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Short communication

Postcolumn fluorescence as an alternative to evaporative light scattering detection for ceramide analysis with gradient elution in non-aqueous reversed-phase liquid chromatography

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Abstract

Ceramide analysis was developed with gradient elution in non-aqueous reversed-phase liquid chromatography with evaporative light scattering detection (ELSD) or postcolumn fluorescence detection. Fluorescence detection (excitation, 360 nm; emission, 425 nm) after postcolumn formation of mixed assemblies between eluted ceramides and 1,6-diphenyl-1,3,5-hexatriene was developed. In comparison with ELSD, fluorescence detection allows a better detection of the minor species ceramide from ceramide type III (commercial mixture of non-hydroxy fatty acid–sphingosine) and appears to be more sensitive for quantitation of ceramides at low concentrations. The fluorescence response is linear over a wide range of injected amount of ceramide III (expressed as stearoyl-phytosphingosine): 10 ng to 1000 ng. The response of ELSD is non linear but can be linearized in double logarithmic coordinates for calculations over a narrow range, e.g. between 10 to 350 ng ceramide III injected. The lower quantitation limits of these two detectors are similar: 5 ng ceramide III was injected.
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1. Introduction

The quantitative and qualitative determination of cutaneous ceramide composition is of prime importance in order to gain insight into their role in skin barrier function [1–3].

In recent years, some high-performance liquid chromatographic (HPLC) techniques devoted to ceramide analysis have been developed [4–10]. Among them, non-aqueous reversed-phase (NARP) HPLC [7] appeared particularly suitable according to the high hydrophobic nature of ceramides. The use of gradient elution was necessary to allow acceptable analysis time due to the large range of polarity of these molecules. However, to develop ceramide analysis, the choice of the mode of detection is decisive. Ceramide detection using ultraviolet (UV) absorption is well known to be insensitive because of their weak chromophoric functionality in the 200–210 nm region [6,8]. However, derivatization of

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ceramides is possible which allows an efficient detection using UV [9,10] or fluorescence [11] detection. The drawback of these derivatization methods is the extensive sample preparation and their time duration. More recently, evaporative light scattering detection (ELSD) has been described as one of the most suitable methods of detection for lipid analysis [5,12–14]. The response of ELSD is known to be non-linear, but the response factor depends only to a very small extent on the nature of the compound. Due to the different phenomena involved in mobile phase nebulization and light scattering, the response (R) is linked to the amount of the solute detected (m) by Eq. (1) [15,16]:

$$R = \mathrm{a}m^{\mathrm{b}} \quad (1)$$

where a and b are constants that depend on the nebulizer design, solvent composition and wavelength of the light source. Deviation from Eq. (1) is encountered for high values of m due to photomultiplier saturation. The non linearity of the ELSD response prompted us to consider other way of detection.

In this work, the use of emission enhancement of a fluorescence polarity probe in the presence of the hydrophobic ceramide compounds was studied. Fluorescence probes may be defined as small molecules which undergo changes in one of more of their properties of fluorescence as a result of non-covalent interaction(s) with a selected molecular species or upon a change of their molecular environment. Among the tested probes, 1,6-diphenyl-1,3,5-hexatriene (DPH) appeared of interest because this molecule does not fluoresce in a polar environment (e.g. in water) but strongly fluoresces in organic solvents or in hydrophobic media. Therefore, this property was exploited in order to enhance the emission of this probe by contact with the apolar moiety of ceramides after postcolumn mixing [17]. This technique has been proposed for analysis of phospholipids eluted by an isocratic HPLC system [18–20]. The aim of this study was to extend this technique to the analysis of ceramides which are more apolar and non-soluble in water, by a gradient NARP-HPLC system [21]. And then, the comparison between fluorescence postcolumn detection and ELSD was studied.

2. Experimental

2.1. Chemicals and solutions

Ceramide III (pure standard of stearoyl phytosphingosine) was a generous gift from Cosmoferm (Delft, Netherlands). Ceramide type III (commercial mixture of non-hydroxy fatty acid–sphingosine), DPH and Brij 35 (30%, w/v) were purchased from Sigma (St. Quentin Fallavier, France). All solvents used for chromatography were of HPLC grade and from Fisher Scientific (Elancourt, France).

A fresh solution of 3 mM DPH in tetrahydrofuran (THF) was prepared monthly and stored in the dark at +4°C. 0.4 mg ml^{-1} stock solution of ceramide III was prepared in THF. One mg ml^{-1} stock solution of ceramide type III was prepared in THF. Methanolic working solutions of ceramide III and ceramide type III were prepared as needed for chromatography.

Ultra pure water provided by an Alpha-Q (Millipore, St. Quentin en Yveline, France) system was used for the preparation of the aqueous solutions.

2.2. Apparatus

The HPLC system consisted of a Thermo Separation Products gradient pump (Model P1000 XR) with a TSP SCM1000 vacuum membrane degasser (Thermo Separation Products, San Jose, California, USA) connected to a Kontron Model 360 autosampler (Kontron Instruments, Milan, Italy) equipped with a 5 μl sample loop injection valve. The flow-rate of mobile phase was set at 0.4 ml min^{-1}. The analytical column (125×2.0 mm I.D.) was a Kromasil C_{18} (Eka Nobel, Bohus, Sweden) with a particle size of 5 μm supplied by Macherey–Nagel (Hoechst, Düren, Germany) preceded by a guard column of the same material. The column was thermostated with a Jetstream 2 temperature controller (Thermotecnics Products, Austria). The chromatograms were recorded with a Kroma System 2000 version 1.60 (Kontron Instruments). Peak areas were used for quantitation.

Two detection systems were alternatively used: first, a Cunow DDL 11 ELSD system (Eurosep, Cergy, France) where the drift tube temperature was set at 35°C and nitrogen pressure at 1 bar; second, a

postcolumn fluorescence detection system which consisted of a second Shimadzu LC-9A pump (Touzart & Matignon, Vitry sur Seine, France) connected via a pulse damper to a Tee piece on line with a 3000×0.5 mm I.D. knitted PTFE tubing (Supelco, Bellafonte, USA), and a Shimadzu Model RF 551 fluorescence spectrometer (Touzart & Matignon, Vitry sur Seine, France). The excitation and emission wavelengths were set to 360 nm and 425 nm, respectively. The optimal postcolumn addition reagent composition 1.60 μmol l^{-1} DPH and 57.50 μmol l^{-1} Brij 35 in ultra-pure water. This solution was prepared freshly and sonicated in a bath sonicator (BRANSCO 2200, Touzart & Matignon, France) before mixed with the chromatographic eluent at a flow-rate of 3.2 ml min^{-1}.

3. Results and discussion

3.1. Optimization of the postcolumn DPH addition

As the DPH probe fluoresces in an apolar environment, the high amount of organic solvent used in NARP chromatography had to be counterbalanced by a sufficient postcolumn addition of aqueous solution containing the probe in order to perform a sensitive detection.

The optimal conditions for the postcolumn detection of ceramides were determined with ceramide III eluted in isocratic mode using the following mobile phase: acetonitrile–1-propanol (60:40, v/v) containing 10 m*M* triethylamine and an equimolar amount of formic acid [21]. The column was thermostated at 30°C.

Firstly, the effect on the fluorescence signal due to each of the three following parameters defining the postcolumn reagent: the amount of DPH, the amount of surfactant (Brij 35) and the flow-rate of postcolumn addition, were studied separately. The use of a surfactant was necessary to prevent the build-up of background fluorescence resulting from adsorption of lipid vesicles in the flow cell [18]. A postcolumn addition solution consisting of 1.2 μmol l^{-1} DPH and 50 μmol l^{-1} Brij 35 and pumped at 3 ml min^{-1} lead to a high peak area of ceramide III. But, the three studied parameters were in fact correlated. Hence, the above obtained results were considered as a starting point for a Simplex procedure in order to determine the optimal conditions of postcolumn fluorescence detection. The peak area (A) and the number of theoretical plates (N) were criteria chosen to assess the detection. The test conditions proposed by the Simplex procedure and the obtained results are presented in Table 1. Among the obtained results in the four initial tests, the peak area varied from 125

Table 1
Conditions of postcolumn DPH aqueous reagent tested in the Simplex and corresponding results

Test No.	Parameters			Results[a]	
	DPH (μmol l^{-1})	Brij 35 (μmol l^{-1})	Flow-rate (ml min^{-1})	Peak area (mV min)	N[b]
1	1.35	37.50	2.8	125	975
2	1.05	62.50	2.8	110	1045
3	1.05	37.50	3.2	125	1115
4	1.35	62.50	3.2	165	1590
5	0.95	70.75	3.3	150	1885
6	0.75	87.50	3.6	NP[c]	NP
7	1.25	45.75	2.9	140	1660
8	1.38	34.75	3.4	135	1770
9	1.55	20.75	3.7	105	1595
10	1.60	57.50	3.2	185	1890
11	1.88	68.00	3.2	180	1990

[a] Mean value ($n=2$).
[b] N = number of theoretical plates.
[c] NP = not performed. See below text for explanation.

to 165 mV min, and N varied from 975 to 1590, respectively. The best results ($A = 165$ mV min, $N = 1590$) were obtained with the highest values of these three parameters. For the seven following tests, test No. 6 appeared unrealizable due to the large amount of surfactant added which rose the fluorescence background signal of the postcolumn reagent itself. The increase of DPH fluorescence by surfactant being at a high concentration has already been observed [17], and can be explained by the micellization of DPH, rendering their molecular environment more hydrophobic. The best results were obtained with test conditions Nos. 10 and 11. However, it should be noted that a large amount of surfactant was added in order to stabilize the baseline in test No. 11. Consequently, the optimal conditions for ceramide analysis by postcolumn DPH fluorescence detection were chosen as the conditions used in test No. 10: The postcolumn addition reagent consisted of 1.6 μmol l^{-1} DPH and 57.5 μmol l^{-1} Brij 35 pumped at a flow-rate of 3.2 ml min^{-1}.

3.2. Comparison between fluorescence detection and ELSD

3.2.1. Analysis of ceramide III by an isocratic NARP-HPLC system

The relative performance of these two detectors was firstly evaluated through their linearity and limit of detection for the determination of ceramide III, tested as a model of ceramide compounds. The chromatographic conditions were modified in comparison with which used in Section 3.1. The mobile phase used was: acetonitrile–1-propanol (70:30, v/v) containing 10 mM triethylamine and an equimolar amount of formic acid. The column was thermostated at 35°C. This change in the chromatographic conditions, increased the number of theoretical plates (N) by about 28% in comparison with the results obtained at the end of the Simplex procedure.

As described above, the response of ELSD is non linear, the response (R) is linked to the amount of solute detected (m) through Eq. (1). But, lineariza-

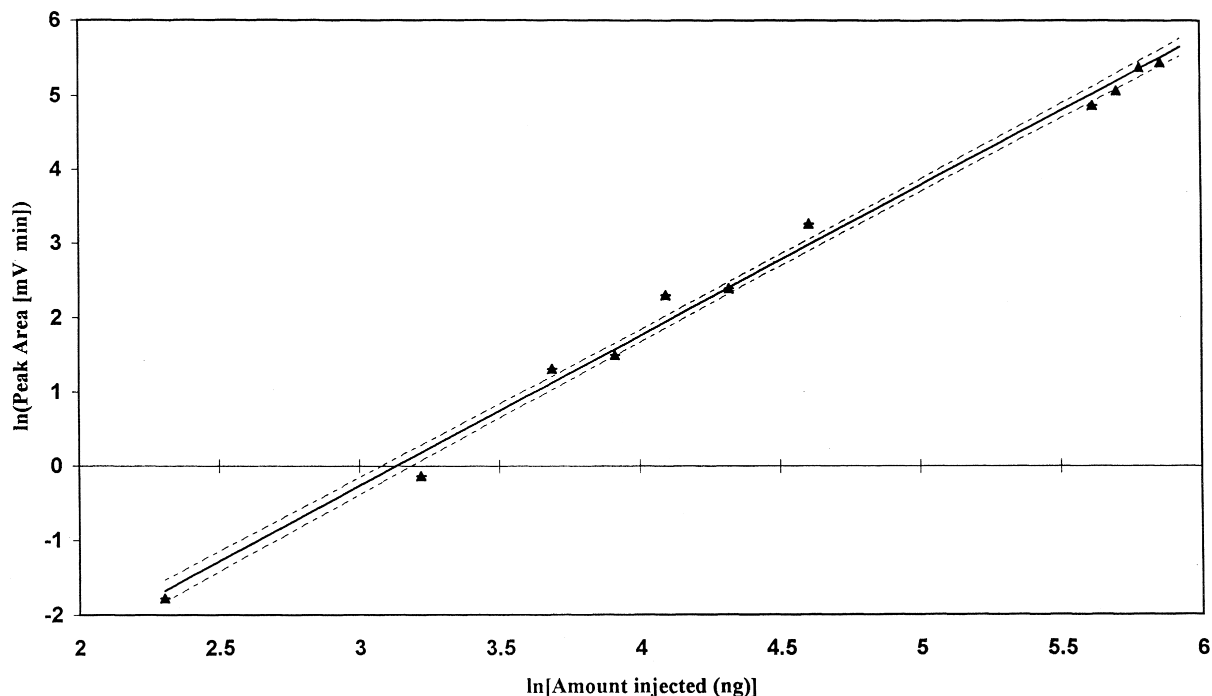

Fig. 1. Regression curve of ELSD response versus injected amount of ceramide III in double logarithmic coordinates.

Table 2
Parameters of linear regressions

Detection	Equation	n	Intercept	Slope	Linearity test (F-test) [a]	r
ELSD	$\ln R = \ln a + b \ln m$	11×3	−6.33±0.15	2.02±0.03	$F^{1}_{31}=3479$	0.9956
Fluorescence	$R = a + bm$	8×3	−0.04±0.28	$0.14\pm7.6\cdot10^{-4}$	$F^{1}_{22}=35\,495$	0.9997

[a] All values are highly significant ($P<0.01$).

tion remains possible using double logarithmic coordinates [16]: Eq. (2):

$$\ln R = \ln a + b \ln m \qquad (2)$$

Fig. 1 shows a plot of peak area versus injected amount of ceramide III for amounts ranging from 10 to 350 ng injected. Values of the coefficients from Eq. (2) are presented in Table 2. The relationship is linear over the full range ($r=0.9956$). Photomultiplier saturation appeared for injected amounts higher than 400 ng leading to truncated peaks and these were not involved in the calculation.

Fluorimetry is known to be linear for diluted samples. The regression curve obtained by fluorescence detection for injected amounts ranging from 10 to 1000 ng of ceramide III ($r=0.9997$) is shown in Fig. 2. The upper amounts were not considered since ceramide III precipitate at higher concentrations.

Compared with the log–log regression line obtained with ELSD, data points from fluorimetric detection appeared to be closer to their estimated

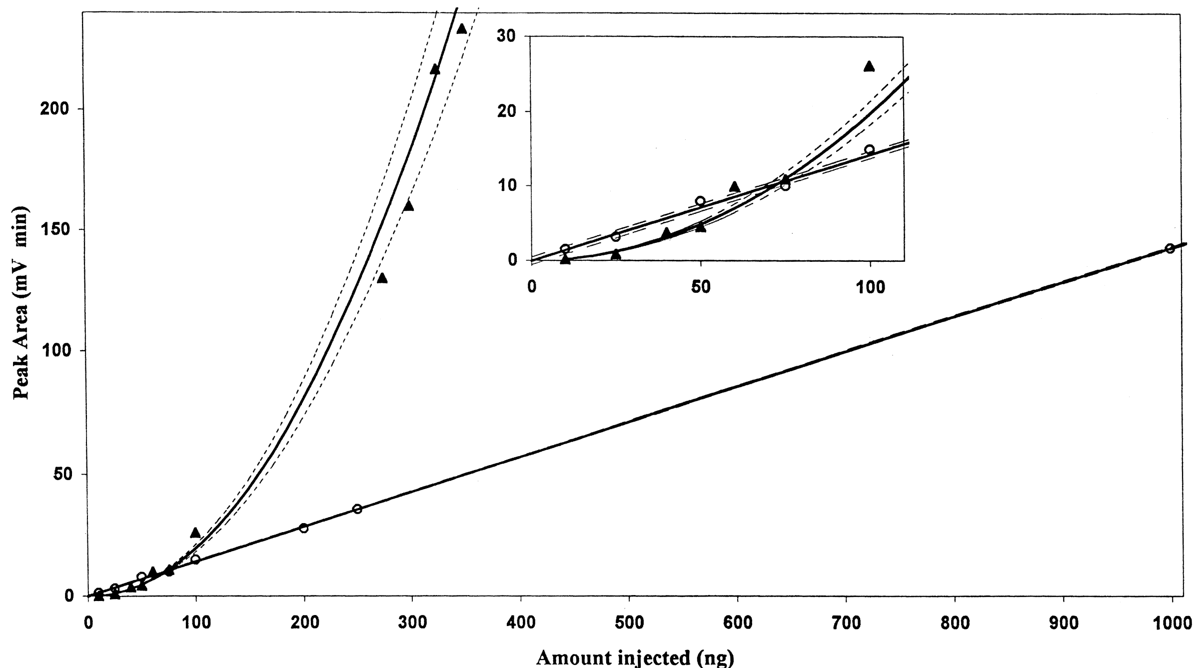

Fig. 2. Detector response versus amount of ceramide III injected in an isocratic NARP-HPLC system (see text for details) with (○) fluorescence detection and (▲) ELSD. Each point is mean±SD of three measurements. When no bar is shown, the SD is smaller than the symbol. Box: lower limit of detection range.

curve suggesting a better precision. From Fig. 2, if we compare the regression line for fluoresence detection with the plots of Eq. (1), it is clear that the confidence limits of the ELSD curve increased with increasing an amount injected whereas it remained constant over the full range for the fluorescence detection. As seen in the box in Fig. 2, ELSD appeared to be not as sensitive as fluorimetry for injected amounts below 75 ng. In contrast, above 75 ng, ELSD is much more sensitive but also less precise than fluorimetry, as demonstrated by the broad confidence limits of the ELSD curve. Detection limits measured from peak intensity higher than three times of the background noise are similar for the two techniques, that is: 5 ng injected ceramide III.

Due to the postcolumn dilution effect obtained with fluorescence detection, a slight reduction of efficiency is observed, as demonstrated by the peak broadening as compared with ELSD. The use of the postcolumn device entailed a loss of about 26% of the number of theoretical plates: $N=3555$ ($n=12$, RSD=8.1%) for fluorescence detection; $N=4470$ ($n=12$, RSD=13.3%) for ELSD. It should be noted that the high postcolumn/main flow-rate ratio (3.2/0.4) yields a more noisy baseline than ELSD does.

3.2.2. Analysis of ceramide type III by a gradient NARP-HPLC system

Subsequently, the compatibility of this fluorescence detection technique with gradient elution, as well as its influence on the efficiency for the separation of a mixture of ceramides (ceramide type III) were studied. The mobile phase used was as described by Gaudin et al. [21]. The gradient program was from 100% acetonitrile to 40% acetonitrile and 60% 1-propanol in 15 min with a concentration of triethylamine and formic acid levels held constant at 10 m*M*. The whole gradient run duration was 17 min. After 15 min equilibration with the initial solvent, the next sample was injected into the system. Fig. 3 shows the chromatograms of ceramide type III with fluorescence and ELSD, respectively. The slight increase in retention times obtained with fluorescence detection was the result of the postcolumn device effect. ELSD is well known to be used in gradient elution without baseline drift. This is clearly shown in Fig. 3B although 4%/min of 1-propanol gradient was used. For fluorescence detection (Fig. 3A), this change of mobile phase composition gives rise to only a little variation of the baseline level (3 mV min^{-1}) measured between 6 and 16 min. The postcolumn fluorescence detection

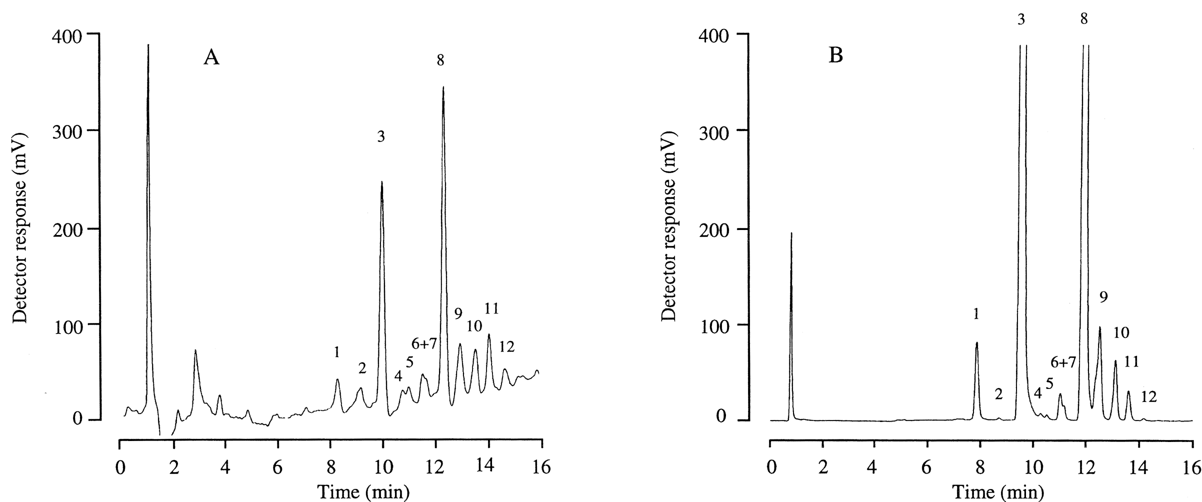

Fig. 3. Typical chromatograms of 0.2 mg ml^{-1} methanolic solution of ceramide type III eluted by gradient NARP-HPLC system with (A) fluorescence detection and (B) ELSD. See text for details.

appeared to also be compatible with gradient elution since the high flow-rate ratio between the post-column reagent and the column elution avoids the non-specific fluorescence of DPH with the increasing amount of organic solvent.

As show in Fig. 3A, fluorescence detection allows better detection of the minor species (peaks 2, 4, 5 and 12). In spite of the loss of efficacy already underlined, the chromatogram obtained with fluorescence detection does not exhibit any significant loss of resolution for the critical pairs of peaks 4, 5 and 6, 7.

In conclusion, ELSD, despite a number of drawbacks including non-linearity over a wide level range, and its inherent intrinsic destructive nature, is a simple and sensitive detection method for ceramide analysis in a narrow range (e.g. from 75 to 350 ng injected ceramide III). In contrast, the postcolumn fluorescence detection method is sensitive for all ceramide components (major and minor) and also linear over a wider dynamic range, (from 10 to 1000 ng injected ceramide III). Thus, fluorescence detection is an alternative for ceramide detection especially in regard to ceramide quantitation at low concentration. In order to complete and refine this first approach, probes other than DPH are presently tested in our laboratory.

References

[1] D.T. Downing, J. Lipid Res. 33 (1992) 301.
[2] D.C. Swartzendruber, P.W. Wertz, D.J. Kitko, D.T. Downing, J. Inverst. Dermatol. 92 (1989) 251.
[3] G. Grubauer, K.R. Feingold, R.M. Harris, P.M. Elias, J. Lipid Res. 30 (1989) 89.
[4] M. Previati, L. Bertolaso, M. Tramarin, V. Bertagnolo, S. Capitani, Anal. Biochem. 233 (1996) 108.
[5] T. Gildenast, J. Lasch, Biochim. Biophys. Acta 1346 (1997) 69.
[6] C.A. Demopoulos, M. Kyrili, S. Antonopoulou, N.K. Andrikopoulos, J. Liq. Chromatogr. Rel. Technol. 19 (1996) 771.
[7] K. Gaudin, P. Chaminade, A. Baillet, D. Ferrier, J. Bleton, S. Goursaud, A. Tchapla, J. Liq. Chromatogr. 22 (1999) 379.
[8] J.I. Teng, L.L. Smith, J. Chromatogr. 322 (1985) 240.
[9] M. Iwamori, C. Costello, H.W. Moser, J. Lipid Res. 20 (1979) 89.
[10] Un Hoi Do, P.T. Pei, R.D. Minard, Lipids 16 (1981) 855.
[11] M. Yano, E. Kishida, Y. Muneyuki, Y. Masuzawa, J. Lipid Res. 39 (1998) 2091.
[12] P. Vander Meeren, J. Vanderdeelen, M. Huys, L. Baert, J. Chromatogr. 447 (1988) 436.
[13] A. Stolyhwo, M. Martin, G. Guiochon, J. Liq. Chromatogr. 10 (1987) 1237.
[14] L. Robinson, R. Macrae, J. Chromatogr. 303 (1984) 386.
[15] G. Guiochon, A. Moysan, C. Holley, J. Liq. Chromatogr. 11 (1988) 2547.
[16] M. Dreux, M. Lafosse, L. Morin-Allory, LC·GC Mag. 9 (1996) 148.
[17] E. London, G.W. Feigenson, Anal. Biochem. 88 (1978) 203.
[18] A.D. Postle, J. Chromatogr. 415 (1987) 241.
[19] D.O.E. Gebhardt, W. Soederhuizen, J.H.M. Feyen, Ann. Clin. Biochem. 22 (1985) 321.
[20] W. Bernhard, M. Linck, H. Creutzburg, A.D. Postle, A. Arning, I. Martin-Carrera, K.-Fr. Sewing, Anal. Biochem. 220 (1994) 172.
[21] K. Gaudin, P. Chaminade, D. Ferrier, A. Baillet, A. Tchapla, Chromatographia 49 (1999) 241.