Journal of Chromatography, 63.5 (1993) 195–202
Elsevier Science Publishers B.V., Amsterdam

CHROM. 24 837

Robustness testing of an optimized reversed-phase high-performance liquid chromatographic system for the separation of six sulphonamides using the rules of error propagation

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(First received October 13th, 1992; revised manuscript received December 21st, 1992)

ABSTRACT

In a previous investigation, the composition of the mobile phase for the reversed-phase HPLC separation of twelve sulphonamides was optimized. The predicted chromatogram showed great similarity with a chromatogram measured under optimum conditions. For routine analysis, it is important to have robust analytical methods, *i.e.*, to have methods that are precise and accurate despite small variations in the measurement conditions. In the experiment described here, a number of routine chromatograms of six sulphonamides were recorded using the HPLC system with the optimum mobile phase to validate the robustness of the system under routine conditions. The variance of the capacity factor was calculated for each sulphonamide and the influence of this variance on the variance of the selectivity and the resolution of each pair of sulphonamides was studied. Considerable variability of the capacity factors was found. However, owing to the high correlation between the variances of the capacity factors of the compounds, relatively small variances of the selectivity $\alpha_{i,j}$ and of the resolution $R_{s_{i,j}}$ of pairs of compounds were found. It was concluded that, owing to the high correlation between the variances of the capacity factor, the chromatographic system was robust with respect to the selectivity and resolution of pairs of sulphonamides.

INTRODUCTION

At the present time, much emphasis is being placed on quality in the laboratory. Accurate and precise measurements are necessary for the consistent determination of the quality of products such as drugs or the quality of the environment. The quality of a product can only be guaranteed if the quality of the analysis is ascertained. Once the quality of laboratory management procedures (e.g., logistics, such as informa-

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0021-9673/93/$06.00

tion flow) has been achieved and ascertained by good laboratory practice (GLP) rules or other compliance programmes, the quality of the chemical procedures may be improved, e.g., the optimization of the robustness of methods or procedures.

Several definitions of quality have been given in the literature. The International Organization of Standardization (ISO) defines quality as "the totality of features and characteristics of a product, process or service that bear on its ability to satisfy stated or implied needs". A very explicit definition has been given by Taguchi and Wu [1]: the quality of a product is expressed by its loss to society. The parameter design procedure of Taguchi and Wu was developed to improve product performance and distinguishes between design variables (controllable variables) and noise variables (non-controllable factors). A particular experimental design is used, the goal of which is to select those settings of the design variables which give optimum results for the performance of a product. Moreover, those settings of the noise factors are selected which have minimal effects on the performance of the product.

Many papers have been published on the optimization of the composition of the mobile phase of reversed-phase high-performance liquid chromatography (RP-HPLC) [2-g]. The applied methods demonstrated good prediction of chromatographic behaviour. Robustness of chromatographic systems has also been described [9-11]. However, the publications concerned dealt with robustness with respect to variables other than the composition of the mobile phase; the influence of small deviations from the optimum settings of variables such as wavelength and flow-rate on the final analytical result was investigated. A similar investigation was performed by Hoogkamer *et al.* [12]. Gooding and Schmuck [13] concluded that through careful method development, the limits on the variability can be defined so that HPLC methods can have the ruggedness required for validation procedures in biotechnological and pharmaceutical situations. De Boer *et al.* [14,15] developed and introduced a robustness coefficient which they illustrated with an application in mixture design optimization strategies. Wieling *et al.* [16] developed a method to estimate the robustness of liquid–liquid extraction procedures of drugs from biological matrices prior to HPLC analysis.

The final aims of the investigation described in this paper were to detect any covariance structure in capacity factors in RP-HPLC and to use robustness parameters in RP-HPLC in future research. Some study of the significance of the implementation of the variance/covariance structure in HPLC separations was needed for this research.

As an example, the robustness of an optimized HPLC system with respect to separation power (expressed by the selectivity $\alpha_{i,j}$ and the resolution R_{s_j}, of peak pairs) under routine conditions was examined. The mobile phase composition that was selected after the optimization procedure [17] was examined with respect to inter-day reproducibility: during a period of 3 weeks the drift of the capacity factors and their influence on selectivity and resolution was investigated. The rules of the propagation of errors were used to determine the validity of the assumption of the presence of covariance between capacity factors.

Detailed discussions on error propagation in chromatography has been given by Ku [18] and Balke [19].

THEORY

The selectivity of a mobile phase for two compounds i and j is defined as the ratio of the capacity factors (k'_i and k'_j) of those compounds in that mobile phase:

$$\alpha_{ij} = k'_i / k'_j \qquad (1)$$

After the recording of a series (n) of chromatograms, the means, $\bar{k}_i$ and $\bar{k}_j$, the variances of the capacity factors of compounds i and j, $S^2_{k_i}$ and $S^2_{k_j}$, and the covariance between the variances of k'_i and k'_j, $S^2_{k_i,k_j}$, are calculated as follows:

$$\bar{k}'_i = \frac{\sum_{p=1}^{n} k'_{i_p}}{n} \quad \text{and} \quad \bar{k}'_j = \frac{\sum_{p=1}^{n} k'_{j_p}}{n}$$

$$S^2_{k_i} = \frac{\sum_{p=1}^{n} (k'_{i_p} - \bar{k}'_i)^2}{n-1} \quad \text{and} \quad S^2_{k_j} = \frac{\sum_{p=1}^{n} (k'_{j_p} - \bar{k}'_j)^2}{n-1}$$

$$S^2_{k_i,k_j} = \frac{\sum_{p=1}^{n} (k'_{i_p} - \bar{k}'_i) \cdot (k'_{j_p} - \bar{k}'_j)}{n-1} \qquad (2)$$

Now, in HPLC systems that show significant variability, a positive or negative drift in capacity factors may arise, which may influence the value of $\alpha_{i,j}$. Under conditions where random variables contribute to the variance of the capacity factors of both compounds i and j and with the same direction and magnitude (i.e., $S^2_{k_i}$ and $S^2_{k_j}$ are completely correlated), no change in $\alpha_{i,j}$ arises. This is demonstrated by the equation

$$S^2_{\alpha_{i,j}} = S^2_{k_i}\left(\frac{\partial \alpha_{i,j}}{\partial k'_i}\right)^2 + S^2_{k_j}\left(\frac{\partial \alpha_{i,j}}{\partial k'_j}\right)^2 + S^2_{k_i,k_j}\left(\frac{\partial \alpha_{i,j}}{\partial k'_i} \cdot \frac{\partial \alpha_{i,j}}{\partial k'_j}\right)$$

$$= \alpha^2_{i,j}\left[\left(\frac{S_{k_i}}{\bar{k}'_i}\right)^2 + \left(\frac{S_{k_j}}{\bar{k}'_j}\right)^2 - 2 \cdot \frac{S^2_{k_i,k_j}}{\bar{k}'_i \bar{k}'_j}\right] \quad (3a)$$

in which the variance of the selectivity, $S^2_{\alpha_{i,j}}$, is expressed as a function of the partial derivatives of the selectivity $\alpha_{i,j}$ to the capacity factors of i and j and as a function of the (co)variances of the capacity factors of i and j.

The correlation coefficient r is a relationship between the covariance and the variances of k'_i and k'_j and expresses the correlation between these capacity factors:

$$r = S^2_{k_i,k_j} / S_{k_i} S_{k_j}$$

The larger the correlation, the more robust the separation of i and j is with respect to small variations of the conditions.

Similarly, these rules of error propagation are valid for the variance of the resolution as a function of the variance of the capacity factors. The resolution of two compounds i and j in an RP-HPLC system is expressed by the equation

$$R_{s_{i,j}} = \frac{\sqrt{N}}{2} \cdot \frac{k'_j - k'_i}{k'_j + k'_i + 2} \qquad (4)$$

where N is the plate number of the column.

Eqn. 5a gives the variance of the resolution of i and j as a function of $S^2_{k_i}$, $S^2_{k_j}$ and $S^2_{k_i,k_j}$:

$$S^2_{R_{s_{i,j}}} = S^2_{k_i}\left(\frac{\partial R_{s_{i,j}}}{\partial k'_i}\right)^2 + S^2_{k_j}\left(\frac{\partial R_{s_{i,j}}}{\partial k'_j}\right)^2 + S^2_{k_i,k_j}\left(\frac{\partial R_{s_{i,j}}}{\partial k'_i} \cdot \frac{\partial R_{s_{i,j}}}{\partial k'_j}\right)$$

$$= \frac{N}{4} \cdot \frac{S^2_{k_i}(2\bar{k}'_i + 2)^2 + S^2_{k_i}(2\bar{k}'_j + 2)^2 - 4S^2_{k_i,k_j}(\bar{k}'_i + 1)(\bar{k}'_j + 1)}{(\bar{k}'_i + \bar{k}'_j + 2)^2}$$

$$= R^2_{s_{i,j}} \cdot \frac{S^2_{k_j}(2\bar{k}'_i + 2) + S^2_{k_i}(2\bar{k}'_j + 2)^2 - 4S^2_{k_i,k_j}(\bar{k}'_i + 1)(\bar{k}'_j + 1)}{(\bar{k}'_j - \bar{k}'_i)^2} \qquad (5a)$$

Often, the covariance terms in the eqns. 3a and 5a are omitted, since it is accepted that there is no covariance between the experimental errors of the capacity factors of two compounds. Eqns. 3a and 5a are then simplified to yield the equations

$$S^2_{\alpha_{i,j}} = S^2_{k_i}\left(\frac{\partial \alpha_{i,j}}{\partial k'_i}\right)^2 + S^2_{k_j}\left(\frac{\partial \alpha_{i,j}}{\partial k'_j}\right)^2$$

$$= \alpha^2_{i,j}\left[\left(\frac{S_{k_i}}{\bar{k}'_i}\right)^2 + \left(\frac{S_{k_j}}{\bar{k}'_j}\right)^2\right] \qquad (3b)$$

$$S^2_{R_{s_{i,j}}} = S^2_{k_i}\left(\frac{\partial R_{s_{i,j}}}{\partial k'_i}\right)^2 + S^2_{k_j}\left(\frac{\partial R_{s_{i,j}}}{\partial k'_j}\right)^2$$

$$= R^2_{s_{i,j}} \cdot \frac{S^2_{k_j}(2\bar{k}'_i + 2) + S^2_{k_i}(2\bar{k}'_j + 2)^2}{(\bar{k}'_j - \bar{k}'_i)^2} \qquad (5b)$$

In eqns. 3b and 5b, the variance of the resolution and the variance of the selectivity are always negatively influenced (increase) by the variance of the capacity factors, whereas their extended forms (eqns. 3a and 5a) show that a positive correlation ($0 < r \leq 1$) positively influences (decreases) the variance of the selectivity and resolution. In fact, these variances are equal to zero when $r = 1$.

Here it is assumed that it is reasonable to expect a correlation between the variances of two capacity factors in the same experiment: random variables probably have more or less the same effect on i and j, especially in this instance, where a set of structurally related compounds

are used. In other words, the variances of the retentions of two compounds are more or less correlated. This leads to the hypothesis that, even if two compounds *i* and *j* have large variances, a separation may be good and robust if these variances of the retention of both compounds are highly correlated, that is, if random variables have identical effects on both compounds.

EXPERIMENTAL

Instruments *and instrumental conditions*

The assay was performed with an HPLC system consisting of a Spectra-Physics (San Jose, CA, USA) Model SP8700 solvent-delivery system used at a flow-rate of 1.0 ml min^{-1} and a Kratos (Ramsey, NJ, USA) Model 757 UV detector, wavelength 260 nm, range 0.005 a.u.f.s., rise time 1 s.

Injections of sulphonamide standard solutions into a Zymark (Hopkinton, MA, USA) Z 310 HPLC injection station, equipped with an electrically controlled Rheodyne valve and a 20-μl sample loop, were performed by a Zymate II robot system. The Zymark Z 310 analytical instrument interface was used to control the HPLC injection station. The analytical column was a 100 x 4.6 mm I.D. Microsphere 3-μm C_{18} cartridge system (Chrompack, Middelburg, Netherlands). Data analysis was performed by means of a Spectra-Physics Chromjet SP4400 computing integrator.

Chemicals and reagents

Six sulphonamides were supplied by Sigma (St. Louis, MO, USA): sulphisomidine (SOMI), sulphathiazole (THIA), sulphapyridine (PYRI), sulphamerazine (MERA), sulphamethoxypyridazine (MEPY) and sulphachloropyridazine (CLPY). Acetonitrile (ACN), tetrahydrofuran (THF) and methanol (MeOH) were supplied by Labscan (Dublin, Ireland) and were of HPLC grade. Acetic acid (100%) (HAc), triethylamine (TEA), phosphoric acid (85%) (H_3PO_4) and potassium dihydrogenphosphate (KH_2PO_4) were all of analytical-reagent grade and supplied by Merck (Darmstadt, Germany). Water was purified by using Milli-RO-15 and Milli-Q water purification systems (Millipore , Bedford, MA, USA). Unless stated otherwise, water of Milli-Q quality was used.

A phosphate buffer (pH 3.0; 0.05 *M*) was prepared by dissolving 6.80 g of KH_2PO_4 in 1000 ml of water. The pH was adjusted at 3.0 using concentrated phosphoric acid. To this buffer 4.15 ml of TEA and *10* ml of HAc were added. The mobile phase was prepared by mixing 1 ml of ACN, 5 ml of THF and 140 ml of MeOH and adding phosphate buffer (pH 3.0; 0.05 *M*) to 1000 ml. This mobile phase composition was the result of an optimization procedure using mixture designs and multicriteria decision making (MCDM) [17]. Before use, the mobile phase was filtered through a Millipore Type HVLP filter (0.45 μm) and degassed before use by ultrasonification for 15 min.

Stock solutions of sulphonamides were prepared by dissolving 100 mg of the compounds in 100 ml of MeOH to give concentrations of 1000.0 mg l^{-1}. These solutions were stored at 4°C. A test solution was prepared containing all six sulphonamides. The concentration of each sulphonamide was 500 $\mu g\,l^{-1}$. The solution was stored at 4°C.

System robustness testing under routine conditions

To test the robustness of the optimized HPLC system in routine analyses, the mixture of the six sulphonamides (SOMI, THIA, PYRI, MERA, MEPY and CLPY) was injected 33 times (eleven injections on three separate days). The 33 chromatograms obtained were used to calculate the mean and variance of the capacity factors and of the resolutions and selectivities for each combination of two sulphonamides.

RESULTS AND DISCUSSION

Fig. 1 gives a representative chromatogram after injection of the test solution. For a mixture of six sulphonamides the variances of the selectivity $\alpha_{i,j}$ and the resolution $R_{s_{i,j}}$ were calculated using eqns. 3a and b and 5a and b using the means and standard deviations determined after the 33 routine analyses (Table I). The ex-

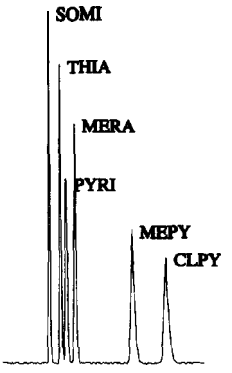

Fig. 1. Chromatogram after injection of the test solution of six sulphonamides.

perimental values of $\alpha_{i,j}$ and $R_{s_{i,j}}$ were determined using eqns. 1 and 4. From the 33 values obtained in this way, the means and the variances of $\alpha_{i,j}$ and $R_{s_{i,j}}$ were also calculated.

The data in Table I demonstrate a high and significant correlation between the experimental errors in the capacity factors. Estimation of the variability in the selectivity $\alpha_{i,j}$ and the resolution $R_{s_{i,j}}$ without the use of a covariance term would lead to overestimated values. This justifies the use of the covariance terms in eqns. 3a and 5a. Further evidence for the use of these terms is given in Tables II and III, where the values for $\alpha_{i,j}$ and $R_{s_{i,j}}$ are given with their standard deviations determined with three methods: (1) by calculating these data from the experimental values of the selectivity $\alpha_{i,j}$ and the resolution $R_{s_{i,j}}$, (2) by calculation by means of eqns. 3a and 5a and (3) by omitting the covariance terms (eqns. 3b and 5b). The tables demonstrate, with the experimentally obtained values for the variance of the selectivity $\alpha_{i,j}$ and the resolution $R_{s_{i,j}}$, that eqns. 3a and 5a are correct. Tables II and III are graphically displayed in Fig. 2. The selected HPLC system is robust with respect to the selectivity and resolution during a long period of analyses: the measured standard deviations of $\alpha_{i,j}$ and $R_{s_{i,j}}$ are equal to the values calculated by eqns. 3a and 5a, whereas the values calculated by eqns. 3b and 5b are much larger. The relative standard deviations (R.S.D.s) of the capacity factors are relatively large (4.9–6.5%, Table I), whereas the values of $\alpha_{i,j}$ and $R_{s_{i,j}}$ which are calculated from these figures, are relatively small; R.S.D.s are 0.5-1.9% for $\alpha_{i,j}$ and 0.5–7.8% for $R_{s_{i,j}}$.

Tables II and III also show the large difference between the standard deviations calculated with eqns. 3b and 5b as compared with the experimentally obtained values; for $\alpha_{i,j}$ these values are 4-16 times too high and for $R_{s_{i,j}}$ 2.5-21 times too high.

CONCLUSIONS

The assumption of the presence of covariance between capacity factors is valid for the separation of six sulphonamides in a mobile phase with constant pH. This conclusions may also be valid for mobile phase systems for the separation of

TABLE I

MEANS AND STANDARD DEVIATIONS OF THE CAPACITY FACTORS OF SIX SLJLPHONAMIDES AND THEIR CORRELATION COEFFICIENTS BETWEEN EXPERIMENTAL ERRORS USING THE OPTIMUM MOBILE PHASE COMPOSITION OVER A PERIOD OF 3 WEEKS (n = 33)

	SOMI	THIA	PYRI	MERA	MEPY	CLPY
Mean	1.5026	2.0970	2.4061	2.8825	5.9790	7.7730
S.D.	0.0799	0.1369	0.1294	0.1404	0.3253	0.4589
R.S.D. (%)	5.3	6.5	5.4	4.9	5.4	5.9
THIA	0.9774					
PYRI	0.9902	0.9952				
MERA	0.9918	0.9946	0.9990			
MEPY	0.9599	0.9956	0.9853	0.9849		
CLPY	0.9514	0.9934	0.9806	0.9800	0.9991	

TABLE II

VALUES OF THE SELECTIVITY $\alpha_{i,j}$ AND THE MEASURED AND CALCULATED STANDARD DEVIATIONS

	SOMI			**THIA**			**PYRI**			MERA			**MEPY**		
	$\alpha_{i,j}$	**S.D.**	R.S.D.	(%) $\alpha_{i,j}$	S.D.	R.S.D.	(%) $\alpha_{i,j}$	S.D.	R.S.D.	(%) $\alpha_{i,j}$	S.D.	R.S.D.	(%) $\alpha_{i,j}$	S.D.	R.S.D. (%)
THIA	1.395[a]														
		0.025[b]	1.8												
		0.024[c]	1.7												
		0.117[d]	8.4												
PYRI	1.601			1.148											
		0.012	0.7		0.015	1.3									
		0.012	0.7		0.015	1.3									
		0.121	7.6		0.097	8.4									
MERA	1.919			1.376			1.198								
		0.015	0.8		0.025	1.8		0.007	0.6						
		0.015	0.8		0.024	1.7		0.007	0.6						
		0.138	7.2		0.112	8.1		0.087	7.3						
MEPY	3.979			2.853			2.485			2.074					
		0.061	1.5		0.034	1.2		0.023	0.9		0.022	1.1			
		0.061	1.5		0.035	1.2		0.023	0.9		0.022	1.1			
		0.303	7.6		0.242	8.5		0.190	7.6		0.151	7.3			
CLPY	5.172			3.708			3.230			2.695			**1.300**		
		0.096	1.9		0.034	0.9		0.040	1.2		0.041	1.5		0.007	0.5
		0.095	1.8		0.035	0.9		0.040	1.2		0.040	1.5		**0.007**	0.5
		0.411	7.9		0.326	8.8		0.258	8.0		0.206	7.6		0.104	8.0

[a] Measured value of the selectivity $\alpha_{i,j}$.
[b] Measured standard deviation of the selectivity $\alpha_{i,j}$.
[c] Standard deviation of the selectivity $\alpha_{i,j}$ calculated with eqn. 3a (**with** correlation terms).
[d] Standard deviation of the selectivity $\alpha_{i,j}$ calculated with eqn. 3b (without correlation terms).

TABLE III

VALUES OF THE RESOLUTION $R_{s_{i,j}}$ AND THE MEASURED AND CALCULATED STANDARD DEVIATIONS

	SOMI			THIA			**PYRI**			MERA			MEPY		
	$R_{s_{i,j}}$	S.D.	R.S.D. (%) $R_{s_{i,j}}$	S.D.	R.S.D. (%) $R_{s_{i,j}}$	S.D.	R.S.D. (%) $R_{s_{i,j}}$	S.D.	R.S.D. (%) $R_{s_{i,j}}$	S.D.	R.S.D. (%) $R_{s_{i,j}}$	S.D.	R.S.D. (%)
THIA	4.204[a]														
		0.290[b]	6.9												
		0.287[c]	6.8												
		1.070[d]	25.5												
PYRI	6.064			1.891											
		0.151	2.5		0.148	7.8									
		0.151	2.5		0.141	7.8									
		0.962	15.9		1.154	61.0									
MERA	8.574			4.473			2.5%								
		0.115	1.3		0.179	4.0		0.049	1.9						
		0.115	1.3		0.178	4.0		0.049	1.9						
		0.912	10.6		1.119	25.0		1.036	39.9						
MEPY	18.727			15.288			13.647			11.306					
		0.285	1.5		0.083	0.5		0.199	1.5		0.233	2.1			
		0.282	1.5		0.083	0.5		0.197	1.4		0.231	2.0			
		0.871	4.7		1.085	7.1		1.052	7.7		1.075	9.5			
CLPY	22.056			18.971			17.477			15.323			4.514		
		0.334	1.5		0.153	0.8		0.273	1.6		0.314	2.0		0.120	2.7
		0.329	1.5		0.150	0.8		0.269	1.5		0.310	2.0		0.119	2.6
		0.840	3.8		1.048	5.5		1.034	5.9		1.073	7.0		1.372	30.4

[a] Measured value of the resolution $R_{s_{i,j}}$.
[b] Measured standard deviation of **the resolution** $R_{s_{i,j}}$.
[c] Standard deviation of the resolution $R_{s_{i,j}}$ **calculated** with eqn. 5a **(with** correlation terms).
[d] Standard deviation of the resolution $R_{s_{i,j}}$ **calculated** with eqn. 5b **(without** correlation terms).

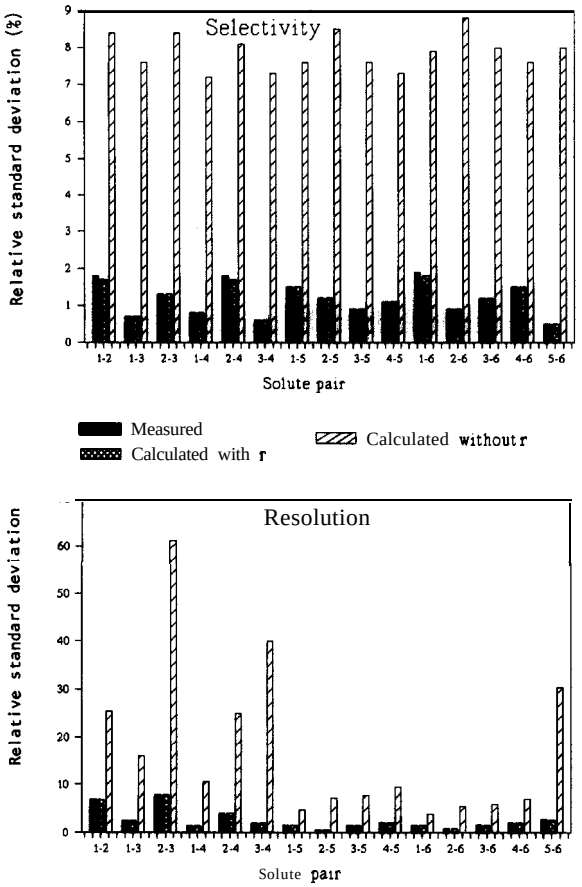

Fig. 2. Graphical comparison between the relative standard deviations of the selectivity and the resolution calculated with the experimental results and with eqns. 3a, 3b, 5a and 5b.

neutral compounds that use water instead of buffer systems. In systems that have small variability in buffer **pH,** e.g., when pumps are used that combine two buffer systems into one mobile phase, the presence of covariance between capacity factors may be less clear, if the pK_a values of the compounds to be separated differ significantly. This may also be the case if pumps are used that combine several pure organic modifiers into one mobile phase; small changes in the polarity or the selectivity of the mobile phase due to small variations in the composition of the mobile phase may affect the change in the capacity factor of one solute differently to the change in the capacity factor of another solute.

The robustness of the separation power (expressed by the selectivity $\alpha_{i,j}$ and the resolution $R_{s_{i,j}}$ of peak pairs) with respect to variations in the capacity factors is very high under routine conditions. At the optimum mobile phase composition the precision of the selectivity and the resolution is much better than the precision of the capacity factors owing to the high correlation of the experimental errors of the capacity factors.

ACKNOWLEDGEMENT

The authors thank the Dutch Technology Foundation (Stichting voor de Technische Wetenschappen, STW) for its support of this project.

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