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MULTIVARIATE CALIBRATION STRATEGY FOR REVERSED-PHASE CHROMATOGRAPHIC SYSTEMS BASED ON THE CHARACTERIZATION OF STATIONARY–MOBILE PHASE COMBINATIONS WITH MARKERS

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SUMMARY

Calibration of chromatographic systems serves several purposes. One of the goals is the correction of retention values in order to make them less dependent on the stationary and mobile phases. Another goal is the transfer of retention values measured on one system to another system. A strategy serving this purpose is reviewed. An example is given which makes use of the latter strategy to correct for batch-to-batch variations of stationary phases. This leaves the way open to update the optimum mobile phase composition. The strategy makes use of special selected compounds: the markers.

INTRODUCTION

Batch-to-batch variation of reversed-phase packing materials

It is widely known that the capacity factor, k', of a specific solute, measured at a specific mobile phase composition, varies between different stationary phase materials of the same type[1,2]. Even stationary phase materials of the same brand differ between batches of the same material[3]. This will be experimentally verified later. Several reasons for these batch-to-batch variations have been given. One of the major causes is the presence of free silanol groups at the surface of the stationary phase materials[4,5]. So far there is no stationary phase material for reversed-phase chromatography that guarantees reproducible results. This points to the necessity of calibration procedures.

Definition of calibration of a chromatographic system

The first step in the calibration of a chromatographic system is the measurement of retention values of specific compounds (standards) on that system, with a specific purpose. The second step depends on the goal of the calibration.

The goal of calibration can be to obtain a "measurement-system-independent" retention value of a solute, measured on a new system. The second step then consists in the correction of the retention value of that solute, using the standards measured on the new system. This correction is particularly useful in the area of identification of unknown compounds by their retention values.

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Another goal of calibration is the transfer of the retention value of a solute from one system to another. The second step then consists in the prediction of that retention value for the new system, with the use of measured standards on that new system. This goal is particularly valuable in the area of mobile phase optimization where an optimum eluent composition must be updated when columns[6] are changed or when a column deteriorates.

Calibration in reversed-phase high-performance liquid chromatography (RP-HPLC)

Calibration with the purpose of obtaining "measurement-system-independent" retention values has been reviewed by Smith[7]. One of the possible calibration schemes uses retention indices: it expresses the retention of a solute relative to that of a homologous series as standard compounds. For details we refer to Smith[7]. It is convenient to summarize briefly some conclusions of this review. Although retention indices are more robust than capacity factors against changes in the content of the organic modifier in the eluent, they were found to be sensitive to changes in the selectivity[8] and in the make of stationary phase material[3,9]. Changes in the proportion of organic modifier are reported to have an effect on retention indices, depending on the kind of modifier[10]. A set of internal standards structurally related to the solutes and used to calculate corrected capacity factors gave the best results in reporting retention when compared with retention times, capacity factors, retention indices and relative adjusted retention times[11]. There is some evidence that batch-to-batch variation does not affect the retention indices considerably[3], but Fig. 9 in ref. 7 does show some small differences between different batches of ODS material, especially with tetrahydrofuran-containing eluents.

Calibration with problem-specific chosen standards (markers) in RP-HPLC

Smilde *et al.*[12] described a calibration procedure with a purpose different from the one described above. Their approach aims at the prediction of the retention of compounds on a new stationary phase, at varying mobile phase compositions, with the use of measurements of specially chosen calibration standards on that new phase. An outline of the procedure is given in the Theory section. The capacity factors of six benzene derivatives are predicted on three types of stationary phase materials (hexyl-, octyl- and phenyl-modified silica material), for six mobile phase compositions (including water–methanol–acetonitrile ternary mixtures). The stationary phases are calibrated with the specially selected compounds, called markers. The average relative prediction error was 6% in k' units.

Some conclusions on calibration in RP-HPLC

We first state some conclusions regarding calibration aimed at obtaining "measurement-system-independent" retention values. The idea of using retention indices, based on homologues, in RP-HPLC finds its root in the use of Kováts retention indices in gas chromatography (GC)[13]. Whereas the retention mechanism in GC can be understood, to a great extent, as a partition mechanism, this is not true for RP-HPLC. The retention process in RP-HPLC is much less defined, and difficult to describe. The retention indices (I) cannot correct completely for the specific interactions between solute, stationary phase and modifier. The I values are still sensitive to column selectivity differences, to the kind of organic modifier and to other

properties of the mobile phase. It is clear that the identification power of an I value of an unknown compound is very sensitive to small variations in the I value due to changing measurement conditions.

A disadvantage of the I values, the inability to correct retention values of compounds that are chemically not related to the homologues used, is partly overcome by using relative capacity factors. A standard is chosen with a similar chemical structure to the compounds that have to be corrected. In this instance some progress is attained compared with the use of I values.

The use of more than one standard compound is more promising. With the notion that there are three principal sample–solvent interactions[14] (electron-donating, electron-withdrawing and dipole), Smith[15] argued that three test compounds, together with the I standards to test polarity effects, should be sufficient to characterize any reversed-phase chromatographic system. Although there seems to be an advantage in using corrected capacity factors based on more than one standard compound, the question of which standards to use is still open.

Note that the calibration along the lines suggested by Smith[15] and performed by Bogusz[16] requires the measurement of at least seven calibration standards (four homologues and three barbiturates).

Conclusions with respect to calibration of RP-HPLC systems aimed at predicting retention values for new chromatographic systems, and ultimately at predicting optimum separation conditions, must be drawn keeping in mind the consequences of small errors in predicting retention values. Small errors in predicting capacity factors may result in poor separations[17]. Relative prediction errors of 6% (in k' units), as reported earlier, are perhaps still too high. However, when it is clear from the statistical evaluation of the calibration that relatively large prediction errors may arise, then one can choose an appropriate mobile phase composition which nevertheless yields an adequate separation.

New calibration strategies in RP-HPLC

Calibration aiming at obtaining measurement-system-independent retention values is a problem that has not yet been completely solved. We focus on calibration with the purpose of predicting retentions on other stationary phases. Although preliminary results[12] indicate that the prediction of retention on other types of stationary phases is possible, we restrict ourself for the moment to stationary phases of the same brand but of different batches. We distinguish between three strategies.

The first strategy is designed for the calibration of a new stationary phase when measurements only on one original stationary phase, at varying mobile phase compositions, are available. To illustrate the scope of this strategy, suppose a separation is optimized with respect to the mobile phase composition on a stationary phase. This stationary phase deteriorates, or breaks down completely. The new stationary phase, either a slightly deteriorated or a fresh one, must be calibrated and the mobile phase optimization has to be updated. The calibration of the new stationary phase is done by measuring the retention of the markers at the same mobile phase compositions as used on the original stationary phase. The retention of the other solutes on that new stationary phase can be predicted at those mobile phase compositions.

The second strategy can be used when measurements on more than one original

stationary phase, at varying mobile phase compositions, are available. The new stationary phase can be calibrated at an arbitrary mobile phase composition where calibration is needed, not restricted to those used for the original stationary phases, by measuring the markers at that mobile phase composition. The retention of the other solutes can then be predicted at that mobile phase composition on the new stationary phase.

The third strategy is appropriate when measurements on more than four original stationary phases, at varying mobile phase compositions, are available. With the use of these measurements it is possible to calibrate new stationary phases by measuring the markers at a few specific mobile phase compositions. The retention of the other solutes can be predicted at every mobile phase composition used on the original stationary phases.

The pay-off between the three strategies is clear. At the price of more experimental effort prior to the calibration, in using the third instead of the second or first strategy, less effort is needed for the calibration of the new stationary phase. Comparing the first and second strategies, it appears that, in contrast to the first strategy, predictions at any mobile phase composition are possible with the second strategy, provided that the markers are measured at that composition on the new stationary phase. Of course, there will also be differences in predictive performance between the strategies, but we shall not pursue this further at the moment. Here we present the results of the second strategy; the results of the other strategies will be presented elsewhere.

Suppose measurements of a set of solutes, defining a separation problem, are available on two stationary phases (different batches of the same brand) at varying mobile phase compositions. The calibration of a new stationary–mobile phase combination should be done with a special subset of these solutes. These special solutes, the markers, should be chosen with statistical techniques to ensure that they are optimum with respect to their calibration power. The markers should reflect the differences between the stationary phases at the measured mobile phase combinations. A procedure will be proposed in the Theory section and illustrated by an example.

THEORY

Choice of markers

Suppose that retention measurements of sixteen solutes are available on two stationary phases, at nine regularly spread mobile phase compositions (see Fig. 1). We can arrange these measurements in a data table as depicted in Fig. 2. The column entries of this matrix, X, (the solutes) can be understood as variables. Strong relationships between those variables are present, hence the choice of the markers comes down to choosing those variables which represent the structure of X best. Stated otherwise, we select those solutes which have the highest mark or calibration power. In the following we describe a procedure, based on the work of McCabe[18], for choosing such variables.

Let x_i be the ith column of X (and thereby the ith variable) representing all retention measurement of solute i on the eighteen ($2 \cdot 9$) stationary–mobile phase combinations. The variance of x_i, s_i^2, can be calculated in a straightforward manner.

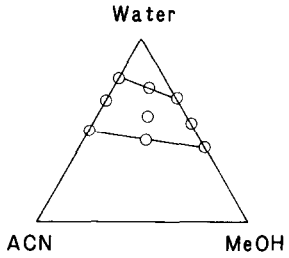

Fig. 1. Mobile phase measurement space. The points are the mobile phase compositions where measurements are taken, ACN and MeOH are acetonitrile and methanol, respectively.

The total variance present in X is then $\sum_{1}^{16} s_i^2$. Suppose four variables are considered appropriate to describe the structure of X. The mark-power of the first four solutes can be assessed in the following way. The variation of solute i can be explained by the variation in the first four solutes by means of multiple regression. A measure of the performance of this regression is $R^2(x_i|x_1,x_2,x_3,x_4)$, the squared multiple correlation coefficient obtained when x_i is regressed on x_1 to x_4. Note that this R^2 value is 1 for $i = 1$–4, because, *e.g.*, x_1 can be explained perfectly by itself.

The amount of variation in x_i which is explained by the multiple regression is $s_i^2 \cdot R^2(x_i|x_1,x_2,x_3,x_4)$, because the R^2 value represents the fraction of explained variation in x_i. When this multiple regression is performed for every i, the percentage of variation in X which is explained by the first four solutes is

$$P = 100 \cdot \frac{\sum_{i=1}^{4} (s_i^2) + \sum_{i=5}^{16} [s_i^2 \cdot R^2(x_i|x_1,x_2,x_3,x_4)]}{\sum_{j=1}^{16} (s_i^2)}$$

The mark-power of the first four solutes is quantitatively assessed by the value P. This whole procedure can be repeated for all possible combinations of four solutes subsequently. The particular combination with the highest P value is chosen. In our

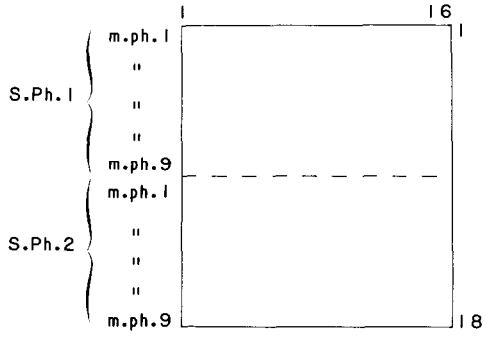

Fig. 2. Matrix X, the training set. Every entry in this matrix is a ln k' value of a solute (out of 16) on a stationary phase (S.Ph.) at a mobile phase composition (m.ph.).

experience four markers, when the best subset is chosen, explains more than 99% of the variation in X and is therefore sufficient for calibration purposes.

Establishing the relationship between markers and non-markers

Four solutes are chosen as markers (M) and the other solutes are labelled non-markers (NM). The data matrix X can be arranged such that the markers are gathered in X[M], and the non-markers in X[NM], see Fig. 3. Using the partial least-squares (PLS) method the measurements in the X[NM] matrix can be related to the measurements in the X[M] matrix. The use of PLS is discussed elsewhere[12,19,20]. It can be viewed as a generalization of multiple regression. When the PLS calculations are finished, a model is available relating the measurements of the non-markers to those of the markers. The matrix X is called the training set for obvious reasons: the matrices X[M] and X[NM] are used to build (train) the model. The selective differences between the stationary phases, at varying mobile phase compositions, of the non-markers (X[NM]) are explained by the selective differences of the markers (X[M]).

Calibration of a new stationary phase

In order to calibrate a new stationary phase, retention measurements of the four markers have to be performed. When the prediction of the retention of the non-markers is needed on the new stationary phase at a specific mobile phase composition, the retention of the markers must be measured on the new stationary phase at that specific mobile phase composition. The retention values of the markers represent the new stationary–mobile phase combination. Stated otherwise, the new stationary–mobile phase combination is described phenomenologically by those marker retention values.

The measured retention values of the markers are used as the input in the previously calculated model and the retention values of the non-markers can be predicted. This can be done for every mobile phase composition, restricted to the range used to build the model (Fig. 1), to avoid extrapolation. This prediction procedure is displayed graphically in Fig. 4.

EXPERIMENTAL

Methanol was of analytical-reagent grade and acetonitrile was of chromatographic quality (both from Merck, Darmstadt, F.R.G.). Water was obtained fresh

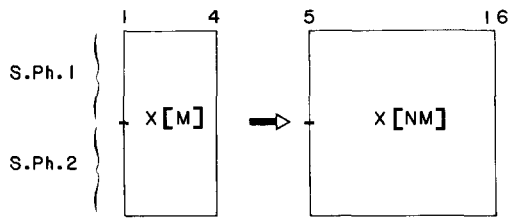

Fig. 3. Rearrangement of X to build a model. In X[M] the ln k' values of the markers (M) on the two stationary phases, at the nine mobile phase compositions, are gathered. The ln k' values of the non-markers (NM), at the same measurement conditions as the markers, are gathered in X[NM].

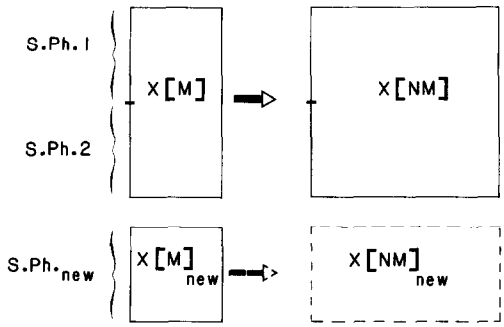

Fig. 4. Calibration of a new stationary phase. ln k' values of the markers on the new stationary phase are gathered in $X[M]_{new}$. Each row of this matrix contains ln k' values of the markers at a specific mobile phase composition on the new stationary phase. The non-markers, at these mobile phase compositions on this new stationary phase are predicted as $X[NM]_{new}$.

from a Milli-Q water purifier (Millipore, Bedford, MA, U.S.A.). The sixteen test solutes were acetophenone (ACP), acetanilide (ACT), anisole (ANS), *p*-cresol (CRE), dimethyl phthalate (DMP), ethyl 4-aminobenzoate (EAB), ethynylestradiol (EE), ethyl 4-hydroxybenzoate (EHB), methyl 4-hydroxybenzoate (MHB), nitrobenzene (NBZ), phenobarbital (PBL), 2-phenylethanol (PE), *n*-propyl 4-hydroxybenzoate (PHB), prednisone (PRE), prednisolone (PRS) and toluene (TOL), obtained from various manufacturers. These test solutes incorporate compounds frequently used in calibration studies[7–11]. The dead time was measured as the retention time of uracil. The concentrations of the injected solutes ranged from 0.02 to 0.08 mg/ml. The flow-rate was 0.5 ml/min.

The three stationary phases were different batches of Chromspher Octadecyl (Chrompack, Middelburg, The Netherlands). All columns were 100 mm × 3.0 mm I.D. Each series of measurements on one stationary phase was performed by a different analyst using different instruments. We label these stationary phase–analyst–instrument combinations as stationary phases A, B and C, respectively.

The first series (stationary phase A) of measurements was performed with an LDC-Milton Roy Mini HPLC pump, a Chromatronix 230 dual-wavelength detector [operated at 254 nm, except for EE (280 nm)], an injection valve (Rheodyne 7125) fitted with a 20-μl loop and a Kipp BD40 recorder. The second series (stationary phase B) was performed with an LDC-Milton Roy Mini HPLC pump, a Shimadzu SPD6A variable-wavelength detector [operated at 254 nm, except for EE (205 nm)], an injection valve (Rheodyne 7125) fitted with a 20-μl loop and a Kipp BD40 recorder. The last series (stationary phase C) was performed with a Waters 6000A HPLC pump, a Kratos Spectroflow 757 variable-wavelength detector (operated at 205 nm), an injection valve (Rheodyne 7010) fitted with a 10-μl loop and an Omniscribe recorder (Houston Instruments).

All k' values are the averages of two repeated measurements. The k' values are reported in Table I, together with the mobile phase compositions at which the measurements were made. The mobile phase compositions were regularly spread over the factor space (see Fig. 1). The reproducibility was tested by measuring at mobile phase composition wm2 three (stationary phases A and B) or four (stationary phase C)

TABLE I

CAPACITY FACTORS OF THE TEST SOLUTES

A, B and C refer to the analyst–instrument–stationary phase combinations. Mobile phase compositions (volume fractions of water–acetonitrile–methanol): wm1 = 0.63:0.00:0.37; wm2 = 0.55:0.00:0.45; wm3 = 0.47:0.00:0.53; wa1 = 0.78:0.22:0.00; wa2 = 0.70:0.30:0.00; wa3 = 0.62:0.38:0.00; am1 = 0.71:0.11:0.18; am2 = 0.62:0.15:0.23; am3 = 0.54:0.19:0.27.

Stationary phase	*Mobile phase*	*Solute*							
		ACP	*ACT*	*ANS*	*CRE*	*DMP*	*EAB*	*EE*	*EHB*
A	wm1	4.44	1.68	9.25	4.36	6.09	4.46	113.78	8.17
	wm2	2.30	0.96	5.16	2.31	2.58	2.02	24.76	3.63
	wm3	1.37	0.62	3.08	1.34	1.31	1.09	8.76	1.78
	am1	6.49	2.25	13.61	6.50	10.81	7.73	181.41	12.50
	am2	3.44	1.30	7.33	3.33	4.69	3.44	28.82	5.03
	am3	1.86	0.72	3.98	1.79	2.26	1.67	10.63	2.32
	wa1	7.21	2.04	17.37	6.48	11.53	8.33	100.19	10.32
	wa2	3.64	1.07	8.84	3.09	4.79	3.59	18.71	3.81
	wa3	2.02	0.62	4.34	1.63	2.33	1.73	5.07	1.74
B	wm1	4.26	1.61	8.83	4.16	5.84	4.29	109.26	7.96
	wm2	2.16	0.89	4.79	2.16	2.37	1.96	24.74	3.34
	wm3	1.26	0.55	2.88	1.25	1.24	1.03	8.83	1.72
	am1	5.61	1.92	12.09	5.75	9.64	6.88	169.95	10.75
	am2	3.24	1.27	6.46	3.01	4.10	3.10	27.20	4.51
	am3	1.66	0.63	3.69	1.86	2.09	1.63	10.30	2.10
	wa1	5.94	1.64	14.42	5.38	9.60	6.97	93.26	8.66
	wa2	3.06	0.83	7.77	2.61	4.21	2.89	16.37	3.28
	wa3	1.47	0.50	3.91	1.43	2.02	1.46	4.51	1.63
C	wm1	4.46	1.66	9.66	4.47	6.43	5.03	108.79	8.57
	wm2	2.41	0.97	5.55	2.51	2.77	2.22	28.46	3.97
	wm3	1.27	0.55	2.98	1.32	1.25	1.02	8.53	1.75
	am1	6.59	2.26	14.49	6.83	10.93	8.01	150.23	12.80
	am2	3.09	1.12	7.09	3.13	4.42	3.26	33.97	4.81
	am3	1.85	0.71	4.08	1.83	2.25	1.70	10.67	2.37
	wa1	6.53	1.80	16.40	6.10	10.54	7.66	87.22	9.53
	wa2	3.61	1.02	8.89	3.14	4.70	3.53	17.70	3.79
	wa3	2.09	0.67	4.69	1.74	2.43	1.82	5.37	1.84
		MHB	*NBZ*	*PBL*	*PE*	*PHB*	*PRE*	*PRS*	*TOL*
A	wm1	3.35	5.39	2.99	3.57	21.63	14.35	20.29	23.56
	wm2	1.66	3.07	1.55	1.97	8.53	4.33	6.46	12.73
	wm3	0.90	1.92	0.84	1.17	3.81	1.85	2.70	7.24
	am1	4.88	9.10	5.58	4.47	34.47	21.56	25.36	33.64
	am2	2.25	5.18	2.43	2.36	12.22	5.99	7.05	17.40
	am3	1.15	2.84	1.16	1.32	4.97	2.17	2.58	8.87
	wa1	4.13	12.70	4.52	3.68	28.11	9.29	8.79	43.54
	wa2	1.83	6.69	1.76	1.75	8.63	2.04	1.78	20.59
	wa3	0.99	3.49	0.67	1.00	3.28	0.78	0.67	9.42
B	wm1	3.24	5.21	2.91	3.44	20.97	14.30	20.04	22.17
	wm2	1.53	2.91	1.42	1.73	7.88	4.16	6.27	11.84
	wm3	0.86	1.77	0.81	1.10	3.70	1.74	2.64	6.80
	am1	4.18	8.07	4.94	4.01	29.97	19.79	23.15	29.41
	am2	1.96	4.92	2.19	2.38	11.00	5.89	6.62	15.73
	am3	1.02	2.54	1.09	1.21	4.56	1.98	2.54	8.57
	wa1	3.44	10.61	3.75	3.03	22.56	7.89	7.50	36.26

TABLE I (*continued*)

Stationary phase	*Mobile phase*	*Solute*							
		MHB	*NBZ*	*PBL*	*PE*	*PHB*	*PRE*	*PRS*	*TOL*
	wa2	1.52	5.70	1.41	1.51	7.50	1.71	1.49	18.01
	wa3	0.87	3.08	0.67	0.86	2.84	0.65	0.51	8.38
C	wm1	3.52	5.58	3.75	3.59	22.70	14.02	20.26	26.33
	wm2	1.80	3.25	1.71	2.06	9.38	4.57	6.95	14.08
	wm3	0.87	1.80	0.79	1.11	3.72	1.68	2.55	7.08
	am1	5.03	9.39	5.70	4.56	35.49	20.82	24.77	35.11
	am2	2.15	4.85	2.30	2.21	11.53	5.25	6.22	17.81
	am3	1.17	2.80	1.17	1.29	5.08	2.02	2.57	9.28
	wa1	3.91	11.80	4.03	3.20	25.60	7.35	7.11	42.58
	wa2	1.85	6.47	1.66	1.72	8.44	1.85	1.65	20.72
	wa3	1.04	3.60	0.84	1.02	3.51	0.77	0.68	10.01

times, on different days, with a regular spacing within the series of nine mobile phase compositions.

The calculations were performed on an IBM PC/AT compatible computer, using standards programs [SIMCA (Sepanova, Enskede, Sweden)], and programs written in Fortran. The ANOVA calculations were performed at a CDC-Cyber 760/260 computer, using the SPSS package.

RESULTS AND DISCUSSION

Preliminary calculations

The reproducibility of the measurement can be calculated for every solute, for each stationary phase, at mobile phase composition wm2. The reproducibility can be reported as coefficients of variation (C.V.). These values are calculated for each stationary phase as $100(s/k_{mean})$, where s is the standard deviation of the three (or four, see stationary phase C) k' values and k_{mean} is the mean value of these capacity factors. The C.V. values for stationary phase A ranged from 0.2 to 2.7% with an average of 1.3%. For stationary phase B the C.V. values ranged from 0.3 to 13.1% with an average of 4.3%. The range of C.V. values for stationary phase C was 0.3–3.2% with an average of 2.0%. Closer examination of stationary phase B showed that the solutes ACT, DMP, EHB, MHB, PBL, PE and PHB were responsible for the high average C.V. No clear evidence was present to question the quality of these measurements, so they were not discarded.

On the assumption that the relative error in k' is constant, a logarithmic transformation of k' produces ln k' values with a constant variance[21]. This property makes the application of linear models easier.

Two analysis of variance (ANOVA) calculations were performed to illustrate the differences between the stationary phases. The first ANOVA is a one-way setup. The (only) factor is the stationary phase (varied at three levels), with three repeated measurements for stationary phases A and B and four repeated measurements for stationary phase C, at mobile phase composition wm2. This ANOVA was done for

every solute separately. With the use of Scheffé confidence intervals, differences between the stationary phases can be visualized for every solute. The results are reported in Table IIa; the level of significance is 5%. When a solute is reported, the null hypothesis that the stationary phases do not differ with respect to the ln k' values of that solute is rejected. Stated otherwise, the variation due to changing stationary phases is significantly larger than the reproducibility of that solute. Two conclusions can be drawn from Table IIa: first, stationary phase C deviates the most of the three, and second, the differences between the stationary phases depend on the solutes.

The second ANOVA is done with two factors: the stationary phases (varied at three levels) and the amount of organic modifier in the mobile phase (varied at three levels). This two-way ANOVA is performed three times, for each type of mobile phase: once with the binary water–methanol mixtures, once with the binary water–acetonitrile mixtures and once with the ternary mixtures as second factor. The results are reported in Table IIb. When a solute is shown, this means that the null hypothesis "no difference between the stationary phases" is rejected at a significance level of 5%. By comparing the results for the two-way ANOVA performed with the different types of mobile phases, it is clear that the differences between the stationary phases are not only dependent on the solutes but also on the type of the mobile phase. The differences between the stationary phases when water–methanol mixtures are considered only show up for *p*-cresol (CRE). The differences between the stationary phases are much more pronounced in water–acetonitrile mixtures. This emphasizes the notion that differences between the stationary phases depend on the solutes, the kind of mobile phase and combinations of these factors. This confirms the remarks made in the Introduction in this respect.

Prediction for a new stationary phase

In order to illustrate the whole calibration procedure, we select stationary phases A and B as the training set (see Fig. 2), because we expect that the predictions for stationary phase C will be the most difficult (see the one-way ANOVA results), thereby burdening the calibration procedure. The four solutes ANS, DMP, EE and PRE explain the highest percentage of variation in X (P = 99.83%), and are therefore

TABLE II

ANALYSIS OF VARIANCE (ANOVA)

(a) One-way ANOVA: solutes that differ between the stationary phases A, B and C:

	A	*B*
B	ACP, ANS, CRE, NBZ, PRE, TOL	
C	ACP, ANS, CRE, EAB, EE, NBZ, PHB, PRE, PRS, TOL	All solutes

(b) Two-way ANOVA: solutes that differ between the stationary phases A, B and C:

Water–methanol	*Water–methanol–acetonitrile*	*Water–acetonitrile*
CRE	ANS, DMP, EHB, MHB, PBL, PHB, TOL	ACP, ACT, ANS, CRE, DMP, EAB, EHB, MHB, NBZ, PE, PHB, TOL

chosen as markers. Two of these solutes, ANS and PRE, were already expected to describe differences between stationary phases A and B (see Table IIa).

The second and third best subsets are DMP, EE, PRE, TOL and ANS, DMP, PRE, PRS. These subsets both explain 99.82%, illustrating the interchangeability of the three subsets of markers. The exchange of ANS and TOL between subsets one and two can be explained, as they belong to the same selectivity group (VII) of Snyder[14], and TOL is also sensitive towards differences between A and B. The solutes EE and PRS are chemically related and can be exchanged.

Note that the three subsets represent moderate- and slow-eluting compounds, and not the fast-eluting ones. This may be a problem when fast-eluting compounds are to be predicted.

The ln k' values are ordered as indicated in Fig. 3. All columns in X[M] and X[NM] were mean centered, no scaling was performed because all ln k' values are measured on the same scale and do not differ greatly in magnitude.

With two dimensions in the PLS model, 99.5% of the variation in X[M] was used to explain 99.2% of the variation in X[NM]. These two dimensions were considered sufficient to reflect the relationship between X[M] and X[NM].

The final step in the prediction procedure is the calibration of the new stationary phase C with the markers. As the measurements of the markers are available on stationary phase C, at the same mobile phase compositions as in the training set, predictions on the new stationary phase were performed at these mobile phase compositions. It is not necessary to predict at the same mobile phase compositions as in the training set, but it is convenient with the data set at hand. The ln k' values of the markers are used to predict the ln k' values of the non-markers, at the nine mobile phase compositions, on the new stationary phase (see Fig. 4).

The results of the calibration of the new stationary phase are given in Table III. The relative errors can be compared with the C.V. for stationary phase C. The relative prediction errors range from 3.0 to 6.3%, with an average of 4.0%. This is roughly double the mean reproducibility, measured in C.V. units. The predictions can be considered good except for the solutes MHB, PBL, PRS and TOL. To obtain an impression of the performance of the calibration, the observed *versus* the predicted capacity factors of ACP and TOL are given (Table IIIb). The solutes represent the best and worst calibration, respectively.

We first discuss the relatively bad predictions of PBL and MHB. This was expected as both compounds are fast eluting. However, other fast-eluting compounds, ACP, ACT, CRE, EAB and PE, are predicted well. Closer examination shows that PBL is predicted worse at the ternary mixtures. This could be expected while the two-way ANOVA showed differences between the measurements of PBL on the stationary phases with respect to the ternary mixtures. Scheffé's simultaneous confidence intervals show that these differences occur between A, B and B, C. The conclusion is that PBL shows selective differences in ternary mixtures which are not completely represented by the markers. The solute MHB is predicted worse for the aml and wa3 mixtures. This may be due to an analogous cause to that of PBL (see Table IIb). For both mobile phase systems (water–acetonitrile and water–methanol–acetonitrile), MHB shows differences between stationary phases A, B and B, C as calculated with Scheffé intervals. Other solutes, *e.g.*, EHB and PHB, also show differences between the stationary phases; for both water–acetonitrile and the ternary

TABLE III

RESULTS OF THE CALIBRATION

The relative prediction errors are calculated as $100 \cdot ABS[(k'_{obs} - k'_{pred})/k'_{obs}]$, where *ABS* is the absolute value.

(a) Relative prediction errors (%)

Solute	*Prediction error*	*Solute*	*Prediction error*
ACP	3.0	NBZ	3.0
ACT	3.6	PBL	5.4
CRE	3.0	PE	3.1
EAB	3.1	PHB	3.6
EHB	3.1	PRS	6.2
MHB	4.6	TOL	6.3

(b) Example of the calibration

Mobile phase	*ACP*		*TOL*	
	Observed k'	*Predicted k'*	*Observed k'*	*Predicted k'*
wm1	4.46	4.58	26.33	24.46
wm2	2.41	2.44	14.08	12.72
wm3	1.27	1.31	7.08	6.64
am1	6.59	6.55	35.11	35.97
am2	3.09	3.22	17.81	17.24
am3	1.85	1.86	9.28	9.77
wa1	6.52	6.65	42.58	37.91
wa2	3.61	3.46	20.72	19.36
wa3	2.09	1.94	10.01	10.55

mixtures differences occur between A, B and B, C. These latter solutes are predicted well, so extrapolation beyond the "markers scale" is probably one of the reasons for the relatively bad predictions of MHB.

We incorporated two slowly eluting markers but nevertheless PRS and TOL are badly predicted. In a related study[22], relatively bad predictions were also observed for slow-eluting compounds. The suggestion was made in that study that the inherent increase in relative error associated with the measurement of long retention times might be a cause. However, the C.V. for both PRS and TOL on stationary phase C is 1.8%, hence no serious relative measurement error is present. Whereas the solutes TOL and PRS are contained in the second and third best marker subsets, respectively, the suggestion is that these solutes are sensitive to the differences between the stationary–mobile phase combinations in the training set. This makes these solutes relatively difficult to predict. The solute TOL is known to be sensitive to stationary phase differences[10].

The predictions for the non-markers, together with the measured k' values of the markers, can be used as the input for a simultaneous optimization scheme[23–25] in order to recalculate the optimum mobile phase composition on the new phase.

Many questions remain to be answered. First, different statistical procedures are available to select the markers. These procedures should be validated with respect to

their power to choose markers which represent best the selective differences between stationary–mobile phase combinations. Second, we realize that it is more elegant to incorporate an optimization update step in the calibration procedure directly. Third, the already mentioned first and third calibration strategies have to be tested. Finally, all three strategies should be tested on calibration problems with different makes or different types of stationary phases. Research on these topics is in progress.

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REFERENCES

1 J. G. Atwood and J. Goldstein, *J. Chromatogr. Sci.*, 18 (1980) 650.
2 L. Hansson and L. Trojer, *J. Chromatogr.*, 207 (1981) 1.
3 R. M. Smith, T. G. Hurdley, R. Gill and A. C. Moffat, *Chromatographia*, 19 (1984) 407.
4 K. K. Unger, *Porous Silica (Journal of Chromatography Library Series*, Vol. 16), Elsevier, Amsterdam, 1979.
5 E. Bayer and A. Paulus, *J. Chromatogr.*, 400 (1987) 1.
6 A. K. Smilde, C. H. P. Bruins, D. A. Doornbos and J. Vink, *J. Chromatogr.*, 410 (1987) 1.
7 R. M. Smith, *Adv. Chromatogr.*, 26 (1987) 277.
8 J. K. Baker and C. Y. Ma, *J. Chromatogr.*, 169 (1979) 107.
9 R. M. Smith, *Anal. Chem.*, 56 (1984) 256.
10 R. M. Smith, G. A. Murilla, T. G. Hurdley, R. Gill and A. C. Moffat, *J. Chromatogr.*, 384 (1987) 259.
11 R. Gill, A. C. Moffat, R. M. Smith and T. G. Hurdley, *J. Chromatogr. Sci.*, 24 (1986) 153.
12 A. K. Smilde, C. H. P. Bruins, P. M. J. Coenegracht and D. A. Doornbos, *Anal. Chim. Acta*, 212 (1988) 95.
13 E. Kováts, *Helv. Chim. Acta*, 41 (1958) 1915.
14 L. R. Snyder, *J. Chromatogr.*, 16 (1978) 223.
15 R. M. Smith, *J. Chromatogr.*, 324 (1985) 243.
16 M. Bogusz *J. Chromatogr.*, 387 (1987) 404.
17 P. J. Schoenmakers and Th. Blaffert, *J. Chromatogr.*, 384 (1987) 117.
18 G. P. McCabe, *Technometrics*, 26 (1984) 137.
19 S. Wold, A. Ruhe, H. Wold and W. J. Dunn, III, *SIAM J. Sci. Stat. Comput.*, 5 (1984) 735.
20 P. Geladi and B. R. Kowalski, *Anal. Chim. Acta*, 185 (1986) 1.
21 G. E. P. Box and N. R. Draper, *Empirical Model Building and Response Surfaces*, Wiley, New York, 1987, p. 284.
22 C. H. Lochmuller, S. J. Breiner, Ch. E. Reese and M. N. Koel, *Anal. Chem.*, 61 (1989) 367.
23 A. K. Smilde, A. Knevelman and P. M. J. Coenegracht, *J. Chromatogr.*, 369 (1986) 1.
24 J. L. Glajch, J. J. Kirkland, K. M. Squire and J. M. Minor, *J. Chromatogr.*, 199 (1980) 57.
25 M. A. Quarry, R. L. Grob, L. R. Snyder, J. W. Dolan and M. P. Rigney, *J. Chromatogr.*, 384 (1987) 163.