

## Multi-Linear Gradient Elution Optimization for Separation of Phenylthiohydantoin Amino Acids Using Pareto Optimality Method

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Multi-linear gradient elution was applied for simultaneous optimization of resolution and analysis times for ten phenylthiohydantoin amino acids (PTH-AAs) in liquid chromatography. Relation of  $\ln K$  upon  $\varphi$  for each analyte was determined using isocratic retention time data, and gradient retention time of analytes was predicted using fundamental equation of gradient elution. Then a grid search program was used to predict retention time of solutes in variable space. Two different chromatographic goals-analysis time and minimum difference between adjacent peaks- were simultaneously evaluated using Pareto optimality method. Gradient program in optimum condition was: initially 24% CH<sub>3</sub>OH/Water for 10 min, linear ramp to 34% over 5 min, to 29% over 5 min, and to 70% over 20 min. The average of calculated relative error in the prediction of the retention time in optimal conditions was -1.67% that shows a good agreement between predicted and experimental values of the chromatographic retention time in optimal condition.

**Keywords:** Multi-linear gradient elution, Optimization, Pareto optimality method, Phenylthiohydantoin amino acids, Separation

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### INTRODUCTION

Protein sequence analysis by Edman degradation chemistry on automated instruments is an important part of the characterization of proteins and peptides in today's analytical laboratory. It is essential to precisely determine the phenylthiohydantoin amino acids (PTH-AAs) after Edman degradation [1]. RP-HPLC mostly has been used as an important separation method of PTH-AAs [2-4].

Gradient elution in reversed-phase liquid chromatography (RP-LC) is based on the programmed change in mobile phase composition, flow rate and column temperature [5-6]. A powerful separation technique can be achieved using changes in mobile phase composition during analysis. These cases of gradient mode can be done stepwise, linear and multi-linear

[7-9]. In multi-linear gradient, separations are mainly affected by slope and time of each step whereby the composition of the mobile phase varies.

The fundamental equation for gradient elution which relates the variation of solute retention to mobile phase composition may be expressed as:

$$\int_0^{t_R-t_0} \frac{dt}{t_\varphi - t_0} = \int_0^{t_R-t_0} \frac{dt}{t_0 k_\varphi} = 1 \quad (1)$$

where  $t_R$ ,  $t_\varphi$  and  $t_0$  are the solute gradient elution time, the isocratic retention time when the mobile phase composition is equal to  $\varphi$  and the column hold up time, respectively.  $K_\varphi$  is the solute retention factor which corresponds to a constant mobile phase composition equal to  $\varphi$ . Nikitas and co-workers [10] have proved that if dwell time ( $t_D$ ) is taken into account, the following equation can be achieved readily.

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$$\int_0^{t_R - t_0 - t_D} \frac{dt}{t_0 k_\varphi} + \frac{t_D}{t_0 k_{\varphi_{in}}} = 1 \quad (2)$$

In practice, common mixers do not produce an ideally smoothed  $t$  vs.  $\varphi$  profile. For this reason, an arbitrary gradient profile is approximated by small  $\delta\varphi$  steps at  $\delta t$  time intervals, instead. Under these conditions, an analyte is eluted when the sum of  $\delta L_a$ , the sum of the subsequent distances are traveled by the analyte inside the column at time interval equal to  $\delta t_c$ , is equal to  $L$ . The derivation of the fundamental equations of gradient elution presented above by Nikitas *et al.* [10] leads to the following very simple numerical solution of Eq. (2):

$$\sum \frac{\delta L_a}{L} = \frac{t_D}{t_0 k_{\varphi_{in}}} + \sum_{i=1}^n \frac{\delta t}{t_0 k_\varphi} \geq 1 \quad (3)$$

where,  $n$  is the least number of terms of the sum that makes the above inequality valid. Now it is evident that when the above expression is fulfilled, the gradient elution time could be obtained by:

$$t_R = t_0 + t_D + n\delta t \quad (4)$$

This equation is the numerical solution of Eq. (2).

However, many factors can affect retention time and cause the optimization of the experimental conditions to be a complicated process. In these cases, chromatographic optimizations require selecting suitable criteria for the evaluation of the results and choosing the optimum conditions. These criteria can be maximum retention time, minimum resolution or compromises between resolution and retention time. Different approaches from multi-criteria making-decision (MCDM) have been used for simultaneous optimization of the criteria in RP-LC methods [11-14].

This paper seeks to evaluate the use of Nikitas's approach to optimize the multi-linear gradients for separation of ten PTH-AAAs acids. It discusses the applicability of some empirical models for the prediction of isocratic retention time. The retention time of solutes is calculated from numerical solution of fundamental equation of gradient elution. Pareto optimality method is used to evaluate the simulated chromatograms for the selection of optimum conditions.

## EXPERIMENTAL

The HPLC grade methanol was purchased from Fluka (Buchs, Switzerland). The water was double distilled deionized and filtered through 0.45  $\mu\text{m}$  Millipore (Bedford, MA 01730) solvent filter. The test solutes including PTH-Systeic acid (PTH-Sys), PTH-Glutamic acid (PTH-Gla), PTH-Serine (PTH-Ser), PTH-Threonine (PTH-Thr), PTH-Glutamine (PTH-Glu), PTH-Alanine (PTH-Ala), PTH-tyrosine (PTH-Tyr), PTH-Methionine (PTH-Met), PTH-Leucine (PTH-Leu) and PTH-Phenylalanine (PTH-Phe) were used as received from Fluka. Stock solutions of PTH-AAAs (0.5-1.0 mg ml<sup>-1</sup>) were prepared in methanol and stored at -20 °C.

The HPLC system consisted of a model 1525 pump, a Spherisorb C18 column (250  $\times$  4.6 mm, 10  $\mu\text{m}$  particle size) and a model 2487 UV detector all from Waters (Waters Assoc., Milford, MA, USA). The column was thermostated at the 25 °C by a water circulator bath. The experiments were performed in isocratic mode in different mobile phase compositions. The flow rate of mobile phase was maintained at 1 ml min<sup>-1</sup> and spectrophotometric detection was employed at 254 nm. The isocratic chromatographic system was conditioned by passing the eluent through the column until a stable base line was observed. Then, repeatable retention times were obtained for three subsequent injections. Dead time value was measured from the time of injection of methanol to the first deviation of the base line.

## RESULTS AND DISCUSSION

Preliminary experiments have shown that two solutes, PTH-Sys and PTH-Gla, have retention time lower than 4 min, and are not used for modeling. The retention time ( $t_R$ ) of solutes in different isocratic conditions are reported in Table 1. In order to obtain the functional dependence of  $\ln K$  upon  $\varphi$  under isocratic conditions several two, three and four-parameters empirical equations were tested [15-17]. The analysis of data was carried out with Microsoft Excel and the results of this fitting procedure can be seen in Table 2. In this table,  $\sigma$ , SSR and S refer to standard deviation of  $\ln K$ , the sum of square of errors for  $\ln K$  and the mean standard errors in calculation of  $t_R$ , respectively. As can be seen, conventional

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**Table 1.** Experimental Retention Times ( $t_R$ ) of PTH-AAs in Aqueous Mobile Phases Modified with Methanol<sup>a</sup>

Solute	$t_R$						
	30 <sup>b</sup>	35	40	45	50	55	60
PTH-Sys	2.6	2.4	2.3	2.2	2.2	2.2	2.2
PTH-Gla	4.0	3.5	3.0	2.5	2.4	2.4	2.4
PTH-Ser	6.9	5.6	4.6	4.1	3.8	3.6	3.5
PTH-Glu	7.7	5.8	5.0	4.4	3.8	3.6	3.5
PTH-Thr	8.1	5.9	5.0	4.4	3.8	3.6	3.5
PTH-Ala	11.9	7.7	7.1	5.6	4.8	4.4	4.1
PTH-Tyr	17.9	13.2	7.2	5.6	4.8	4.4	4.1
PTH-Met	26.0	19.2	13.4	8.9	6.9	5.1	4.1
PTH-Phe	51.4	33.6	20.2	12.5	8.7	5.9	4.4
PTH-Leu	61.1	37.4	24.2	14.6	9.7	6.5	4.6

<sup>a</sup>Conditions: 4.6 mm × 250 mm Waters C18 column, 10 μm particle size, 25 °C. <sup>b</sup>%Methanol in H<sub>2</sub>O-methanol mobile phase.

**Table 2.** Fitting Criteria for Different Empirical Equations

Equation	Adjustable parameters	$\sigma^a$	SSR <sup>b</sup>	S <sup>c</sup>	Ref.
Two parameter					
$\ln k = m + nE_T^N$	m, n	0.124	0.074	0.074	[15,16]
$\ln k = a - \ln(1 + b\varphi) - \frac{c\varphi}{1 + b\varphi}$	a, c	0.104	0.052	0.061	[17]
$\ln k = a - \frac{c\varphi}{1 + b\varphi}$	a, c	0.087	0.036	0.055	[17]
Three parameters					
$\ln k = a - \ln(1 + b\varphi) - \frac{c\varphi}{1 + b\varphi}$	a, b, c	0.093	0.041	0.058	[17]
$\ln k = a - \frac{c\varphi}{1 + b\varphi}$	a, b, c	0.088	0.036	0.055	[17]
$\ln k = m' + \frac{n'}{1 + b, \varphi} + q'\varphi$	m', n', q'	0.063	0.019	0.038	[17]
$\ln K = a + b\varphi + c\varphi^2$	a, b, c	0.047	0.011	0.024	[18]
Four parameters					
$\ln k = a - \frac{c\varphi}{1 + b\varphi} + d\varphi$	a, b, c, d	0.049	0.011	0.026	[19]

<sup>a</sup>Standard deviation for  $\ln K$ . <sup>b</sup>Sum of square of errors for  $\ln K$ . <sup>c</sup>Mean standard error for calculation of retention time.

quadratic equation ( $\ln K = a + b\varphi + c\varphi^2$ ) shows better ability for the prediction of retention time and in comparison with four-parameter equation has lower adjustable parameter and lower complexity. The parameters of quadratic equation for all PTH-AAAs were calculated and are shown in Table 3. Figure 1 shows the residuals calculated from the experimental  $t_R$  values under various isocratic elution conditions. The propagation of residuals in both sides of zero line indicates that no systematic error exists in the prediction of retention time of solutes using quadratic equation. Also, the maximum deviation of the predicted and the experimental retention times are less than 1.5 min.

A program has been written in Excel for the application of

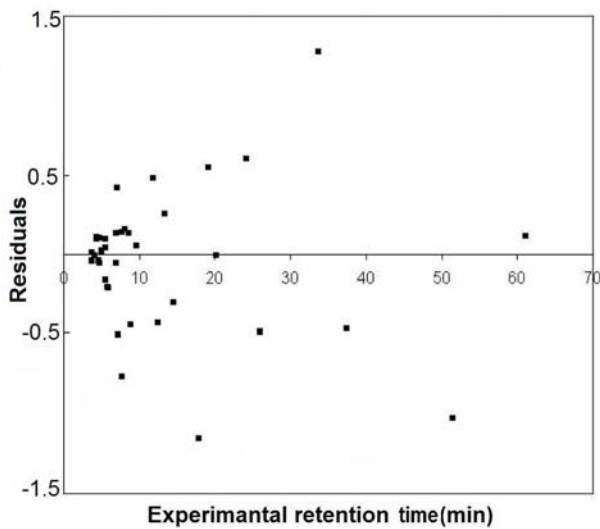
Nikitas's approach for calculating the gradient retention time of mixture of PTH-AAAs that can calculate gradient retention time of each solute for the following gradient profile:

$$\varphi = \begin{cases} \varphi_1 = \varphi_{in} & t \leq t_1 \\ \varphi_2 = \varphi_1 + b_1 t & t_1 < t \leq t_2 \\ \varphi_3 = \varphi_2 + b_2 t & t_2 < t \leq t_3 \\ \varphi_4 = \varphi_3 + b_3 t & t_3 < t \leq t_4 \\ \varphi_5 = \varphi_{max} & t > t_4 \end{cases} \quad (1)$$

where  $\varphi_{in}$  and  $\varphi_{max}$  are initial and final mobile phase composition and  $b_1$ ,  $b_2$  and  $b_3$  are the slope of each linear portion, respectively. Note that this gradient is applied after

**Table 3.** Parameters of Quadratic Equation ( $\ln K = a + b\varphi + c\varphi^2$ ) for PTH-AAAs

	PTH-Ser	PTH-Glu	PTH-Thr	PTH-Ala	PTH-Tyr	PTH-Met	PTH-Phe	PTH-Leu
a	4.214	4.294	3.643	4.818	7.397	4.365	6.150	6.071
b	-15.001	-13.952	-11.046	-14.233	-22.806	-5.386	-9.842	-8.582
c	12.002	9.713	6.496	10.066	16.657	-3.667	-0.574	-2.160

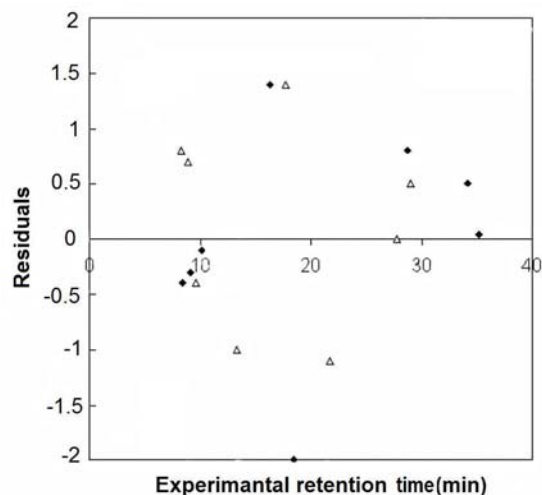


**Fig. 1.** Differences between experimental and predicted retention times of PTH-AAAs under various isocratic elutions in aqueous mobile phases modified with methanol using quadratic equation.

the dwell time. Therefore, the total gradient consists of five parts: an initial isocratic part, due to dwell time, three linear parts with different slopes and finally isocratic part with the maximum value of  $\varphi$  ( $\varphi_{max}$ ). The parameters that should be optimized for separation of PTH-AAAs were:  $\varphi_{in}$ ,  $\varphi_{max}$ ,  $b_1$ ,  $b_2$ ,  $b_3$ ,  $t_1$ ,  $t_2$ ,  $t_3$  and  $t_4$ .

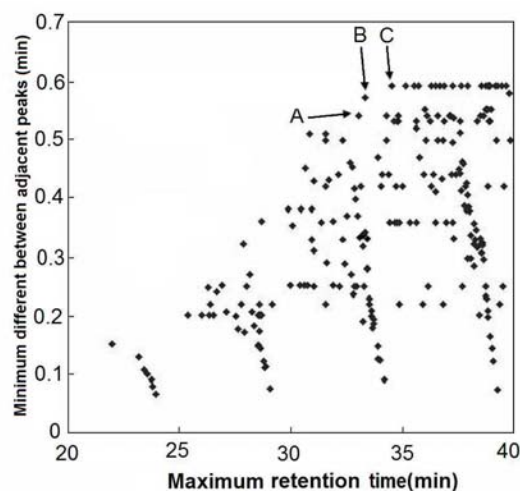
For the evaluation of the program, two gradient patterns were applied and the retention time of solutes was calculated and compared with experimental retention time that can be seen in Fig. 2. Good agreement between predicted and experimental  $t_R$  reveals the credibility of this approach. Therefore, the above approach can describe satisfactorily the multi-linear gradient elution.

In order to optimize the separation of the PTH-AAAs, a grid search program was written in Excel that calculated gradient retention times of all analytes for every mobile phase composition within the feasible factor space. The retention time of the last solute to be eluted and the minimum  $t_R$  difference ( $\delta t$ ) between adjacent peaks were predicted for each



**Fig. 2.** Differences between experimental and predicted retention times of PTH-AAs under various multi-linear gradient elution in aqueous mobile phases modified with methanol. (◆) Condition 1:  $\varphi_{in} = 0.26$ ,  $t_{in} = 10$  min,  $\varphi_1 = 0.36$ ,  $t_1 = 5$  min,  $\varphi_2 = 0.31$ ,  $t_2 = 5$  min,  $\varphi_3 = 0.6$ ,  $t_3 = 15$  min. (△) Condition 2:  $\varphi_{in} = 0.27$ ,  $t_{in} = 7$  min,  $\varphi_1 = 0.375$ ,  $t_1 = 7$  min,  $\varphi_2 = 0.415$ ,  $t_2 = 8$  min,  $\varphi_3 = 0.6$ ,  $t_3 = 8$  min.

mobile phase composition. Then, the corresponding Pareto-optimal plot was plotted that can be seen in Fig. 3. It should be emphasized that this plot does not represent a direct functional dependence of minimum  $\delta t$  on maximum  $t_R$ . Investigation of the obtained Pareto-optimal points reveals that conditions A, B and C can be Pareto optimal. Experiment in these conditions showed that the optimal values of the experimental variables were  $\varphi_{in} = 0.24$ ,  $\varphi_{max} = 0.7$ ,  $b_1 = 0.02$ ,  $b_2 = -0.01$ ,  $b_3 = 0.021$ ,  $t_1 = 10$ ,  $t_2 = 5$ ,  $t_3 = 5$  and  $t_4 = 20$  min. The efficiency of this algorithm was evaluated by performing the experiment under the predicted optimal conditions. Figure 4, shows the chromatogram obtained under these conditions. As can be seen a good resolution for all analytes in analysis time of lower than 40 min was achieved. The experimental and predicted gradient retention time of PTH-AAs in optimum conditions is reported in Table 4. It is apparent that a good agreement was observed between predicted and experimental values of the retention time ( $R^2 = 0.98$ ). The average of calculated relative error in prediction of the retention time in optimal conditions was -1.67%.



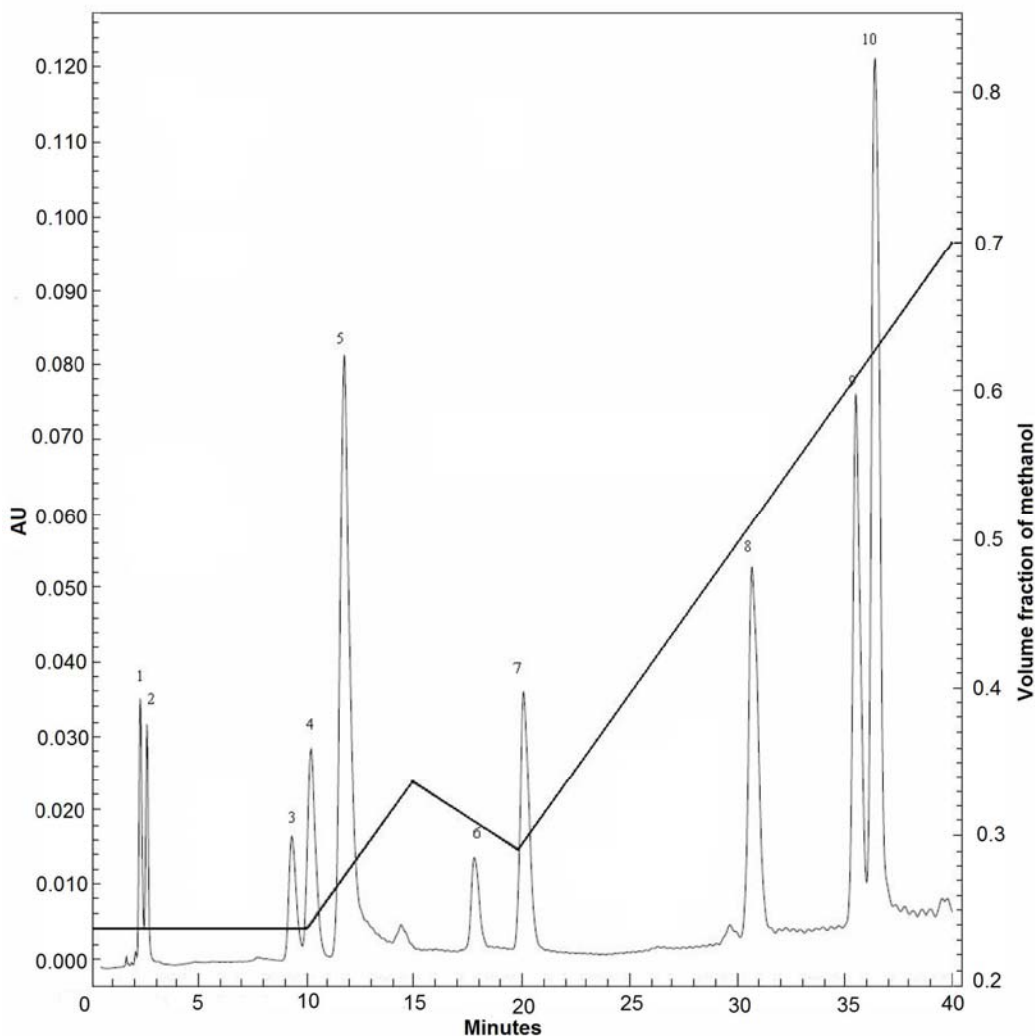
**Fig. 3.** The multi-criteria decision-making plot for separation of PTH-amino acids under constrained condition  $t_{max} < 40$ .

**Table 4.** Predicted and Experimental Retention Times in Optimal Condition Described in Text

Solutes	$t_{R(Exp)}$	$t_{R(Pred)}$	%Error
PTH-Ser	9.3	10.2	-9.67
PTH-Glu	10.2	10.7	-4.90
PTH-Thr	11.7	11.9	-1.71
PTH-Ala	17.8	16.4	7.86
PTH-Tyr	20.1	22.3	-10.94
PTH-Met	30.7	29.3	4.56
PTH-Phe	35.5	35.1	1.13
PTH-Leu	36.4	36.3	0.27

## CONCLUSIONS

The multi-linear gradient elution is a powerful technique for the separation of complex mixture. Nikitas' approach in multi-linear gradient elution was applied for the optimization of the separation of ten PTH-AAs. The different theoretical expressions of  $\ln K$  vs. organic modifier content were tested and the quadratic equation was selected for the prediction of  $t_R$  values. Since several variation schemes of  $\varphi$  could be used to



**Fig. 4.** Chromatogram of PTH-AAs mixture in optimum condition described in text. Mixtures consist of: (1) PTH-Sys, (2) PTH-Gla, (3) PTH-Ser, (4) PTH-Glu, (5) PTH-Thr, (6) PTH-Ala, (7) PTH-Tyr, (8) PTH-Met, (9) PTH-Phe and (10) PTH-Leu.

achieve the best separation, the Pareto optimality method was chosen as MCDM method. Results showed that the proposed optimization technique afforded good performance and great flexibility.

## REFERENCES

- [1] G. Allen, *Laboratory Techniques in Biochemistry Molecular Biology Sequencing of Proteins and Peptides*, Elsevier/North-Holland, Amsterdam, 1981.
- [2] K. Hayakawa, M. Hirano, K. Yoshikawa, N. Katsumata, T. Tanaka, *J. Chromatogr. A* 846 (1999) 73.
- [3] M.R. Seifar, J.C. Kraak, H. Poppe, W.T. Kok, *J. Chromatogr. A* 832 (1999) 133.
- [4] F. Safa, M.R. Hadjmohammadi, *J. Chromatogr. A* 1078 (2005) 42.
- [5] P. Jandera, J. Churacek, *Gradient Elution in Liquid Chromatography. Theory and Practice*, Elsevier, Amsterdam, 1985.
- [6] C.F. Poole, *the Essence of Chromatography*, Elsevier,

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- Amsterdam, 1985
- [7] M.R. Hadjmohammadi, K. Kamel, M.H. Fatemi, *J. Sep. Sci.* 30 (2007) 2687.
- [8] L.R. Snyder, J.W. Dolan, J.R. Gant, *J. Chromatogr.* 165 (1979) 1.
- [9] P. Nikitas, A. Pappa-Louisi, A. Papageorgiou *J. Chromatogr. A* 1157 (2007) 178.
- [10] P. Nikitas, A. Pappa-Louisi, *Anal. Chem.* 77 (2005) 5670.
- [11] B. Bourguignon, D.L. Massart, *J. Chromatogr.* 586 (1991) 11.
- [12] J.L. Glajch, J.J. Kirkland, *J. Chromatogr.* 485 (1989) 51.
- [13] M.R. Hadjmohammadi, F. Safa, *J. Sep. Sci.* 27 (2004) 997.
- [14] F. Safa, M.R. Hadjmohammadi, *J. Chromatogr. A* 1078 (2005) 42.
- [15] B.P. Johnson, M.G. Khaledi, J.G. Dorsey, *Anal. Chem.* 58 (1986) 85.
- [16] B.P. Johnson, M.G. Khaledi, J.G. Dorsey, *J. Chromatogr.* 384 (1987) 221.
- [17] P. Nikitas, A. Pappa-Louisi, P. Agrafiotou, *J. Chromatogr. A* 946 (2002) 33.
- [18] P. Nikitas, A. Pappa-Louisi, P. Agrafiotou, *J. Chromatogr. A* 946 (2002) 9.
- [19] A. Pappa-Louisi, P. Nikitas, P. Balkatzopoulou, C. Malliakas, *J. Chromatogr. A* 1033 (2004) 29.