode; k_2 is a constant dependent on the type of cell geometry.

The effective volume of the amperometric cell is determined by the space above the electrode surface. Ballancing the volumetric flow-rates in the cell and in the column, the following relationship is obtained between the mobile phase linear velocity in the column, u, and that in the cell, u_{det}:

$$\frac{V_{det} l u_{det}}{A} = k_3 \frac{V_M \cdot u}{L} \tag{9}$$

where L is the column length and k_3 is a constant dependent on the type of cell geometry.

By combining equations (8) and (9), the dependence of the limiting current on the effective cell volume is obtained in the form:

$$I_{lim} = k_2 zFcA \left\{ \frac{V_M \cdot uDk_3}{V_{det} \cdot L} \right\}^{1/2} . \quad (10)$$

It follows from this relationship that the detector response, i.e. the limiting current, is a linear function of the cell surface area. Since electrode noise depends on the surface area in the same way, the signal-to-noise ratio is not determined by the electrode surface area. Moreover, a reduction in the effective cell volume can also lead to an improvement in the signal-to-noise ratio, as has been shown earlier [20].

From the foregoing comparison of the two detection principles, it appears that as regards the minimum detectable concentration, amperometric detectors are more advantageous for micro-columns than spectrophotometric detectors.

The high sensitivity of the amperometric detector [13] combined with micro-columns (150 × 0.7 mm i.d.) has already been demonstrated for the analysis of drugs, natural substances and some aromatic compounds [21, 22]. Methods involving the oxidation of analytes at the working electrode and also the formation of complexes with ions of the electrode material have been reported [23, 24]. Examples of amperometric detection are shown in Figs 3–6, where the injected amounts of solutes are very small. The minimum detectable concentrations (Table 1) are of the order of $\mu g\ l^{-1}$, which in many cases is

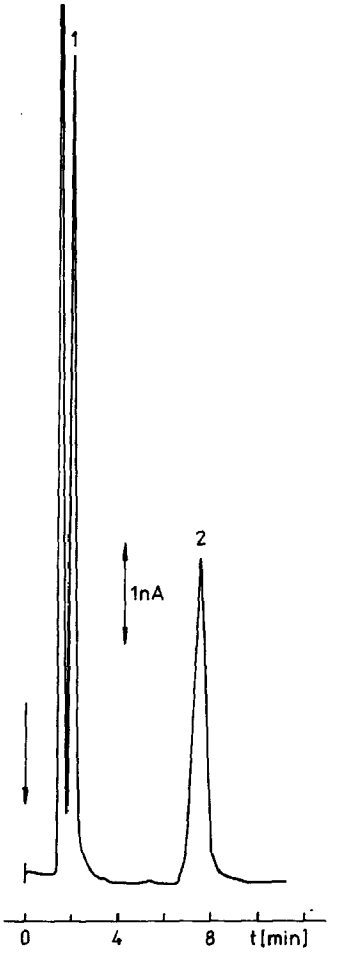

Figure 3
Chromatogram of penicillamine and cysteine. Column: CGC 150 × 0.7 mm i.d., packed with 10-μm Separon Si-C_{18}; mobile phase: 10^{-1} M $NaClO_4$ + 10^{-3} M $HClO_4$ + 10^{-3} M EDTA + 10^{-4} M $C_8H_{17}SO_3H$ in water; flow-rate, 0.5 μl s^{-1}; sample volume, 0.2 μl. Detector: EMD 10, Au electrode; working potential +1.0 V. Key: 1, cysteine (15 ng); 2, penicillamine (24 ng).

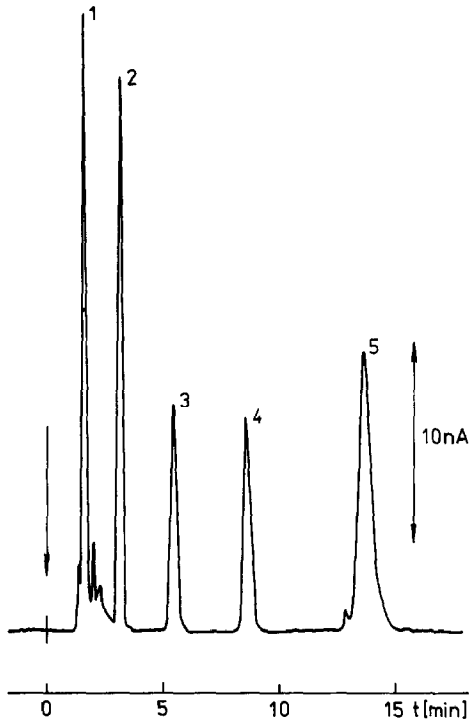

Figure 4
Separation of sulphonamides. Column: CGC 150 × 0.7 mm i.d., packed with 7-μm LiChrosorb RP-18; mobile phase, acetonitrile–water (3:97, v/v) with 0.1 M $NaClO_4$; linear velocity, 1.7 mm s^{-1}; sample volume, 0.2 μl. Detector: EMD 10, Pt electrode, working potential +1.2 V. Key: 1, sulphanilic acid (1.4 ng); 2, sulphanilamide (1.7 ng); 3, sulphacetamide (2.3 ng); 4, sulphathiazole (1.4 ng); 5, sulphamethoxydiazine (8.4 ng).

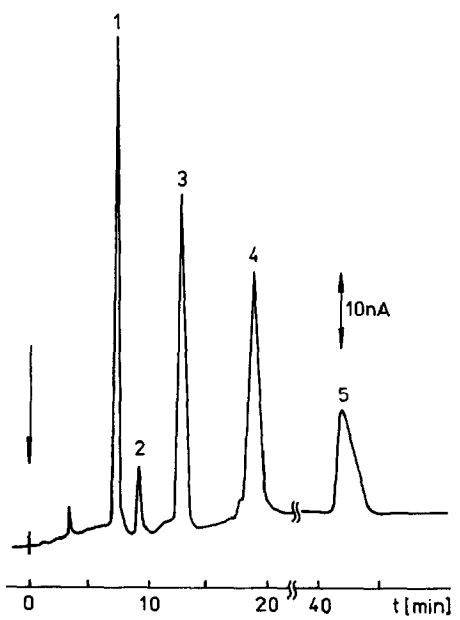

Figure 5
Chromatogram of a mixture of phenothiazines. Column: CGC 150 × 0.7 mm i.d., packed with 5-μm LiChrosorb SI-100; mobile phase, acetonitrile with 0.001 M $NaClO_4$ and 0.01 M NH_4OH; linear velocity, 0.75 mm s^{-1}; sample volume, 0.2 μl. Detector: see Fig. 4. Key: 1, levopromazine (22.8 ng); 2, chlorprothixene (43.6 ng); 3, chlorpromazine (18.4 ng); 4, thioridazine (20.4 ng); 5, prochlorperazone (19.2 ng).

Table 1
Minimum analysable amounts and minimum detectable concentrations for solutes detected amperometrically after separation by microbore LC*

		Minimum analysable amount (pg)	Minimum detectable concentration (μg/l)
Sulphonamides	Sulphanilic acid	5	1.3
	Sulphanilamide	6	1.5
	Sulphacetamide	20	3
	Sulphathiazole	10	6
	Sulphamethoxydiazine	60	4
Thiaxanthen	Chlorprothixene	500	44
Phenothiazines	Levopromazine	40	3.5
	Chlorpromazine	40	2.5
	Thioridazine	60	2.7
	Prochlorperazine	100	3.5
Tetracyclines	Rolitetracycline	500	47
	Tetracycline	1000	49
Chlorinated phenols	4-Chlorophenol	20	3
	2,4-Dichlorophenol	30	4
	2,4,6-Trichlorophenol	50	5
	2,3,4,6-Tetrachlorophenol	200	–
	Pentachlorophenol	200	11
Polycyclic aromatic hydrocarbons	Anthracene	30	3.3
	Pyrene	100	7
	Perylene	40	1.7
	1,2-Benzpyrene	40	1.5
	20-Methylcholanthrene	90	2.5
Parabens	4-Hydroxybenzoic acid	300	83
	Methyl-4-hydroxybenzoate	300	68
	1-Propyl-4-hydroxybenzoate	900	140
Vitamins	Vitamin A-acetate	200	23
	Vitamin D_2	800	50
	Vitamin E	12 700	409
	Folic acid	200	20
Flavonoids	Quercetin	30	2.3
Azo-dyes	4-Aminoazobenzene	20	4
	2-Aminoazotoluene	30	5
	N,N-Dimethyl-4-aminoazobenzene	20	3.3
Aromatic amines	Benzidine	3	0.9
	1-Naphthylamine	6	1.0
	2-Naphthylamine	5	1.2
	Carbazole	9	1.4
	Diphenylamine	6	0.8

*Noise = 50 pA.

considered as trace analysis. The selectivity of the amperometric detector can be adjusted by varying the applied potential and the type of electrode material employed.

Conclusion

The theoretical assumptions for a successful application of the micro-column to trace analysis are examined and techniques for trace-enrichment illustrated by reference to a number of separations of interest in pharmaceutical and biomedical analysis. The capability of micro-columns combined with low-volume detectors has been shown to

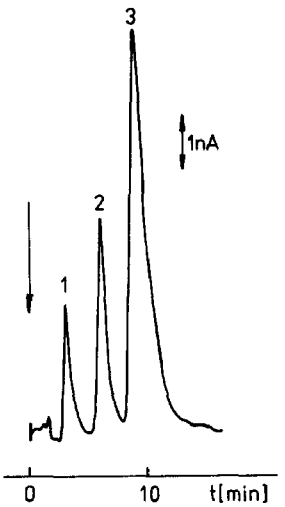

Figure 6
Separation of dipeptides. Column and packing: see Fig. 3. Mobile phase: 5.10^{-3} M phosphate buffer (pH = 7.5) in water. Volumetric flow-rate and sample volume, see Fig. 3. Detector: EMD 10, Cu electrode, working potential +0.2 V. Key: 1, Gly-Val (35 ng); 2, Gly-Leu (56 ng); 3, Leu-Gly (94 ng).

extend to very low concentrations of analyte, as in trace analysis, and to very low volumes of sample, as often encountered in clinical analysis. The simplicity and universality of these approaches should make a considerable contribution to trace analysis in the pharmaceutical and biomedical sciences.

References

[1] R. P. W. Scott and P. Kucera, *J. Chromatogr.* **169**, 51–92 (1979).
[2] T. Takeuchi and D. Ishii, *J. Chromatogr.* **190**, 150–155 (1980).
[3] K. Šlais and D. Kouřilová, *Chromatographia* **16**, 265–266 (1982).
[4] M. Krejčí, K. Šlais and D. Kouřilová, *Chem. Listy* **78**, 469–486 (1984).
[5] D. Kouřilová, K. Šlais and M. Krejčí, *Coll. Czech. Chem. Commun.* **49**, 764–771 (1984).
[6] P. Guinebault and M. Broquiare, *J. Chromatogr.* **217**, 509–522 (1981).
[7] M. Broquiare and P. Guinebault, *J. Liq. Chromatogr.* **4**, 2039–2061 (1981).
[8] K. Šlais, D. Kouřilová and M. Krejči, *J. Chromatogr.* **282**, 363–370 (1983).
[9] D. Kouřilová, K. Šlais and M. Krejčí, *Chromatographia* (in press).
[10] D. Kouřilová, K. Šlais and M. Krejčí, *Coll. Czech. Chem. Commun.* **48**, 1129–1136 (1983).
[11] D. Ishii, T. Tonda, K. Hibi, T. Takeuchi and T. Nakanishi, *J. High Resolut. Chromatogr. Chromatogr. Commun.* **2**, 371–377 (1979).
[12] F. J. Yang, *J. High Resolut. Chromatogr. Chromatogr. Commun.* **4**, 83–85 (1981).
[13] K. Šlais and M. Krejčí, *J. Chromatogr.* **235**, 21–29 (1982).
[14] Y. Hirata, P. T. Lin, M. Novotný and R. M. Wightman, *J. Chromatogr.* **181**, 287–294 (1980).
[15] K. Šlais and D. Kouřilová, *J. Chromatogr.* **258**, 57–63 (1983).
[16] M. Goto, Y. Koyanagi and D. Ishii, *J. Chromatogr.* **208**, 261–268 (1981).
[17] R. L. Stevenson, in *Liquid Chromatography Detectors* (T. M. Vickrey, Ed.), pp. 23–86. M. Dekker, New York (1983).
[18] K. Štulík and V. Pacáková, *Chem. Listy* **73**, 795–820 (1979).
[19] K. Štulík and V. Pacáková, *Československ. Farm.* **30**, 241–247 (1981).
[20] S. G. Weber and W. C. Purdy, *Anal. Chim. Acta* **100**, 531–544 (1978).
[21] M. Vespalcová, K. Šlais, D. Kouřilová and M. Krejči, *Československ. Farm.* (in press).
[22] K. Tsuji and R. B. Binns, *J. Chromatogr.* **253**, 227–236 (1982).
[23] W. Th. Kok, H. B. Hanekamp, P. Bos and R. W. Frei, *Anal. Chim. Acta* **142**, 31–45 (1982).
[24] W. Th. Kok, U. A. Th. Brinkman and R. W. Frei, *J. Chromatogr.* **256**, 17–26 (1983).

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