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Reliability of the retention factor estimations in liquid chromatography

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Abstract

The retention factor is one of the most universally used parameters in chromatography. However, large differences in the experimental retention factor values are observed when the same compound is injected in a given stationary/mobile phase system under intermediate precision conditions. Conventional protocols for estimating retention factors have problems that mainly arise from difficulties in the hold-up time measurements and the omission of the existence of extra-column times by practicing chromatographers. In the present paper, three different approaches for estimating retention factors are tested: (i) classical retention factor estimations based on the gross hold-up time, (ii) based on the real hold-up time (taking into account the extra-column time), and (iii) a new approach that uses 'relative' retention factors based on the use of an external standard. Assays are performed in micellar liquid chromatography (MLC) under intermediate precision conditions (different days, equipments, columns lengths, and mobile phase flow rates). The reliability of the three approaches tested is evaluated by means of precision studies, analysis of factors affecting retention factors, and uncertainty calculations. The approach based on 'relative' retention factors was found to be the most precise, reliable, and robust strategy for estimating retention factors.

Keywords: Relative retention factors; Retention factors; Hold-up time; Extra-column time correction; Intermediate precision; Uncertainty

1. Introduction

The retention factor (k) is one of the most internationally used parameters in chromatography. The use of k permits the comparison between retention data obtained in different chromatographic systems. Also, it is used in models like QSRRs (quantitative structure retention relationships) and QRARs (quantitative retention activity relationships). In the QRAR models, the retention factor is the principal parameter for activity estimation of compounds (i.e. local anesthetics [1], non-steroidal anti-inflammatory drugs [2], anticonvulsant drugs [3], etc.).

Retention factor calculation involves the use of hold-up volume (or time) values, which can affect k values severely. Some methods have been reported and reviewed [4–6] for hold-up volume estimation. Most of these procedures have been applied to chromatographic systems equipped with refractive index detectors and are not appropriate if sample components have to be detected with a light absorption detector [7]. For these cases, an unretained compound should be injected for a direct measurement of the hold-up volume, but no ideal unretained compounds exists [5]. The ambiguity, controversy, and difficulties of the use of the hold-up time (t_M) in chromatography have been discussed [6].

On the other hand, the experimental retention time (gross retention time; t_R^g) is the sum of the retention time (t_R) and the extra-column time (t_{ext}), which is the retention time contribution due to the injector, detector and connections. In this case, the retention factor may not be obtained with the expression $k = (t_R^g - t_M^g)/t_M^g$, where t_M^g is the gross hold-up time, except in the case in which the hold-up time, $t_M \sim t_M^g$ (i.e. t_{ext} is carefully minimized) [5]. For instance, Wilson et al. [8] reported errors in retention factor data around 5–10% related to large t_{ext}, in repeatability conditions. Unfortunately, this extra-column time has been ignored in the past [5].

Torres-Lapasió et al. [9] worked with micellar liquid chromatography (MLC) and due to the wide and variable perturbations that appear at the heads of the chromatograms, they proposed and compared four different criteria for the 'dead time' (in reality the gross hold-up time) determination. They concluded that the measurement of the gross hold-up time at the start of the main first perturbation (a subjective

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decision) on the chromatograms gave satisfactory retention factors. Anyway, such measurements are not easy to automate and deserve careful inspection by operator. Additionally, they may vary for different equipments, sample-mobile phase composition and time.

In this paper, the reliability of three different approaches for estimating retention factors is tested by means of precision studies, analysis of factors affecting retention factors and uncertainty calculations. Assays are performed in micellar liquid chromatography under intermediate precision conditions (inter-day assays, different equipments, column lengths and mobile phase flow rates).

2. Experimental

2.1. Instrumental and measurements

Two Hewlett-Packard HP 1100 chromatographs with an isocratic pump and an UV-Vis detector (variable wavelength detector) were employed. One of them was equipped with a column thermostat with a capacity for at least three columns (9 μl extra-column volume is available for preheating mobile phase prior to the column) and an autosampler with a 20 μl loop. The other one had a Rheodyne value (Cotati, CA) with also a 20 μl loop for manual injection of samples. Data acquisition and processing were performed by means of an HP Vectra XM computer (Amsterdam, The Netherlands) equipped with an HP-Chemstation software (A.07.01 [682] ©HP 1999). Two Kromasil C18 columns (5 μm, 150 mm × 4.6 mm i.d.; Scharlab S.L., Barcelona, Spain) and (5 μm, 50 mm × 4.6 mm i.d.; Scharlab) and two guard columns of similar characteristics (5 μm, 35 mm × 4.6 mm i.d.; Scharlab) were used. The mobile phase flow rate was 1.0 or 1.5 ml min^{-1}. The detection was performed in UV at 254 nm for acetanilide, aniline, caffeine, and pyrene, 220 nm for atenolol, 2,4-dimethylphenol, flunarizine, and salicylic acid. The columns were thermostatted at 36.5 °C for all assays.

2.2. Reagents and standards

A micellar mobile phase of Brij35 0.06 M at pH 7.40 was prepared by dissolving polyoxyethylene(23)lauryl ether (Brij35, Acros Chimica, Geel, Belgium) in 0.05 M phosphate buffer solution. The buffer solution was prepared with sodium dihydrogen phosphate (reagent grade, Scharlab, Barcelona, Spain). The pH was potentiometrically adjusted at 7.4 by addition of sodium hydroxide (97%, purissimum, Panreac, Barcelona, Spain) aqueous solution.

Compounds used in this study were obtained from different sources. Standard of atenolol was kindly donated by Zeneca–Farma (Madrid, Spain). Standards of acetanilide, aniline, and pyrene were obtained from Scharlab S.L. (Barcelona, Spain), caffeine from Guinama S.L. (Valencia, Spain), salicylic acid from Panreac (purissimum, Barcelona, Spain), 2,4-dimethylphenol from Dr. Ehrenstorfer (Augsburg, Germany), and flunarizine from Sigma (St. Louis, Missouri, USA). Stock standard solution of every compound was prepared by dissolving 10 mg of each compound in 10 ml of 0.06 M Brij35 solution (pH 7.4) or acetonitrile. Working solutions were prepared by dilution of the stock standard solutions using the mobile phase solution. The solutions were stored under refrigeration at 5 °C.

Barnstead E-pure, deionized water (Sybron, Boston, MA, USA) was used throughout. The mobile phase and the solutions injected into the chromatograph, were vacuum-filtered through 0.45 μm nylon membranes (Micron Separations, Westboro, MA, USA).

2.3. Nomenclature

The nomenclature and abbreviations for chromatography has been adapted to the last revision of terms in the International Union of Pure and Applied Chemistry (IUPAC Recommendations 2001) [5].

See Appendix A for further details about the nomenclature used.

3. Results and discussion

3.1. Precision study of retention factor data

The retention factor definition includes the hold-up time. However, t_M is usually estimated experimentally as a gross hold-up time by means of the gross retention time of an unretained compound or the first disturbance on the chromatogram. This assumption is only acceptable when the extra-column time is negligible. Otherwise, there is a need of accurate experimental measurements to approximate to the real hold-up time.

The bad practice of assuming t_M^g as t_M without confirmation, together with the intrinsic difficulties of hold-up measurements (t_M^g or t_M) leads to unreliable retention factors estimations.

3.1.1. Approach 1: classical retention factor estimations (k^g)

A laboratory cannot determine reproducibility as such (because this has to be done in interlaboratory experiments), but it can determine intermediate precision conditions. The ISO-standard [10] recognizes what is called *M*-factor different intermediate precision conditions, where *M* (=1, 2, 3, ...) factors (i.e. equipment, day, operator, ...) differ between successive determinations. Such measurements are useful for long-term data comparability when the *M* factors included in the their study can vary.

In order to check the reliability of classical retention factor data estimations (k^g, estimated from t_M^g), a study under intermediate precision conditions (factors: time, equipment, and column length) was performed. For this purpose, acetanilide was used as test compound because it is a molecule

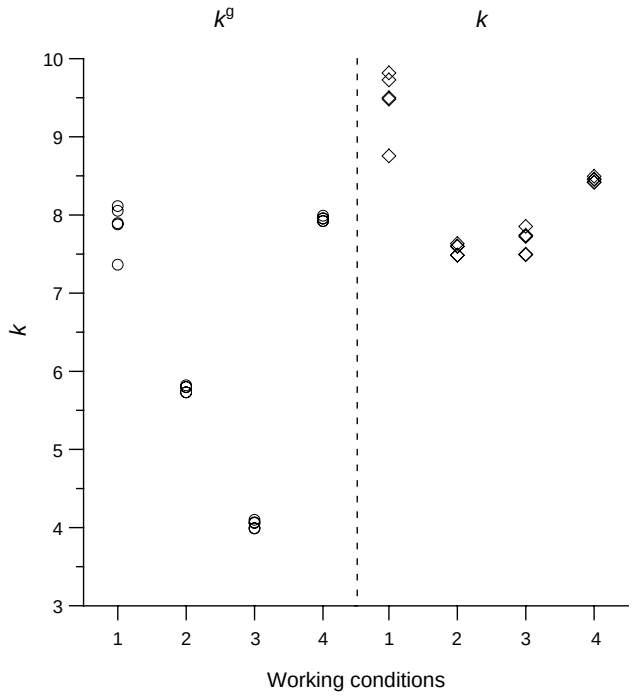

Fig. 1. Retention factors obtained for acetanilide in the four different working conditions assayed (five replicates per condition). Classical k^g (○) and k (◇) estimations. Working conditions: (1) DAY-1, EQ-2, COL-2. (2) DAY-1, EQ-1, COL-1. (3) DAY-2, EQ-1, COL-2. (4) DAY-2, EQ-2, COL-1. COL-1: 150 mm; COL-2: 50 mm; EQ-1: equipment with a manual Rheodyne valve; EQ-2: equipment with an autosampler.

with intermediate hydrophobicity (log $P = 1.16$) and can be injected in short and long columns with acceptable t_R^g.

A two-factor fully-nested experimental design was performed in four runs where the factors equipment, column, and day varied between each run. Five replicates were performed in each run in order to account for repeatability. The t_M^g were measured in a classical way at the beginning of the first perturbation on the chromatograms. In this paper, t_M^g estimations were performed for each injection.

Under these conditions, the retention factor should be constant. However, as can be seen in Fig. 1 (left part) a large variability in the k^g values obtained was observed. Along the four different working conditions in Fig. 1, repeatability (five replicates) of t_R^g, in terms of relative standard deviation (R.S.D.), was adequate (R.S.D., 0.06–0.084%). However, the large variability in t_M^g (R.S.D., 0.27–3.4%), determined the final k^g variability (R.S.D., 0.35–4.33%) in repeatability conditions. A different question is the global R.S.D. associated to intermediate precision conditions. Table 1 shows the precision statistics obtained for these 20 k^g determinations (four runs × five replicates). The global R.S.D. obtained for acetanilide was 26%, which should be considered unacceptable. Here, besides the t_M^g variability, the errors introduced by the omission of the t_{ext} (extra-column time) are also included. Therefore, the possibility of introducing the real hold-up time in the k estimation was evaluated.

Table 1
Precision statistics for acetanilide retention factors obtained in four different working conditions (runs) with five replicates per condition

Statistic	k^g	k
Number of data	20	20
Mean	6.4	8.3
s	1.7	0.8
R.S.D. (%)	26.0	9.8
Range ($k_{max} - k_{min}$)	4.1	2.3

The same experiment was also repeated at 25 °C (where possible thermal gradients are minimized). The results were similar to those in Fig. 1 and Table 1. A similar variability in repeatability conditions was observed and the global R.S.D. was 22.9% (as a result of a similar standard deviation, but a larger k^g mean).

3.1.2. Approach 2: retention factor estimations (k) based on the extra-column time correction

t_M was estimated as the difference between the gross hold-up time and the extra-column time. t_{ext} was measured in an independent experiment without the column at the beginning of the first perturbation on 0.05 M phosphate buffer chromatogram. Under these conditions, the k values were calculated from t_R^g, t_M^g, and t_{ext} according to Eq. (1):

$$k = \frac{t_R - t_M}{t_M} = \frac{t_R^g - t_M^g}{t_M} = \frac{t_R^g - t_M^g}{t_M^g - t_{ext}} \quad (1)$$

Fig. 1 (right part) displays the k values obtained for acetanilide after t_{ext} correction. As can be seen, smaller differences between the k values (i.e. R.S.D. = 9.8%) than those previously observed in k^g values were found. Nevertheless, there were some variations in the k values yet, which suggested the influence of some factors (besides the extra-column time) on k values. Therefore, a thoughtful statistic study on the effects contributing to retention factors estimations would be convenient (Section 3.2). In addition, the use of k values has some practical/experimental disadvantages because of the necessity of measuring t_{ext} to calculate t_M for a given chromatographic system. Such measurements have to be performed for every chromatograph and when changes in the tubing of equipment (an usual practice) or in the configuration of the system are performed.

3.1.3. Approach 3: retention factor estimations (k_R) based on an external standard

An alternative strategy has been tested: to relate the retention factor of compounds to a reference retention factor value (k_{REF}), which is considered constant. k_{REF} is established (as a gross retention factor) from a reference compound and a given experimental condition close to an optimal system where $t_{ext} \sim 0$, $t_M^g \sim t_M$, $t_R^g \sim t_R$, and $k^g \sim k$. From the conventional equation of the gross retention factor corresponding to the reference and a new compound i, the 'relative' retention factor for this new compound i

(k_{Ri}) can be re-calculated as follows:

$$k_{Ri} = \frac{t^{g}_{Ri}}{t^{g}_{R(REF)}}(1 + k_{REF}) - 1 \quad (2)$$

where t^{g}_{Ri} is the gross retention time of the new compound i and $t^{g}_{R(REF)}$ is the gross retention time of the reference compound. Therefore, when both the reference and the new compound are injected in the same working session, k_{Ri}, which is in fact a gross retention factor, can be directly estimated from t^{g}_{Ri} and $t^{g}_{R(REF)}$, without need of estimating any hold-up (and extra-column) times. A practical advantage of the use of a reference compound (external standard) is that it is based on t^{g}_{R} measurements, which can be considered more reliable and easier than t^{g}_{M} or t_{ext} measurements used in the previous approaches.

Wilson et al. [8] suggested the use of a reference compound to normalize the retention factor values for long-term operations of a HPLC system, admitting that the ratio between retention factors of the test solute and reference compounds (the selectivity factor) must be constant along the time. This is in the same philosophy used here, however, in Eq. (2) t^{g}_{R} values (not retention factors) are normalized based on a reference compound.

Acetanilide was selected as reference compound to calculate k_{Ri} for the new chromatographed compounds for several reasons: (i) aqueous solutions of acetanilide are stable for a long time (>2 months). (ii) Acetanilide is present in its neutral form in the whole operating pH range of silica bonded phases (basic compound with $pK_a = 0.5$). (iii) This compound showed adequate (non-extreme) gross retention times under the experimental conditions assayed in both, the short (~2.4–2.7 min) and long (~6.9–7.2 min) columns. (iv) Standards of acetanilide are commercially easy available and economical.

The chromatographic system that corresponds to working condition 4 in Fig. 1 was selected as reference system to establish k_{REF}. This system showed some special features: (i) the chromatograph (EQ-2) showed the smallest t_{ext} values and therefore the k^{g} values closest to the corresponding k values as shown in Fig. 1. (ii) The longest column (COL-1) avoided the coincidence of the first perturbation with the beginning of the chromatogram, which allowed for a better measurement of t^{g}_{M}, so then for calculating k_{REF}. (iii) The t^{g}_{M} values obtained using COL-1 were next to unity in both chromatographs, so then, for mathematical reasons provided more precise k^{g} values than COL-2.

In contrast, the low t^{g}_{M} values measured for COL-2 (0.2–0.3 min) made k^{g} values more sensible to small variations in t^{g}_{M}. In addition, in EQ-1 the t_{ext} values measured in COL-2 accounted for the ~50% of t^{g}_{M}, which produced k^{g} values unacceptably far from the corresponding k values.

For all these reasons, the average of five replicates in EQ-2 and COL-1 for acetanilide was used as k_{REF} value ($k_{REF} = 7.95$). All future calculations with this approach (Eq. (2)) use this reference as a constant value.

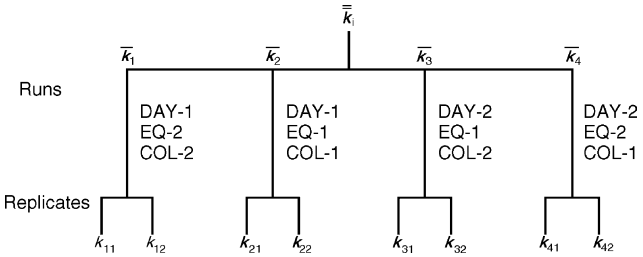

Fig. 2. Two-factor fully-nested design for salicylic acid, aniline, and 2,4-dimethylphenol. $\bar{k}_j$ is the mean of the two replicate measurements performed on run j. The grand mean, $\bar{\bar{k}}_i$, is calculated as the mean of the mean values obtained on the different runs ($\bar{k}_j$).

3.2. Comparative study of effects of the experimental variables affecting the retention factor data

3.2.1. Combined study of time, equipment, and column length factors

In order to evaluate and compare the main factors affecting the precision of the three above-mentioned approaches, three compounds (salicylic acid, aniline, and 2,4-dimethylphenol) were used as test compounds. Each compound was chromatographed in combination with acetanilide (external standard) according to the two-factor fully-nested design [10,11] shown in Fig. 2. Every day, the experiments of test compounds were randomized. For k^{g} estimations (approach 1), t^{g}_{M} values in every chromatogram were measured taking into account the first disturbance. For k estimations (approach 2), besides t^{g}_{M} values of approach 1, t_{ext} measurements were performed at the ending of every working session (day). To calculate k_{Ri} of test compounds (approach 3), acetanilide was injected at the beginning, during, and at the ending of a working session.

The evaluation of effects involving the following factors, equipment (EQ, factor A), column (COL, factor B), and day (DAY = block; confounded with a possible interaction EQ–COL), was performed by means ANOVA. The interaction was assumed negligible. In the experimental design, two code levels (1, −1) for each factor (EQ, COL, and block) were used, instead of 1 and 2, respectively.

Table 2 shows the estimated effects for response variables k^{g}, k, and k_{R}. As can be seen, the major factor affecting the retention factors values was the equipment in all cases, although the effect of the column and day (~AB + block) cannot be ignored. In general, k^{g} was the response variable showing the most accused effects ($k^{g} > k > k_{R}$). For k^{g} and k, the estimated effects increased as retention of compounds increased (retention order: salicylic acid > aniline > 2,4-dimethylphenol). In the case of k_{R}, aniline (the compound with a retention time closest to the one of acetanilide) was the compound showing the lowest effects. On the other hand, k showed the highest uncertainties expressed as confidence intervals ($1/2\ CI_{95\%}$: $k > k^{g} > k_{R}$) except for 2,4-dimethylphenol, the most retained compound ($k \sim k_{R} > k^{g}$). The highest uncertainties of k data could be attributed

Table 2
Estimated effects of response variables k^g, k, and k_R for compounds studied

Variable	Factor	Effect ± 1/2 $CI_{95\%}$[a]		
		Salicylic acid	Aniline	2,4-Dimethylphenol
k^g	Average	2.41 ± 0.02	7.56 ± 0.03	22.5 ± 0.2
	AB + block (∼DAY)	0.29 ± 0.04	0.70 ± 0.05	2.5 ± 0.4
	A: EQ	−1.14 ± 0.04	−3.53 ± 0.05	−9.9 ± 0.4
	B: COL	0.29 ± 0.04	1.27 ± 0.05	4.6 ± 0.4
k	Average	3.21 ± 0.06	9.99 ± 0.04	29.9 ± 0.4
	AB + block (∼DAY)	0.07 ± 0.11[b]	−0.02 ± 0.09[b]	0.6 ± 0.9[b]
	A: EQ	−0.39 ± 0.11	−1.24 ± 0.09	−2.7 ± 0.9
	B: COL	−0.36 ± 0.11	−0.70 ± 0.09	−0.9 ± 0.9[b]
k_R	Average	3.149 ± 0.016	9.356 ± 0.013	27.0 ± 0.5
	AB + block (∼DAY)	−0.08 ± 0.03	0.04 ± 0.03	0.9 ± 0.9[b]
	A: EQ	0.25 ± 0.03	−0.06 ± 0.03	−1.2 ± 0.9
	B: COL	−0.33 ± 0.03	0.06 ± 0.03	1.3 ± 0.9

ANOVA screening design: 2^2 + block, two replicates; factors: A (EQ, equipment); B (COL, column); block (DAY); AB (interaction of factors A and B).
[a] 95% confidence intervals based on total error with four degrees of freedom ($t = 2.77645$).
[b] Non-statistically significant.

to the two hold-up time estimations (t_M^g and t_M) involved in k calculations. The large 1/2 $CI_{95\%}$-k values explain that some of the effects were statistically non-significant.

Recently, Martens et al. [12] proposed PLS regression as an alternative strategy to ANOVA for checking the significance of predictor variables ($\boldsymbol{X}$, factors) in the prediction of a response variable ($\boldsymbol{y}$), based on crossvalidation/jack-knifing. Fig. 3 shows the estimated PLS regression coefficients (b) for the first two latent variables (optimal number of latent variables, LVs, explaining ∼99% of $\boldsymbol{y}$-variance) of $\boldsymbol{X}$-variables—day (DAY, here used as a variable), equipment (EQ), and column (COL)—together with their error range ($\pm 2s_b$; [12]). For 2,4-dimethylphenol the regression coefficients for the first latent variable of k are shown

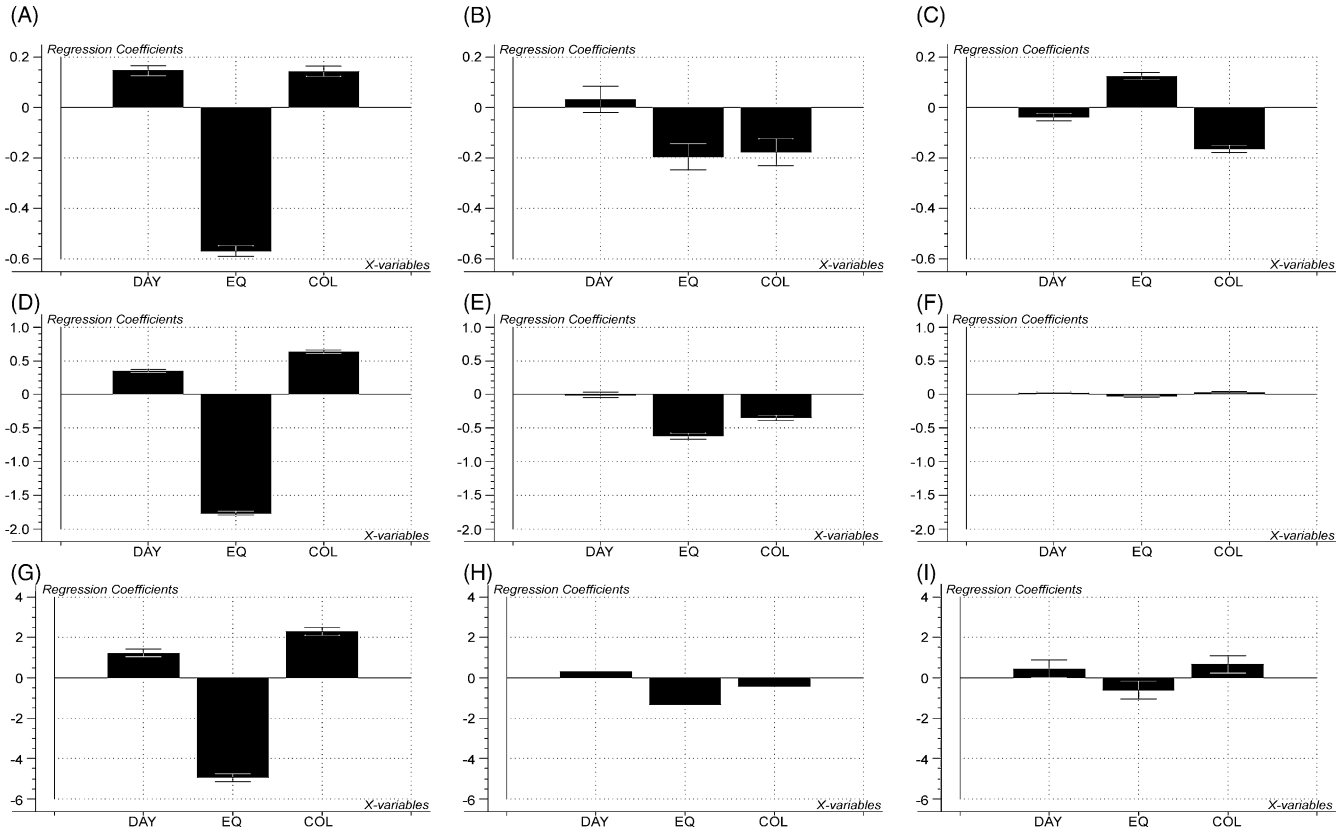

Fig. 3. PLS analysis of effects in screening designs. Regression coefficients (b) for the two-latent variable PLS models with reliability ranges ($\pm 2s_b$). $\boldsymbol{X}$-matrix variables: DAY, EQ (equipment), and COL (column). Response $\boldsymbol{y}$-variables: (A, D, G) k^g, (B, E, H) k, and (C, F, I) k_R. Test compounds: (A–C) salicylic acid, (D–F) aniline, and (G–I) 2,4-dimethylphenol.

because the two LVs-PLS model did not converge. As can be observed, the conclusions derived from the PLS model are close to those derived from ANOVA (PLS-coefficients ~1/2 ANOVA effects and PLS-error interval ~1/2 $CI_{95\%}$). The agreement between both strategies, PLS and ANOVA, improves the reliability of the estimations. In conclusion, we may expect that k_R (approach 3) provides the most reliable estimations of retention factors in view of working under intermediate precision conditions, since it is the response variable less affected by the effects studied and/or with narrower confidence intervals.

3.2.2. Study of the mobile phase flow rate

The mobile phase flow rate is not expected to be an important factor on retention factor estimations. However, an independent experiment was performed to verify this statement. For this assay, salicylic acid, aniline, and 2,4-dimethylphenol were injected under the DAY-2, EQ-2, and COL-2 working conditions at two different mobile phase flow rates (1 and 1.5 ml min^{-1}). Two non-consecutive replicates per compound were performed and acetanilide was injected at the beginning, during, and at the ending of each working session to calculate k_R. Measurements of t_{ext} were performed at the ending of every working session.

The corresponding k^g, k, and k_R values for each compound were calculated and their resultant means obtained for each flow rate were compared (compound to compound and retention factor estimation to retention factor estimation) by the adequate hypothesis t-tests. To select the adequate t-test for means, their corresponding variances were previously compared by F-tests. k^g and k showed in some cases statistically significant differences between the means obtained from each flow rate ($P = 0.02$ for k of salicylic acid; $P = 0.046$ and 0.04 for k^g and k of aniline, respectively). Changes in retention factor due to flow rate effect could be caused by changes in pressure [13].

In contrast, k_R was the only variable having statistically comparable means at 95% confidence level ($P > 0.05$; unaffected by the change in the flow rate) for all compounds. Therefore, k_R was the most robust retention factor estimation when working at several mobile phase flow rates.

3.3. Precision statistics

Precision statistics as repeatability (s_r) and run-different intermediate (s_i) standard deviations, as well as their relative standard deviations (R.S.D.$_r$ and R.S.D.$_i$, respectively) can be calculated from one-way ANOVA by means of the following equations [10,11]:

$$s_r = \sqrt{MS_{w\text{-group}}} \quad (3)$$

$$s_{run} = \sqrt{\frac{MS_{b\text{-group}} - MS_{w\text{-group}}}{N_r}} \quad (4)$$

$$s_i = \sqrt{s_r^2 + s_{run}^2} \quad (5)$$

where $MS_{w\text{-group}}$ and $MS_{b\text{-group}}$ are the within-group and between-group mean square values of ANOVA table, respectively, s_{run} the between-run standard deviation, and N_r is the number of replicates performed in every run. When $MS_{w\text{-group}}$ is higher than $MS_{b\text{-group}}$, s_{run} is usually accepted to be equal to 0 and therefore $s_i = s_r$.

Besides salicylic acid, aniline, and 2,4-dimethylphenol, two hydrophilic (atenonol and caffeine) and two hydrophobic compounds (pyrene and flunarizine) were included in this study in order to account with the hydrophobicity factor (i.e. precision-log k study). In this case, hydrophilic compounds were only chromatographed in COL-1 (150 mm; to avoid extreme low t_R^g) and hydrophobic compounds in COL-2 (50 mm; to avoid extreme high t_R^g) in different days and both equipments (only equipment and day were varied between runs). Two non-consecutive replicates per compound in each run were performed. For salicylic acid, aniline, and 2,4-dimethylphenol three independent calculations were performed, using the data corresponding to COL-1, COL-2, and both columns.

Figs. 4 and 5 show the precision statistics of k^g, k, and k_R retention factor data estimations versus $\log k^g$, $\log k$, and $\log k_R$, respectively. As can be observed in Fig. 4A, the repeatability standard deviation increased as hydrophobicity increased. For hydrophilic compounds, all approaches showed low s_r values. However, k data showed the highest s_r values, the worst repeatability, for all compounds mainly for hydrophobic compounds. It might be due to the two hold-up time measurements involved in k calculations. The run-different intermediate standard deviation values (Fig. 4B) also increased with the hydrophobicity being in all cases $s_i(k^g) > s_i(k) \geq s_i(k_R)$ as it could be expected according to the study of effects on retention factor data estimations (Section 3.2).

Regarding the relative standard deviation for repeatability, k data showed the highest R.S.D.$_r$ values (<3.5%) followed by k^g data (<3%) and finally k_R (<2%), which showed the best repeatability in all cases. Fig. 5 shows the relative standard deviation for intermediate precision. For salicylic acid, aniline, and 2,4-dimethylphenol three independent R.S.D.$_i$ values were calculated for each compound using retention factor data from COL-1 or COL-2 or using the data of both columns. As can be expected, the k^g estimations showed the highest R.S.D.$_i$ values (up to 50%). For k_R data, a parabolic dependence between the R.S.D.$_i$ values and $\log k_R$ was observed. The highest R.S.D.$_i$ values were found at the outskirts of the parabola (mainly for hydrophilic compounds, R.S.D.$_i$ < 18%) and the lowest for aniline (the compound with the closest retention to acetanilide). The R.S.D.$_i$ values for k data showed a similar behavior and values to k_R data.

In conclusion, k_R data globally showed the best precision statistics (repeatability and intermediate precision), therefore

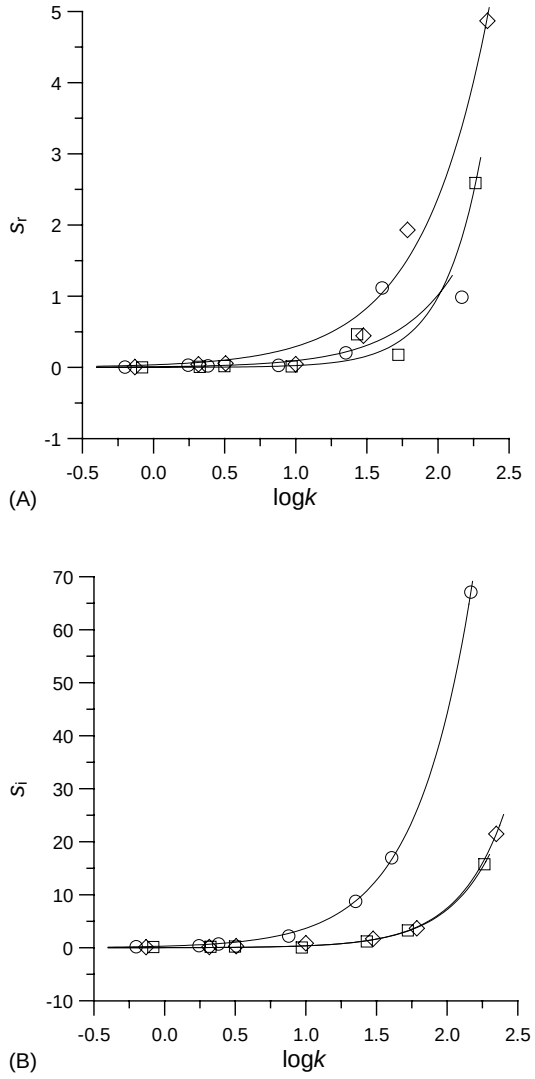

Fig. 4. Precision statistics vs. $\log k$ for k^g (○), k (◇), and k_R (□) retention factor estimations: (A) Repeatability standard deviation (s_r) and (B) run-different intermediate standard deviation (s_i).

it can be considered the most reliable strategy for retention factors estimations.

3.4. Uncertainty estimations of k_R data (approach 3)

In the present case study, whose result is an estimation of retention factors in liquid chromatography, the main source of uncertainty of k_{Ri} is assumed to be the precision of the analytical procedure. Other possible sources of uncertainty associated to k_{Ri} are either neglected mainly respect to the contribution of the intermediate precision or are not quantifiable (i.e. the typical contribution related to the assessment of accuracy is not quantified because of the lack of reference retention factors for acetanilide or the test compounds).

If every compound is analyzed in N_{run} different runs and, in each run N_r replicates are carried out, an estimation of the uncertainty ($U_{\bar{\bar{k}}_{Ri}}$) of the grand mean, $\bar{\bar{k}}_{Ri}$, of these measurements is given by the following expression [11]:

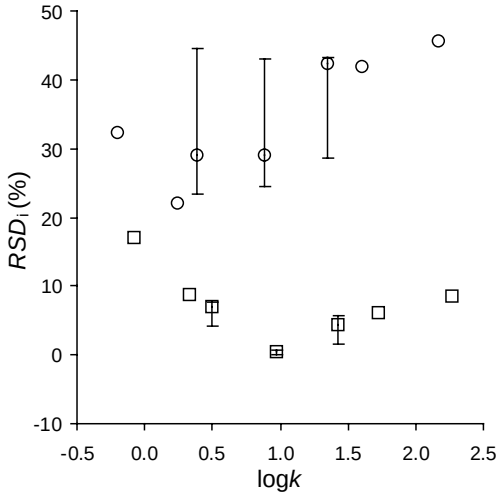

Fig. 5. Relative standard deviation for intermediate precision conditions (R.S.D.$_i$) vs. $\log k^g$ (○) and $\log k_R$ (□). For salicylic acid, aniline, and 2,4-dimethylphenol, the median values are shown and the error bars represent the extreme values.

$$U_{\bar{\bar{k}}_{Ri}} \approx 2\sqrt{\frac{s_{run}^2}{N_{run}} + \frac{s_r^2}{N_r}} \quad (6)$$

where 2 is an approximation of $t_{\alpha/2,\nu_{eff}}$, the two-sided t_{eff}-tabulated value at a given α probability and ν_{eff} degrees of freedom and s_{run} and s_r are obtained as depicted in Section 3.3. Normally, the retention factor of a compound is obtained in a given working condition (one run, $N_{run} = 1$) as the mean of two or three replicates (in the present study $N_r = 2$). Therefore, to calculate the uncertainty associated to future measurements $N_{run} = 1$ and $N_r = 2$ must be introduced in Eq. (6).

Uncertainties of $\bar{\bar{k}}_{Ri}$ values for test compounds (atenolol, caffeine, salicylic acid, aniline, 2,4-dimethylphenol, pyrene, and flunarizine) were estimated by means of Eq. (6). For salicylic acid, aniline, and 2,4-dimethylphenol three independent uncertainties were calculated for each compound using k_R values obtained from COL-1 or COL-2 or using the data of both columns.

Fig. 6A displays an exponential $U_{\bar{\bar{k}}_R}$ –$\log \bar{\bar{k}}_R$ relationship where $U_{\bar{\bar{k}}_R}$ values remain lower than 0.5 from low to medium retained compounds ($\log k_R < 1$) and then increases as $\log k_R$ increases (mainly for $\log k_R > 2$).

As shown in Fig. 6B, a parabolic ($U_{\bar{\bar{k}}_R}/\bar{\bar{k}}_R$)–$\log \bar{\bar{k}}_R$ relationship was found. The highest $U_{\bar{\bar{k}}_R}/\bar{\bar{k}}_R$ values were found at the extremes of the parabola (mainly for the most hydrophilic compound, $U_{\bar{\bar{k}}_R}/\bar{\bar{k}}_R \sim 0.35$). The compound with the closest retention to acetanilide was found at the vertex of the parabola (the lowest $U_{\bar{\bar{k}}_R}/\bar{\bar{k}}_R$). The equation of the fitted model was:

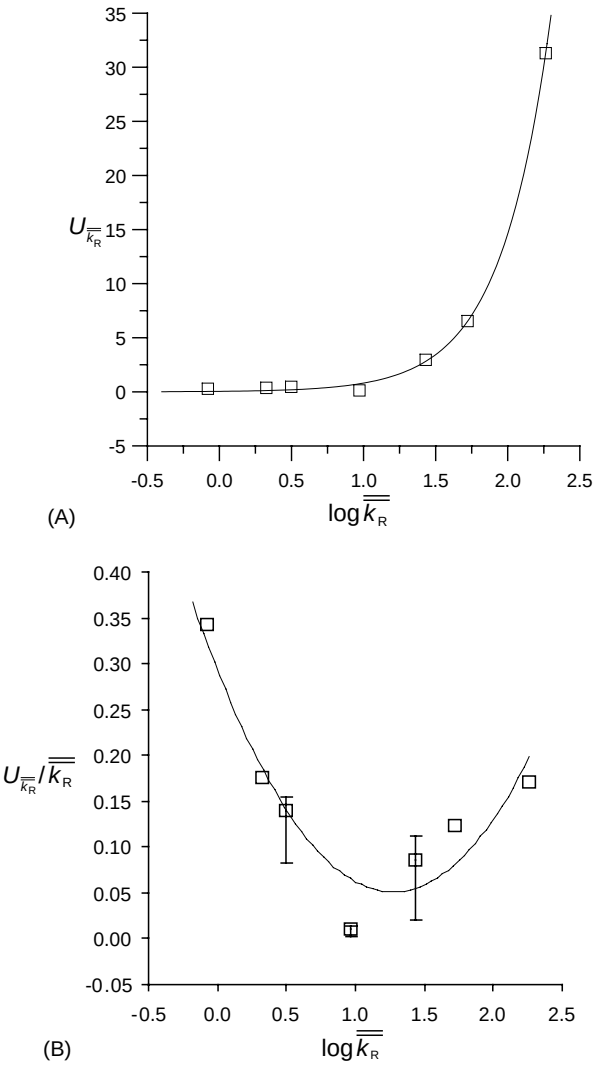

Fig. 6. (A) Overall uncertainty of $\bar{\bar{k}}_R$ ($U_{\bar{\bar{k}}_R}$) and (B) relative uncertainty ($U_{\bar{\bar{k}}_R}/\bar{\bar{k}}_R$) vs. $\log \bar{\bar{k}}_R$. For salicylic acid, aniline, and 2,4-dimethylphenol, the median values are shown and the error bars represent the extreme values.

$$\frac{U_{\bar{\bar{k}}_R}}{\bar{\bar{k}}_R} = (0.15 \pm 0.08)(\log \bar{\bar{k}}_R)^2 - (0.38 \pm 0.19) \log \bar{\bar{k}}_R + (0.29 \pm 0.09) \quad (7)$$

where $N = 7$, $S.E. = 0.04$, $r^2 = 0.89$, $r^2_{adj} = 0.84$, $F = 16.24$, and $P = 0.012$

This equation can be used to estimate $U_{\bar{\bar{k}}_R}/\bar{\bar{k}}_R$ and $U_{\bar{\bar{k}}_R}$ for new compounds chromatographed in a given working condition by duplicate.

4. Conclusions

Retention factor estimations based on the use of an external standard (k_R, approach 3) have proven to be more precise than classical retention factor estimations (k^g, approach 1), even when the extra-column time correction is introduced (k, approach 2), under the intermediate precision conditions assayed (different days, equipments, column lengths, and mobile phase flow rates). Classical retention factor estimations can provide unacceptable values if cautions to minimize the extra-column time are not taken. The inclusion of the extra-column time into the retention factor calculations does not avoid the tedious, difficult, and imprecise measurements of the gross hold-up times on the chromatograms. In addition, the extra-column time measurement, which has the same drawbacks of the gross hold-up times, must be performed at least for every chromatograph and for every change performed in the configuration or in the connections of the chromatograph.

In contrast, once established a reference retention factor value under optimal conditions, k_R calculation (Eq. (2)) only involves the measure of the gross retention times for the test and reference compounds. Therefore, 'relative' retention factor estimation does not require the estimation of hold-up time and extra-column time values, which are the cause of poor reliability and largely contribute to the uncertainty in the retention factor estimation.

On the other hand, PLS has proven to be an attractive alternative to ANOVA for the evaluation of effects involving screening designs. Since PLS is a graphical approach, their results are more illustrative than those derived from ANOVA.

Finally, the use of 'relative' retention factor estimations ensures the reliability of long-term studies (collection of data along the time), where changes in the chromatographic system (i.e. extra-column time, column lengths) can occur. In addition, k_R should correct small changes in mobile phase preparation or packing material differences between commercially available columns. On the other hand, the gross retention time of the reference compound can be used to check the condition and establish the life-time of columns. All these features are of great importance, mainly in those studies that use retention factor as a response or descriptor variable, such as in quantitative structure–retention relationships and quantitative retention–activity relationships.

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Appendix A

ANOVA	analysis of variance
b	regression coefficients of PLS model

Brij35	polyoxyethylene(23)lauryl ether
$CI_{95\%}$	confidence interval at 95% probability level
COL	column
EQ	equipment
F	F-ratio (residual to modeled variance ratio)
F-test	hypothesis test for comparison of variances
k	retention factor ($k = (t_R^g - t_M^g)/(t_M^g - t_{ext})$)
k^g	gross retention factor ($k^g = (t_R^g - t_M^g)/t_M^g$)
k_R	'relative' retention factor (retention factor for new compounds calculated from k_{REF} by means of Eq. (2))
k_{REF}	reference retention factor ($k_{REF} = (t^g_{R(REF)} - t_M^g)/t_M^g$)
$\bar{k}$	mean of the replicate measurements performed on a given run
$\bar{\bar{k}}$	grand mean (mean of the mean values obtained on the different runs)
$\log P$	logarithm of the octanol/water partition coefficient
LV	latent variable of PLS model
$MS_{b\text{-group}}$	between-group mean square of ANOVA
$MS_{w\text{-group}}$	within-group mean square of ANOVA
N	number of data included in a model
N_r	number of replicates per run
N_{run}	number of runs (working conditions)
PLS	partial least squares
P-value	probability, measure of significance of a hypothesis test, ANOVA or a model
QRARs	quantitative retention activity relationships
QSRRs	quantitative structure retention relationships
r^2	correlation coefficient
r^2_{adj}	correlation coefficient adjusted for degrees of freedom
R.S.D.	relative standard deviation
$R.S.D._i$	relative standard deviation for intermediate precision
$R.S.D._r$	relative standard deviation for repeatability
s	standard deviation
s_b	standard deviation of b-coefficients of PLS regression
S.E.	standard error of estimate of a model
s_i	run-different intermediate standard deviation
s_r	repeatability standard deviation
s_{run}	between-run standard deviation
$t_{\alpha/2,\nu_{eff}}$	two-sided t_{eff}-tabulated value at a given α probability and ν_{eff} degrees of freedom
t_{ext}	extra-column time
t_M	hold-up time ($t_M = t_M^g - t_{ext}$)
t_M^g	gross hold-up time
t_R	retention time ($t_R = t_R^g - t_{ext}$)
t_R^g	gross retention time
$t^g_{R(REF)}$	gross retention time of reference compound
t-test	hypothesis test for comparison of means
U	overall uncertainty

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