Journal of Chromatography, 502 (1990) 305–315
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 052

Sensitive, indirect photometric detector for high-performance liquid chromatography using a light-emitting diode

ALAIN BERTHOD[a], MARK GLICK and JAMES D. WINEFORDNER*
University of Florida, Department of Chemistry, Gainesville, FL 32611 (U.S.A.)
(First received June 15th, 1989; revised manuscript received October 3rd, 1989)

SUMMARY

A low-noise detector for indirect photometric detection has been constructed using a highly stable source —a light-emitting diode (LED). Use of the detector is demonstrated for reversed-phase liquid chromatography by adding methylene blue to the mobile phase to make a background signal. The indirect determination of alcohols by their effect on methylene blue concentration distribution is demonstrated, and an investigation is made into the conditions for high sensitivity. Because the source exhibits low noise, the detection limits for alcohols are as low as more complex and expensive detection methods, despite the lower radiant power of the LED. Detection limits for nine alcohols are below μg injected amounts.

INTRODUCTION

The main problem in liquid chromatography (LC) is detection. In gas chromatography there are different universal detectors such as the flame ionization detector, the electron-capture detector or the mass spectrometer. In LC the column effluent contains trace amounts of solutes of interest that can be difficult to detect. Pre- or post-column derivatization can solve some specific problems, but these techniques are time consuming and are not universal. Another approach is to use a mobile phase containing an additive with an inherent detector response. Analytes can be indirectly detected by their displacement of the additive. This approach is often referred to as indirect detection, and has been demonstrated for a wide variety of chromatographic systems[1]. The most successful use of indirect detection, and one of the earliest, has been for ionic compound LC detection. An absorbing ion is paired with transparent ionic solutes of the opposite charge. The ion pairs can be detected by the counter-ion absorbance[2]. Indirect detection was later extended to non-charged solutes.

There are several problems associated with indirect detection. All chromatograms obtained using indirect methods contain one or more extraneous peaks, called

[a] Permanent address: Laboratoire des Sciences Analytiques, UA CNRS 435 (J. M. Mermet), Université de Lyon 1, 69622 Villeurbanne Cedex, France.

0021-9673/90/$03.50

system peaks[3]. These peaks may result in incorrect peak identification, or coelution with solutes and erroneous quantitation. Baseline instability is another inherent drawback of indirect detection methods.

In previous papers we demonstrated methods for indirect fluorescence detection and proposed a possible mechanism of response[4–6]. We also recently showed that it is possible to construct a high-precision fluorimeter by using a light-emitting diode (LED)[7]. In this work we describe an inexpensive, sensitive indirect photometric detector using an LED. By monitoring the absorbance of a colored additive in the mobile phase, non-absorbing solutes could be indirectly detected. The separation of aliphatic alcohols, using methylene blue as the additive, was chosen as a test system for evaluation of the detector performance. This chromatographic system was first studied by Gnanasambandan and Freiser[8,9].

EXPERIMENTAL

Chemicals

Methylene blue ($C_{16}H_{18}N_3SCl$, mol.wt. 319.9) was purchased from Kodak. The purity was listed as 84%, and it was used as received. Methanol, ethanol, 1-propanol, 2-propanol, 2-methyl-2-propanol, 1-butanol, 2-butanol, 2-methyl-1-butanol, 1-pentanol, 3-pentanol, 1-hexanol, 1-heptanol, 1-octanol and 1-decanol were obtained from Burdick and Jackson, Fisher, Aldrich and Kodak. All were analytical grade and were used as received. Water was deionized and filtered to a resistivity greater than 14 MΩ with a Barnstead Ultrapure system.

Chromatography

An Altex high-performance liquid chromatograph was used to pump the methylene blue–methanol–water mobile phase through a 25 cm × 4.6 mm I.D. Altex Ultrasphere column. The packing material was 5 μm particle size bonded with octadecyl chains and end-capped with trimethyl chlorosilane. The silica pore size was 8 nm mean diameter. The surface area was approximately 200 m^2/g. The carbon loading was approximately 12% (w/w), and the calculated surface coverage is about 2.96 $\mu mol/m^2$. The dry stationary phase column content was estimated to be 2.5 g (column surface area: 620 m^2, column organic stationary phase content: 1.2 mmole). A pulse dampener (Alltech Free-Flow) was used to improve baseline stability. The column was immersed in a water bath for temperature stability. All measurements were made at 30 ± 0.5°C.

Detection system

The LC detector was a modification of the LED fluorometer described in an earlier paper[7]. A diagram of the detection system is shown in Fig. 1.

The peak wavelength of the LED (Hewlett-Packard, HLMP-3950) was 565 nm. Rather than use lenses to transfer the light through the cell, the LED was placed directly on the face of the flow cell. A 12-V motorcycle battery in series with a variable resistance (100–500 Ω) was used to power the LED for long periods of time without recharging. To reduce long-term intensity drift of the LED, the LED was "aged" by operating at high current (>20 mA) for about a week. Urethane foam was used throughout the detector to keep out stray light and to thermostat the LED. A photodiode (EG & G, UV-100BG) was placed next to the LED to monitor the intensity.

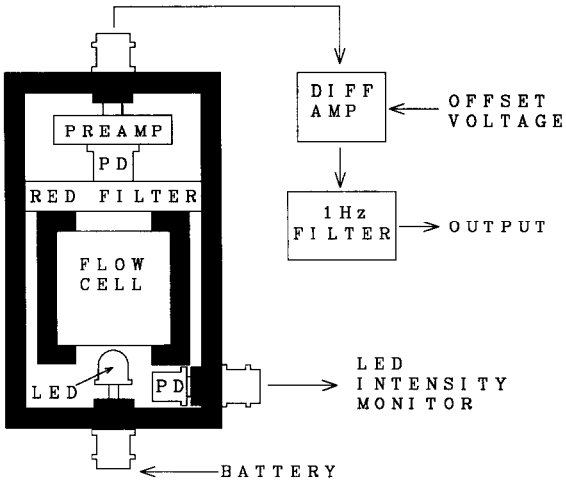

Fig. 1. Diagram of the LED detector. A photodiode (PD) was used to monitor the LED light intensity. A second integral photodiode/preamplifier (PREAMP) detected the transmitted light.

The flow cell was a 1-cm cuvette with an internal volume of 8 μl (Hellma, 176.753). A red glass filter was used to reduce stray light. A combination photodiode/preamplifier (EG & G, TCN 1000-93) was used to detect the light transmitted through the flow cell and the filter. Several 9-V batteries were used to power the detector.

The voltage output of the detector was amplified by a differential amplifier (Princeton Applied Research, 113) with respect to a constant-voltage source (laboratory constructed). The voltage source could be varied to negate the large offset that occurs with indirect photometry. All chromatograms were obtained with an instrumental bandwidth of 1 Hz.

RESULTS

Methylene blue adsorption

The column was conditioned with all of the mobile phases including the dye additive according to the procedure described by Gnanasambandan and Freiser[8,9]. The mobile phase was passed through the column until the effluent absorbance was equivalent to that of the original mobile phase. The mobile phase volume was measured and used to calculate the amount of adsorbed dye (break-through method).

To remove the dye, the column was flushed with 20 internal volumes of pure methanol (1 ml/min for 30 min) and 20 internal volumes of chloroform–methanol (20:80, v/v)[8]. The column was then rinsed overnight with pure methanol (0.5 ml/min for 12 h). All effluents were collected and the dye concentration was spectrophotometrically determined, corresponding to a desorbed quantity of dye. After this treatment, the dye was considered to be removed, although it was still possible to see a very pale blue color in the eluting methanol mobile phase. A maximum dye concentration of $1.2 \cdot 10^{-6} M$ was measured in the case of the water–methanol (95:5) mobile phase after the desorption treatment. The adsorbed amount of dye obtained with the

TABLE I

METHYLENE BLUE ADSORPTION ON ULTRASPHERE ODS

Mobile phase water–methanol (%,v/v)	*Methylene blue concentration* (M)	*Adsorbed amount* mass (g)	*Adsorbed amount* mole (μmole)	*Stationary phase*[a] *concentration* (molar ratio)
95:5	$6.7 \cdot 10^{-4}$	14.8	46	0.035
95:5	$1 \cdot 10^{-4}$	8.3	26	0.020
95:5	$3 \cdot 10^{-5}$	4.0	12.5	0.0096
90:10	$1 \cdot 10^{-4}$	3.5	11	0.0085
40:60	$1 \cdot 10^{-4}$	0.54	1.7	0.0013
30:70	$1 \cdot 10^{-4}$	0.51	1.6	0.0012

[a] The active stationary phase is the ODS layer estimated to be 1.3 mmoles inside the column. The stationary phase concentration is the molar ratio of the adsorbed moles of dye over the ODS moles (1.3) in the column.

break-through method was in agreement within 10% with the corresponding desorbed amount. The average values are listed in Table I.

Separation of aliphatic alcohols

Fig. 2 presents the chromatogram obtained after injecting a mixture of six alcohols. Table II lists the chromatographic parameters and analytical figures of merit for the mobile phases studied. Fig. 3 shows a chromatogram obtained after injecting a mixture of nine alcohols. Peak heights were used for quantitation. The detector response was linear over three orders of magnitude of injected concentration

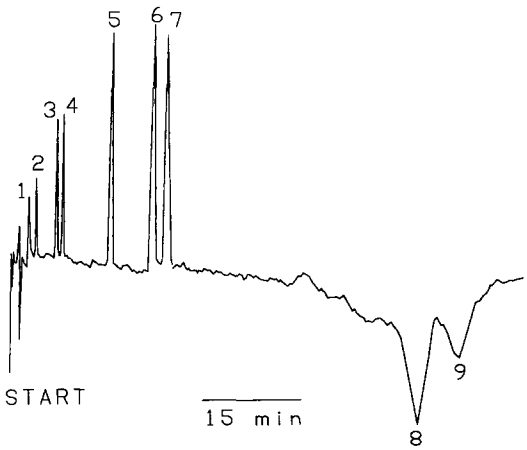

Fig. 2. Indirect chromatogram of six alcohols. Mobile phase water–methanol (95:5, v/v) with methylene blue 10^{-4} *M*, 1 ml/min, 30°C. Peaks: 1 = methanol; 2 = ethanol; 3 = 2-propanol; 4 = 1-propanol; 5 = 2-methyl-2-propanol; 6 = 2-butanol; 7 = 1-butanol; 8, 9 = system peaks. Injection: 8 μg of each alcohol but methanol (injection of a 20 μl 0.05%, v/v, solution).

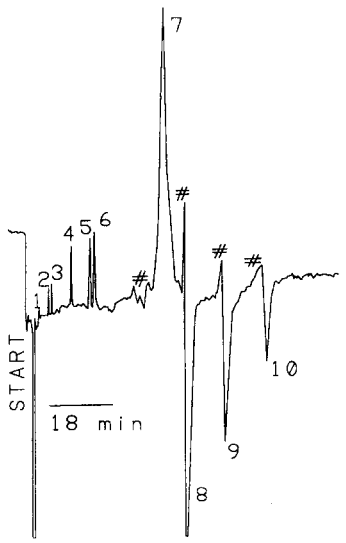

Fig. 3. Indirect chromatogram of nine alcohols. Mobile phase water–methanol (90:10, v/v) with methylene blue $10^{-4}M$, 1 ml/min, 30°C. Peaks: 1 = ethanol (6 μg); 2 = 2-propanol (6 μg); 3 = 1-propanol (6 μg); 4 = 2-methyl-2-propanol (6 μg); 5 = 2-butanol (6 μg); 6 = 1-butanol (6 μg); 7 = 3-pentanol (16 μg); 8 = system peak; 9 = 2-methyl-1-butanol (16 μg); 10 = 1-pentanol (16 μg); # = shoulder peaks, see text.

(0.005% to 5%, v/v, injected solution) for the water–methanol (95:5) mobile phase. The linearity was only two orders of magnitude for the other mobile phases.

Peak efficiencies, N, were determined using the classical plate count equation for Gaussian peaks, $N = 4\,(V_R/w_{0.6h})^2$, in which V_R is the retention volume and $w_{0.6h}$ is the peak width, expressed in volume units, at 60% of the peak height.

DISCUSSION

LED detector

The capabilities of the simple LED detector presented in this paper are demonstrated by the low limits of detection (LOD) obtained for aliphatic alcohols (Table II). As described by Takeuchi and Yeung[10], the dynamic reserve, the concentration of the absorbing additive, and the displacement ratio play important roles in determining the sensitivity that can be achieved with indirect detection. The dynamic reserve of a detector is defined as the ratio of the background signal to its noise level[10]. Although the light power of an LED is low, in the 50 mW range, the emitted light is so stable, with root mean square noise fluctuation in the 0.0004% range[7], that the signal-to-noise ratio is better than many other light sources.

The concentration of the absorbing species —the mobile phase additive— is responsible for the background signal. However, it also has a strong influence on the amount of dye adsorbed on the stationary phase (Table II). In our case, we found that the amount of methylene blue adsorbed in the Ultrasphere ODS column followed a Langmuir isotherm, *i.e.* the ratio 1/(mobile phase dye concentration) is proportional

TABLE II

CHROMATOGRAPHIC RETENTION PARAMETERS AND ANALYTICAL FIGURES OF MERIT

Mobile phase water–methanol	*Methylene blue concentration (M)*	*Alcohol*	V_R *(ml)*	k'	N *(plates)*	*LOD*		*Response factor (a.u./mmol)*
						μg	*nmole*	
95:5	$1 \cdot 10^{-4}$	Ethanol	3.72	1.25	3900	1.6	29	130
		2-Propanol	6.84	3.15	5100	0.9	15	290
		1-Propanol	7.80	3.73	5200	0.9	15	310
		2-Methyl-2-propanol	15.2	8.20	6200	0.6	8	730
		2-Butanol	21.8	12.2	6800	0.5	6.8	750
		1-Butanol	23.9	13.5	6600	0.5	6.8	660
		System I	63.2[a]	37.3	2900	–	–	–
		System II	69.3[a]	41.0	2900	–	–	–
95:5	$3 \cdot 10^{-5}$	Ethanol	3.62	1.19	3500	2.0	44	51
		2-Propanol	6.75	3.09	4200	1.4	24	100
		1-Propanol	7.65	3.64	4200	1.3	12	110
		2-Methyl-2-propanol	15.0	8.09	4900	1.0	13.5	230
		2-Butanol	21.3	11.9	5700	0.9	12.2	240
		1-Butanol	23.2	13.1	6000	0.9	12.2	260
		System I	63.0[a]	37.1	2800	–	–	–
		System II	69.1[a]	40.8	2800	–	–	–

90:10	$1 \cdot 10^{-4}$	Ethanol	3.42	1.07	7000	3.0	65	29
		2-Propanol	5.88	2.56	9500	1.5	25	83
		1-Propanol	6.72	3.07	9500	1.5	25	84
		2-Methyl-2-propanol	12.4	6.49	11000	0.7	9.5	210
		2-Butanol	17.7	9.73	13000	0.7	9.5	210
		1-Butanol	19.3	10.7	11000	0.6	8.2	240
		3-Pentanol	38.1	22.1	2800	0.4	4.5	360
		2-Methyl-1-butanol	55.5[a]	32.6	11000	0.9	9.9	170
		1-Pentanol	72.1[a]	42.7	12000	1.1	12	140
		System peak	45.3[a]	26.4	–	–	–	–
40:60	$1 \cdot 10^{-4}$	1-Pentanol	4.82	1.91	–[b]	60	680	6.1
		1-Hexanol	7.41	3.45	4400	110	1080	3.9
		1-Heptanol	12.0	6.27	4200	30	260	13
		1-Octanol	21.6[a]	12.1	4000	30	230	13
		1-Decanol	76.5[a]	45.5	4300	100	630	2.2
		System peak I	5.1[a]	2.09	–[b]	–	–	–
		System peak II	15.0[a]	8.09	–[b]	–	–	–
		System peak III	17.1[a]	9.36	–[b]	–	–	–

[a] Negative peak

[b] Efficiency cannot be calculated due to peak overlap.

to the ratio 1/(stationary phase dye concentration) with a regression coefficient r = 0.9997. The same relationship was observed by Vigh and Leitold[11] with benzyl alcohol as the absorbing additive and a Merck RP-18 stationary phase.

The displacement ratio is the number of dye molecules displaced by one solute molecule. The higher the displacement number, the higher the detection sensitivity. Clearly the displacement ratio is dependent on the amount of adsorbed dye on the stationary phase. Table II shows that the response factors are lower when the adsorbed amount of dye is lower (Table I). The LOD values of the water–methanol (95:5) mobile phase containing only $3 \cdot 10^{-5}$ M of methylene blue were 50% higher than the corresponding value with the same mobile phase containing 10^{-4} M of dye (Table II). Decreasing the dye concentration in the mobile phase produced a decrease in background signal and noise, which should increase the sensitivity. However, a concurrent reduction in the amount of adsorbed dye produced a decrease in the displacement ratio and in the response factor, which should decrease the sensitivity. The global effect was negative, producing higher LOD values with lower mobile phase dye concentrations.

The chromatograms of Figs. 2 and 3 and the analytical figures of merit listed in Table II compare quite well with those of similar studies found in the literature[2,8,9,12]. These figures of merit demonstrate the utility of the simple LED detector.

Chromatographic efficiency

The column efficiency was tested using a commercial UV LC detector with a classical mobile phase (methanol–water 70:30) and was found to be in the 12 000 plate range (height equivalent to a theoretical plate = 20 μm or 4 × particle diameter). The efficiencies measured with the LED detector and the water–methanol (95:5) mobile phase were 50% lower (Table II), while those obtained with the water–methanol (90:10) mobile phase were in the 12 000 plate range. We think that the high water content (95%) of the first mobile phase is responsible for the lower efficiency. It has been shown that water-rich mobile phase does not properly wet the monolayer ODS stationary phases. The C_{18} hydrocarbon chain of the "brush" type ODS phase can be agglomerated by water (hydrophobic repulsion)[13]. Such a water collapsed state is destroyed by about 10% methanol which can wet the C_{18} chains and restore the efficiency (Table II). A low efficiency was also obtained with a methanol-rich mobile phase, but the corresponding experiments were performed last and we suspected column aging. The detector flow cell volume (8 μl) was suitable for classical chromatographic analysis and did not induce excessive band broadening.

System peaks

Figs. 2 and 3 and Table II show system peaks. As stated already, such peaks always appear in indirect detection methods. System peaks have been intensively studied in the literature[3,11,14–16]. They are produced by a disruption of the adsorbed mobile phase components (dye additive, methanol and water) by the injected sample. It was found that system peaks carried much useful information about adsorption isotherms, void volumes and retention mechanisms[3,14]. In indirect detection chromatography, one of the system peaks corresponded to the retention of the absorbing additive. In our case, we observed two system peaks with the water–methanol (95:5) (Fig. 2) and only one with the water–methanol (90:10) mobile phase (Fig. 3). As

system peaks are not really useful for quantitative analysis, it is best to minimize them by dissolving all injected solutes in the mobile phase with the same amount of dye. In Fig. 3a symbol marks shoulders or associated peaks that appeared before each peak that was close to the system peak. Similar artifacts were observed by Parkin and Lau[17] who suggested that they were due to refractive index changes. We think the artifact peaks could be due to a local methylene blue concentration perturbation. They may also arise from an absorbing impurity in the methylene blue, which was only 84% pure. An impurity would explain the second system peak observed with some mobile phase compositions (Fig. 2).

Chromatographic mechanism

The alcohol–methylene blue complex formation advanced by Gnanasambandan and Freiser[8] was questioned by several authors[11,17,18]. The mechanistic interpretation of induced peaks described by Stranahan and Deming[16] more likely explains the alcohol–methylene blue induced peaks obtained in our case. The injection of a solute disturbs the steady-state concentration distribution of methylene blue in the injection zone and this disturbance travels through the column. This leads to an induced peak that coelutes with the solute and a peak in the reverse direction eluting at the dye retention time. For solutes with retention times shorter than the system peak, the local disturbance is a solubility enhancement of the dye in the mobile phase due to the presence of the alcohol. Conversely, if the alcohol retention time is greater than the system peak, then the disturbance is a decrease in the solubility of the dye in the mobile phase (Fig. 3).

Table III lists the slopes and intercepts and regression coefficients of the plots $\log k' = f(n_c)$, in which n_c is the carbon number of linear aliphatic alcohols. The logarithm of the capacity factor k' can be related to the free energy of phase transfer of the solute, ΔG^0, by:

$$\ln k' = \Delta G^0/RT + \ln \varphi$$

where R, T and φ are the gas constant, the absolute temperature and the phase ratio of the column[19]. For members of a homologous series of solutes, such as the linear primary alcohols, the previous equation can be written as:

$$\ln k' = \Delta G_e^0/RT + \ln \varphi + n_c \Delta G_i^0/RT$$

TABLE III

METHYLENE FREE ENERGY OF TRANSFER FOR LINEAR ALCOHOLS

Mobile phase water–methanol	*log k' = f(n_c)*	*n_c*	*Correlation coefficient*	*$\Delta G^0_{CH_2}$ (kJ/mol)*
95:5	$-0.950 + 0.517\ n_c$	3	0.9989	3.0
95:5[a]	$-0.983 + 0.522\ n_c$	3	0.9993	3.0
90:10	$-1.238 + 0.571\ n_c$	4	0.9996	3.3
40:60	$-1.116 + 0.276\ n_c$	5	0.9996	1.6
30:70	$-0.985 + 0.209\ n_c$	5	0.9996	1.2

[a] Methylene blue concentration $3 \cdot 10^{-5}$ M, all other mobile phase contained $1 \cdot 10^{-4}$ M methylene blue.

in which the subscripts e and i refer to the retention energy contribution of the end group (OH) and the incremental (CH_2) group, respectively.

The first two entries listed in Table III show that there was no significant change in the retention mechanism when the methylene blue concentration was changed. The dye does not seem to interfere in the retention process. The methylene free energy increased from 3.0 to 3.3 kJ/mol when the methanol mobile phase content increased from 5 to 10%. This is evidence of the better wetting of the stationary phase by the 10% methanol mobile phase. Because the "collapsed" state is destroyed by the 10% methanol, the ODS chains have an enhanced mobility and they can interact more easily with the alcohol alkyl chains. At higher methanol content, the polarity difference between the stationary and the mobile phase decreased and so did the methylene blue free energy of transfer (Table III).

Some long-term noise was observed in all separations. Typically, the baseline fluctuated by 1% full scale with a period of several hours. Figs. 2 and 3 show chromatograms lasting more than an hour where the long term baseline drift can be seen. We found such baseline drifts in most papers showing indirect detection chromatograms lasting for more than one hour[1,2,8,12,17,18]. The short-term, peak–peak noise of the detection system was 0.004% —a factor of ten poorer than the noise of the LED. Pressure and temperature instabilities were believed to be responsible for this degradation. The steady-state dye distribution is very sensitive to any variation. The whole system must be carefully temperature regulated in order to minimize such noise. It is also possible that each injection induced "second order" or higher order systems peaks that would explain the long-term noise observed.

CONCLUSIONS

A simple, compact low-noise LED-filter-photodiode indirect photometric detector for reversed-phase high-performance LC using methylene blue in the mobile phase is shown to have use for non-absorbing solutes. Because of the low noise of both the LED source and the photodiode detector, the detection limits for alcohols are similar to more expensive, more complex commercial systems based on various spectrometric principles. The detector could easily be miniaturized further and operated in the field. The potential use of this detector in a mobile system appears considerable.

ACKNOWLEDGEMENT

This research was supported by NIH-RO1-GM11373-26.

REFERENCES

1 G. Schill and J. Crommen, *Trends Anal. Chem.*, 6 (1987) 111.
2 M. Denkert, L. Hackzell, G. Schill and E. Sjögren, *J. Chromatogr.*, 218 (1981) 31.
3 S. Levin and E. Grushka, *Anal. Chem.*, 58 (1986) 1602.
4 S. Y. Su, A. Jurgensen, A. Bolton and J. D. Winefordner, *Anal. Lett. Part A.*, 14 (1981) 1.
5 S. Y. Su, E. P. C. Lai and J. D. Winefordner, *Anal. Lett. Part A.*, 15 (1982) 439.
6 E. L. Torres, F. Van Geel and J. D. Winefordner, *Anal. Lett. Part A*, 16 (1983) 1207.
7 B. W. Smith, B. T. Jones and J. D. Winefordner, *Applied Spectrosc.*, 42 (1988) 1469.

8 T. Gnanasambandan and H. Freiser, *Anal. Chem.*, 54 (1982) 1282.
9 T. Gnanasambandan and H. Freiser, *Anal. Chem.*, 53 (1981) 909.
10 T. Takeuchi and E. S. Yeung, *J. Chromatogr.*, 366 (1986) 145.
11 G. Vigh and A. Leitold, *J. Chromatogr.*, 312 (1984) 345.
12 L. Hackzell, T. Rydberg and G. Schill, *J. Chromatogr.*, 282 (1983) 179.
13 R. K. Gilpin, *J. Chromatogr. Sci.*, 22 (1984) 371.
14 S. Levin and E. Grushka, *Anal. Chem.*, 59 (1987) 1157.
15 G. Brandt, P. Vógler and A. Kettrup, *Fresenius Z. Anal. Chem.*, 325 (1986) 252.
16 J. J. Stranahan and S. N. Deming, *Anal. Chem.*, 54 (1982) 1540.
17 J. E. Parkin and H. T. Lau, *J. Chromatogr.*, 314 (1984) 488.
18 W. Kang, H. B. Oh and Y. J. Moon, *J. Korean Chem. Soc.*, 31 (1987) 184.
19 G. Guiochon, in Cs. Horváth (Editor), *High-Performance Liquid Chromatography —Advances and Perspectives*, Vol. 2 Academic Press, New York, 1980.