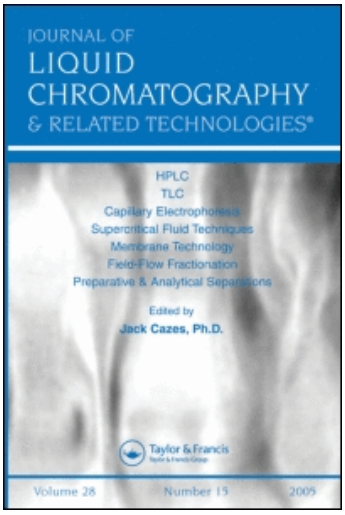

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ABSTRACT

This study presents an example of method validation, applied to an IIR-RP-HPLC method, developed for the separation of nine PPPs (Priority Pollutant Phenols). The parameters of both primary validation (accuracy, precision, response linearity, detection. and quantitation limits,) and of secondary validation (method ruggedness) are evaluated. The glossary used in literature for *ruggedness* and *robustness* is discussed. The *robustness* test is carried out with respect to five chromatographic conditions, both depending on analyst accuracy (concentration of the ion-interaction reagent, concentration of the organic modifier, and pH of the mobile phase) and on instrumentation (column temperature and flow-rate).

The effect of the experimental factors is studied by a fractional factorial design and mathematical models are built, that correlate the chromatographic retention to the experimental factors and to their interactions. The cross-validated models can usefully be employed to evaluate for each studied variable the region of acceptable *ruggedness* for any given confidence level.

INTRODUCTION

Each new analytical method must be validated, that means that its ability to give reliable results must be proved. Only a method that has undergone a validation process can be easily transferred to other laboratories (1–10).

The so called primary validation is usually always performed when developing a new method, and consists mainly of evaluating accuracy, sensitivity, specificity, precision, range linearity, detection, and determination limits (11–13).

Besides the primary validation process, a second process of validation must be performed that mainly consists in the evaluation of the ruggedness method. The *ruggedness* of an analytical procedure is defined as "its capacity to yield exact results in the presence of small changes of the experimental conditions, such as might occur during the utilisation of the procedure," where a "small change" is "any deviation of a parameter of the procedure compared to its nominal value, as described in the method of analysis (8)". The possible deviations from the nominal values (identified in the process of development and optimisation) can not be avoided or controlled because they are due to natural variations of the whole system. They could be, for instance, due to inaccuracy of the analyst, to deviations of the instrumental performances, to a low stability of the reagents, to variations of the atmospheric conditions in the laboratory, etc. and in general, to all the possible indeterminate system and laboratory errors that can affect the results of analysis.

In the most recent literature, a unified terminology for this property of the method does not exist. *Ruggedness* of a method is, for example, defined as "an intra-laboratory experimental plan, used before undertaking an inter-laboratory study, to examine the behaviour of an analytical process when small changes in the environmental and/or operating condition are made, akin to those likely to arise in different laboratories (14)." Other authors refer to the same property as *robustness* (15), and others indicate the two terms as equivalent to each other (16).

In this paper, we choose to use the terminology proposed by Jenke (17), in accordance with which *ruggedness* is the "reproducibility of test results obtained by the analysis of samples under a variety of normal test conditions such as different laboratories, analysts, instruments, reagent lots, elapsed assay time, temperature, etc. Thus, *ruggedness* addresses *unintentional* variation in the method introduced by its application, at different times by different people at different locations using different instrumentation and materials".

In turn, the *robustness* is "a measure of a procedure's capacity to remain unaffected by small but deliberated variations in method parameters and, thus, is a measure of the procedure's reliability during normal usage (17)." So, we perform *robustness* tests to evaluate the *ruggedness* of a method.

Robustness tests must be applied to any new method and often represents an intra-laboratory test that is essential for easy transferability of the method to

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other laboratories. Up to now, no uniform *robustness* testing procedure has been defined and the approaches proposed by different authors (1,20–28) depend on the requirements of the method.

Of primary importance is the identification of the response that must be tested; for instance, the main aim of a chromatographic method could be to achieve the best resolution of the components of a mixture, or the best sensitivity, or a short analysis time. Then, the variables that mainly affect the response are selected and to these variables are intentionally imposed known and prefixed variations.

Among the different applications of the LC technique, ion-interaction reagent chromatography (IIR-HPLC) is particularly suitable to simultaneously separate (without cumbersome sample pre-treatments or derivatization reactions) cationic, anionic, and neutral species (29–34). Solute retention is affected by a number of factors (chemical properties and concentration of the ion-interaction reagent, concentration of the organic modifier, and pH of the mobile phase, temperature) and a careful tuning of all of them makes the technique very versatile to solve many chromatographic separations.

In this work, a *robustness* test is performed to evaluate the *ruggedness* of a RP-IIR-HPLC method for the separation of nine priority pollutant phenols (35) towards possible variations of five experimental factors (the IIR concentration, the organic modifier concentration, the mobile phase pH, the column temperature, and the elution flow-rate).

In order to consider the simultaneous effect of all the variables involved, multivariate methods of experimental designs are used.

EXPERIMENTAL

Apparatus

HPLC analyses were carried out with a Merck-Hitachi (Tokyo, Japan) Lichrograph Chromatograph Model L-6200, equipped with a two-channel D-2500 Chromato-integrator and interfaced with a UV-visible detector L-4200 of the same firm.

A Metrohm 654 pH-meter (Switzerland) equipped with a combined glass-calomel electrode was employed for pH measurements and a Varian Cary 1E UV-Vis spectrophotometer for absorbance measurements.

Chemicals and Reagents

Ultrapure Milli-Q water (Millipore) was used for the preparation of solutions. Hexylamine was Fluka analytical grade chemical. Ortho-phosphoric acid was C. Erba chemical and acetonitrile BDH analytical grade chemical.

Phenol, 2-nitrophenol, 4-nitrophenol, 2,4-dinitrophenol, 2-chlorophenol, 3-chlorophenol, 2,4-dichlorophenol, 2,4-dimethylphenol, and 4-chloro-3-methylphenol were supplied by Supelco chemicals.

Ion-interaction RP-HPLC Analysis

The technique makes use of a Merck Superspher 100 RP-18, 4 μm, (250 $\times$ 4 mm), fully endcapped, reversed-phase stationary phase and of a Lichrospher RP-18, 5 μm (50 $\times$ 4 mm) pre-column.

The chromatographic system is conditioned by passing, under isocratic conditions, the mobile phase through the column until a stable baseline signal is reached and reproducible retentions are obtained for three subsequent injections (about an hour, at flow-rate of 1.0 mL/min, is usually enough). After use and between the use of the different mobile phases employed, the column is washed by flowing water (0.50 mL/min, 15 min), 50/50 v/v water/acetonitrile mixture (flow-rate 0.5 mL/min, 30 min), and 100% acetonitrile (0.50 mL/min, 5 min).

The mobile phases required to perform the experiments planned in the experimental design are prepared with different combinations of C_M (acetonitrile % concentration), C_{IIR} (hexylamine molar concentration), and pH, by adding to the required water/acetonitrile solution of hexylamine the amount of o-phosphoric acid to obtain the desired operational pH value.

Factorial Design

The chemometric treatment of the experiments planned according to a 2-level factorial design, allows the evaluation of the effect of the experimental factors and of their interactions. The full factorial design (36–38) contains all the possible combinations of the 2 levels of the p experimental factors, so that a 2-level full factorial design requires 2^p experiments. Fractional factorial designs are more often used, since they require less experiments than the correspondent full factorial ones; but the advantage of the lower number of experiments is offset with the loss of some information, under the form of aliasing between some of the calculated effects. The statistically relevant factors can be selected on the basis of a t test, the uncertainty of the effect being:

$$s_b^2 = \frac{4 \cdot s_{pe}^2}{n_F} \tag{1}$$

where $s^2{}_{pe}$ is the variance of the experimental error, estimated in our case as the pooled experimental variance (36), and n_F is the total number of the experiments performed (taking also into account the replications).

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RESULTS AND DISCUSSION

The separation of the nine considered phenols can be considered a critical analysis and, for the point of view of both resolution and sensitivity, can be used as a System Suitability Test (1,10–17). The method, already optimised and validated for the primary validation parameter in a previous work (35) for a Merck RP-18 LichroCart (250 × 4 mm) column, was easily transferred to a Merck Supersphere 100 RP-18 (250 × 4 4 μm) fully end-capped reversed phase stationary phase (that is considered to offer advantages of greater reproducibility and time stability).

The results indicate the *ruggedness* of the method with respect to the two material packings. The mobile phase is a 2.00 mM solution of hexylamine o-phosphate in water/acetonitrile 67/33 v/v brought to pH 4.0 for o-phosphoric acid, flowing under isocratic conditions at 1.0 mL/min. The temperature is 30°C and the spectrophotometric detection is performed at 285 nm (the average wavelength at which all the analytes show appreciable absorbance values).

Figure 1 presents the separation of the nine phenols: a) phenol (1.0 mg/L), b) 4-nitrophenol (0.2 mg/L), c) 2,4-dinitrophenol (0.2 mg/L), d) 2-chlorophenol (5.0 μg/L), e) 2-nitrophenol (0.2 mg/L), f) 3-chlorophenol (3.0 μg/L), g) 2,4-dimethylphenol (0.5 mg/L), h) 4-chloro-3-methylphenol (1.0 mg/L), and i) 2,4-dichlorophenol (1.0 mg/L) under these conditions.

Primary Validation Parameters

The accuracy of the IIR-HPLC method has been previously checked (35) with respect to the standard EPA GC-FID (39) method and to a modified GC-MS method proposed by the Public Health Association Standard Methods (40), by performing inter-calibration statistical tests both parametric (t-test, F-test, paired t-test, t-test with multiple samples) (41–42) and non parametric (Mann-Whitney U-test and Wilcoxon matched-pair signed-rank test) tests (42).

The reproducibility of retention times, evaluated as the standard deviation of 5 different preparations of the mobile phase in the nominal conditions, always resulted within 3%. The reproducibility in peak area, evaluated for concentrations of 0.20 mg/L for each analyte, is always within 5%.

In order to verify the linearity of the response (peak area vs concentration) for each analyte, calibration plots are built reporting the peak area (relative units as given by the integrator) vs. standard concentration (at least five values). The solutions were injected as a function of increasing concentration, in order to overcome possible memory effect. The concentrations range between the determination limit and 5.00 mg/L. The regression parameters and the correlation coefficients R^2 (always >0.9885) are reported in Table 1.

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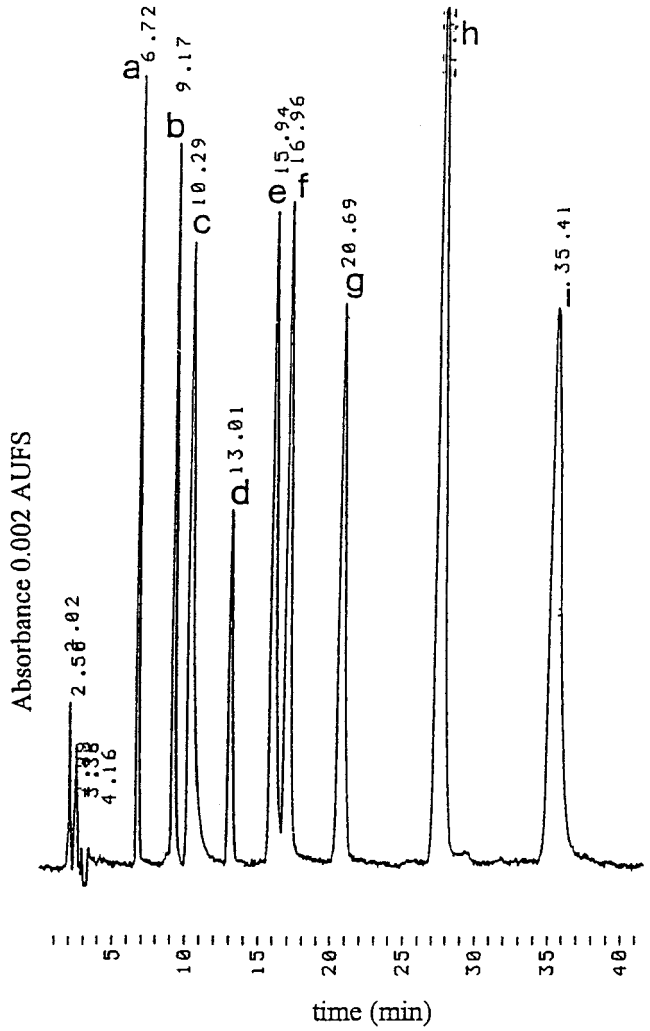

Figure 1. Optimised separation of the mixture of the following nine priority pollutants phenols, at the indicated concentrations: *a*) phenol (1.0 mg/L), *b*) 4-nitrophenol (0.2 mg/L), *c*) 2,4-dinitrophenol (0.2 mg/L), *d*) 2-chlorophenol (5.0 μg/L), *e*) 2-nitrophenol (0.2 mg/L), *f*) 3-chlorophenol (3.0 μg/L), *g*) 2,4-dimethylphenol (0.5 mg/L), *h*) 4-chloro-3-methylphenol (1.0 mg/L) and *i*) 2,4-dichlorophenol (1.0 mg/L). Conditions: Stationary phase: reversed-phase Superspher column (250 $\times$ 4 mm), 4 μm fully end-capped. mobile phase: 2.00 mM solution of hexylamine o-phosphate in water/acetonitrile 67/33 v/v brought at pH 4.0 for o-phosphoric acid. Flow-rate 1.0 mL/min. Column temperature 30°C. Spectrophotometric detection at 285 nm. Injection volume 100μL.

Table 1. Calibration Plot Equations: Peak Area (y, Relative Units) vs. Standard Concentrations (x, mg/L), Correlation Coefficients R^2, Detection Limits LOD (μg/L) and Determination Limits LOQ (μg/L) in the Optimised Conditions as in Figure 1

Analyte	Calibration Plot	R^2	LOD μg/L	LOQ μg/L
phenol	y = 45613.18 x + 135.94	0.9953	47.4	151.1
4-nitrophenol	y = 85646.23 x + 795.99	0.9839	24.3	75.5
2,4-dinitrophenol	y = 324139.52 x + 486.41	0.9804	9.3	30.2
2-chlorophenol	y = 2.9812E7 x + 143.79	0.9976	0.1	0.5
2-nitrophenol	y = 148302.6 x + 121.05	0.9885	14.0	45.5
3-chlorophenol	y = 3.1506E7 x + 375.78	0.9938	0.1	0.4
2,4-dimethylphenol	y = 56610.48 x + 353.4	0.9950	36.8	102.5
4-chloro-3-methylphenol	y = 45551.67 x + 365.85	0.9913	47.5	148.2
2,4-dichlorophenol	y = 76590.52 x + 377.62	0.9987	27.2	75.8

In order to express the sensitivity (given as the peak area for 1.0 mg/L concentration as given by the slopes of the calibration plots) into concentration units, in the chromatogram, an area which corresponds to a signal to noise ratio around 3 is identified and used to proportionally transform sensitivity into the LOD values in concentration units (ppm). LOD values, reported in Table 1, are always lower than 50 μg/L and, in particular for 2- and 3-chlorophenol, they are close to the threshold concentration of phenols in waters (0.1 μg/L).

Quantitation limits (LOQ_s) (Table 1) are evaluated by the calibration plots as the concentration that can easily be quantified and are around a signal to noise ratio equal to 10.

Secondary Validation

The factors that, on the basis of previous results (33,43,44), mostly affect the retention in IIR mode and are here selected for the *robustness* test are both: i) parameters that depend on the manual skill of the operator as the concentration of the ion-interaction reagent C_{IIR}, the organic modifier percentage in the mobile phase C_M, the pH of the mobile phase ii) parameters that are automatically set by the instrumentation, as the column temperature T and the elution flow rate F.

The ranges of variation of the five experimental factors, with respect to the optimised (nominal) values, are selected by designating two extreme levels of the factors that must be larger than the changes that would be expected under normally changing conditions. The two levels were fixed by imposing ±20% variations (surely higher than those that can naturally occur), with respect to the nominal conditions (Table 2a).

Table 2a. Nominal, Low and High Values for Ion-interaction Molar Concentration (C_{IIR}), CH_3CN %, Concentration (*CM*) in the Mobile Phase, Mobile Phase *pH*, Temperature *T* and Elution Flow-rate *F*

Factor	Nominal Value	Low Value (−20%)	High Value (+20%)
C_{IIR} (mM)	2.0	1.6	2.4
CM (%CH_3CN)	33	26	40
pH	4.0	3.2	4.8
T (°C)	30	24	36
F (mL/min)	1.0	0.8	1.2

To evaluate the effect of the five variables, the test is first performed by a 2 (5–2) fractional factorial design, that requires 8 experiments, instead of the 32 required by the corresponding full factorial design. The experimental conditions are reported in Table 2b.

The centre of the experimental domain, i.e. the experiment that corresponds to the nominal experimental settings (experiment n.0), was replicated 5 times to obtain an estimate of the experimental error. Four replicates of each experiment of the fractional factorial design were performed: Table 3 reports the average retention times obtained. The results indicate that: i) retention is greatly affected by the experimental conditions, ii) resolution is generally maintained but, iii) the elution sequence order can vary as a function of the experimental conditions. In particular, it can be noticed that elution sequence variations are observed when 2,4-dinitrophenol is involved. The behaviour can be explained by its pK_a value ($pK_a = 4.1$), that is the only one comprised in the pH range (3.2–4.8) explored in the robustness study. For all the other analytes pK_a values are >7.

From the experiments of Table 2b), the following regression models, correlating the retention times to the experimental factors and to some of their second

Table 2b. Table of the Experiments Planned According to the Fractional Factorial Design

Exp.	C_{IIR} (mM)	*CM* (%)	*pH*	*T* (°C)	*F* (mL/min)
1	1.6	40	4.8	24	0.8
2	2.4	26	3.2	24	0.8
3	1.6	26	4.8	24	1.2
4	2.4	40	3.2	24	1.2
5	1.6	40	3.2	36	0.8
6	2.4	26	4.8	36	0.8
7	1.6	26	3.2	36	1.2
8	2.4	40	4.8	36	1.2

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Table 3. Results of the *Fractional Factorial Experimental Design*. Average Retention Time from 4 Replicates of the *fractional Design* (Experiments 1–8), and 5 Replicates of the Central Experiment (Experiment 0).

Exp.	Phenol	4-nitro phenol	2,4-dinitro phenol	2-chloro phenol	2-nitro phenol	3-chloro phenol	2,4-dimethyl phenol	4-chloro-3-methylphenol	2,4-dichloro phenol
0	6.56	8.90	10.12	12.66	15.53	16.37	20.04	26.46	34.02
1	6.40	7.87	5.49	10.49	13.04	12.78	15.12	18.18	22.50
2	11.54	18.49	29.01	27.29	31.60	38.52	47.75	72.36	95.70
3	8.14	13.23	10.41	19.57	22.33	27.73	34.53	52.80	69.85
4	4.47	5.57	7.89	7.67	9.30	9.30	11.06	13.57	16.96
5	6.08	7.27	9.95	9.95	12.08	11.86	14.17	16.81	20.76
6	11.28	17.47	15.49	26.76	30.39	37.29	47.75	71.41	94.25
7	7.65	11.94	18.41	18.41	20.59	25.40	32.52	48.84	64.40
8	4.05	4.77	3.57	6.42	7.85	7.76	9.18	10.85	13.36

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order interactions, were calculated:

$$
\begin{aligned}
t_{R\ \text{phenol}} &= 7.45 + 0.38\ C_{IIR} - 1.37F - 2.20\ CM - 0.19\ T \\
t_{R\ \text{p-nitrophenol}} &= 10.82 + 0.75\ C_{IIR} - 1.90\ F - 4.45\ CM - 0.46\ T \\
t_{R\ \text{2,4-dinitrophenol}} &= 12.53 + 1.46\ C_{IIR} - 2.45\ F - 5.80\ CM - 0.67 \\
&\qquad T - 3.78\ pH + 1.59\ CM^{*}pH \\
t_{R\ \text{2-chlorophenol}} &= 15.82 + 1.21\ C_{IIR} - 2.80\ F - 7.19\ CM \\
t_{R\ \text{o-nitrophenol}} &= 18.40 + 1.39\ C_{IIR} - 3.38\ F - 7.83\ CM - 0.67\ T \qquad (2) \\
t_{R\ \text{3-chlorophenol}} &= 21.33 + 1.88\ C_{IIR} - 3.78\ F - 10.90\ CM \\
t_{R\ \text{2,4-dimethylphenol}} &= 26.51 + 2.42\ C_{IIR} - 4.68\ F - 14.12\ CM \\
t_{R\ \text{4-chloro-3-methylphenol}} &= 38.10 + 3.94\ C_{IIR} - 6.59\ F - 23.25\ CM \\
t_{R\ \text{2,4-dichlorophenol}} &= 49.72 + 5.34\ C_{IIR} - 8.58\ F - 31.33\ CM
\end{aligned}
$$

The plots of the. experimental t_R vs. the t_R predicted by the models show R^2 values always >0.9916, to indicate that the cross-validated models containing the relevant factors allow a meaningful interpretation of the effects. A similar pattern of statistically relevant factors works for all the analytes. The factors are: i) the flow-rate (*F*), which shows the expected negative effect on the retention times; ii) the organic modifier concentration (*CM*) which shows a strong negative effect, according to the expected mechanism (a larger organic solvent concentration both increases the eluotropic strength of the mobile phase and decreases the extent of the column surface modification); iii) the concentration of the ion interaction reagent (C_{IIR}), that plays a positive effect on retention, since (in the studied concentration range) higher values of C_{IIR} provide a more effective modification of the column; (iv) the temperature (*T*), which exhibits a negative effect on retention times, according to the thermodynamic aspects of ion-interaction HPLC chromatography (45).

A somewhat different behaviour can be observed for 2,4-dinitrophenol model, that, in addition, shows a relevant effect of *pH* and of the interaction *CM***pH*: the behaviour is explained by the pK_a value of this analyte that is the only one that lays in the range of *pH* explored by the experimental design. Retention is therefore, affected by variations, as a function of *pH*, of the molar fraction of the analyte dissociated form.

In conclusion, for all the analytes, the models indicate that the factor which has to be set with particular care is the concentration of the organic modifier, whose effect is always the largest.

The use of the models also allows one to predict, in the variable dominion investigated, i) possible variations in the elution sequence order and ii) the *ruggedness* of the method with respect to any set of experimental factors and at any given confidence level. For each analyte, an experimental region of *ruggedness* of the

method centred on the nominal conditions can be defined by the equation:

$$(t_{R\text{analyte}}(C_{IIR}, CM, pH, T, F) - t^{\circ}_{R\ \text{analyte}})/ \leq t_{24,0.95}.s_{pe'\ \text{analyte}} \quad (3)$$

which states that the difference (absolute value) between the retention time t_R predicted by the regression model and the experimental retention time t_R^o in the nominal conditions must be lower than the critical value $t_{24,0.95}.s_{pe'\ \text{analyte}'}$ where $t_{24.0.95}$, is the t-student reference value and $s_{pe'\ \text{analyte}}$ is the pure experimental error for each analyte.

The region described by the equations (3) is hyper-ellipsoidal: the main axes are parallel to the experimental axes (C_{IIR}, F, CM and T), with the exception of 2,4-dinitro-phenol, whose axes are bent with respect to *CM* and *pH,* since these factors exhibit a relevant interaction.

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