COMPUTER-ASSISTED OPTIMISATION METHOD FOR MODELLING AND PREDICTION THE RETENTION BEHAVIOUR OF ACIDIC COMPOUNDS ON A FLUORINATED PACKING

Part 1 of this article appeared in the January 2008 issue of International Labmate and gave the background to the work carried out by the authors alongside experimental detail and a discussion of some of the Results obtained using the methodology of screening significant factors using the orthogonal array (OA)design. In Part 2 the article discussion centres on the use of Optimisations with the aid of RSM and central composite design,specifically Polynomial mathematical models of retention time and resolution and Optimisation of separation with the aid the overlapping contour plot. Conclusions are then drawn on the results obtained and presented.

OPTIMISATIONS WITH THE AID OF RSM AND CENTRAL COMPOSITE DESIGN

Polynomial mathematical models of retention time and resolution

Many mathematical models like first, second or non-linear models are utilised to describe the retention behaviour of substances on high performance liquid chromatography (HPLC) [19-23]. A second-order model was applied in this study The objective of using a central composite design was to provide enough tests to fit the second-order equations to predict the responses at any point within factors domain. For three factors, the model takes the form [24, 25]:

$$y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{11} x_1^2 + \beta_{22} x_2^2 + \beta_{33} x_3^2 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \varepsilon$$

where y is the response variables, i.e. retention times of individual compounds and resolutions between two peaks; x1 the percentage of methanol in the mobile phase, x2 pH value and x3 concentration of buffer; and β_j terms are the regression coefficients 1. The interaction between the factors is described by β_{ij}xix$_j$, and curvature by $\beta_{ii}x_{i^2}$. εi is the deviation between the fitted curve and the calculated response in design point i and n the number of design evaluations. The CCD provides information about the importance of curvature and interaction between the factors.

Table 3. Experimental matrix of the central composite design used for the separation of acidic compounds on Fluorinated stationary phase: real value of variables.

Block	Expt. no.	Conc. of MeOH (x1, % v/v))	pH (x2)	Conc. of buffer (x3, mM)
q	1	48	3	15
	2	48	3	35
	3	48	5	15
	4	48	5	35
	5	58	3	15
	6	58	3	35
	7	58	5	15
	8	58	5	35
t	9	53	4	25
	10	53	4	25
r	11	46.6	4	25
	12	59.4	4	25
	13	53	2.7	25
	14	53	5.3	25
	15	53	4	12.1
	16	53	4	37.9

The experimental runs and levels of the critical parameters are listed in *Table 3*. The value of star points, α was calculated by α2= (√nq-q)/2 [26]. (N: number of factorial runs).

The data from the experiments was examined by multivariate regression analysis by an edited MATLAB program at each design point to build the second-order polynomial model. For each model, the experimental data showed a good fit with the models because R^2 is more than 0.992 for retention time and more than 0.920 for resolution, which were statistically acceptable at the p<0.05 level.

In order to describe the geometric nature of models, 2-D and 3-D response surface diagrams are plotted to describe the relationship between responses and the independent parameters. The response plots are mapped with the vertical axes showing the responses, and each of the two horizontal axes representing parameter x1 and x2 whilst keeping x3 constant.

The 2-D response models are mapped with the plots of the test compounds where the logarithm of retention factor is used as the measure of the function against the content of methanol in the mobile phase or pH of buffer. At every plot, the two factors not represented by the horizontal axes were fixed at the constant levels (*Figure 2*.)

The 3-D response surface plot was visualised with the plot of the test compounds where retention time is used as measure of the function as against the variation of the concentration of methanol and pH value whilst keeping x3 (the concentration of buffer) at a constant level. At the same time, in order to describe the influence of x3, three surfaces are plotted for the same retention time by locating x3 at lowest, medium and highest level, respectively and they were overlapped in one diagram (*Figure 3*). The plot shows that the variation of x3 didn't affect the response surface, which indicates its influence was not significant on the retention time of acidic analytes on the PFP phase.

Figure 2 shows that the elution order for most compounds keeps the same under different mobile phase conditions. At the lower pH, all analytes were uncharged and the elution order of the neutral compounds follows the reversed-phase distribution mode according the hydrophiblity.

Therefore, protocatechuic acid was the first peak in the chromatogram and flubiprofen eluted last. The elution order for the peak of tolbutamide and indoprofen changed at pH 4-5. This may be because of the competitive influence of the factors x1 and x2 are more significant when the buffer pH increased.

Effects of parameters for retention time and resolutions

In order to elucidate the retention mechanism of acidic analytes on the PFP phase, the regression analysis from SPSS and MATLAB was applied to investigate the significance of the coefficients in the equations and to describe the relative effects of parameters on the responses. *Figure 4* shows that the responses were influenced by the terms related with x1 (the content of methanol in the mobile phase) and x2 (pH value of buffer). Among them, the linear terms of x1 and x2 were the most significant factors (*Figure 4*).

Based on the coefficient plots of the independent parameters and the mathematical models, the response models can be divided into 3 groups.

The first group includes the retention time of a1, a5 and resolution of R^2. In this group, x1 and x22 had negative effects whilst x2 had a positive effect (for a1) or no significant influence (for a5) on the responses. The competitive effect of the two parameters explains the retention behaviour of a1, a5 and elucidates the reversal elution order of tolubamide and indoprofen. For acidic analytes, interaction between the molecules with the stationary phase reduces at higher pH values. Therefore, the retention times for the test analytes should be decreased when the buffer pH increased.

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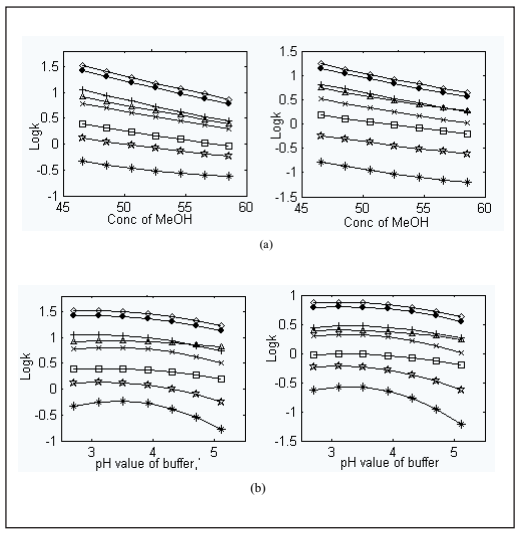

Figure 2. Response plots representing the relationship between the logarithm of the retention factor and independent variables on the FluoroSep-RP Phenyl HS packing, where log k vs. (a) content of MeOH, (b) pH of buffer by keeping the other two variables at constant level. Temperature, 38°C, flow rate 1.0 ml/min. Solute: (*) protocatechuic acid, (☆) syringic acid, (□) p-coumaric acid, (×) chlorpropamide, (Δ) tolbutamide, (+) indoprofen, (●) fenoprofen, (◊) flubiprofen.

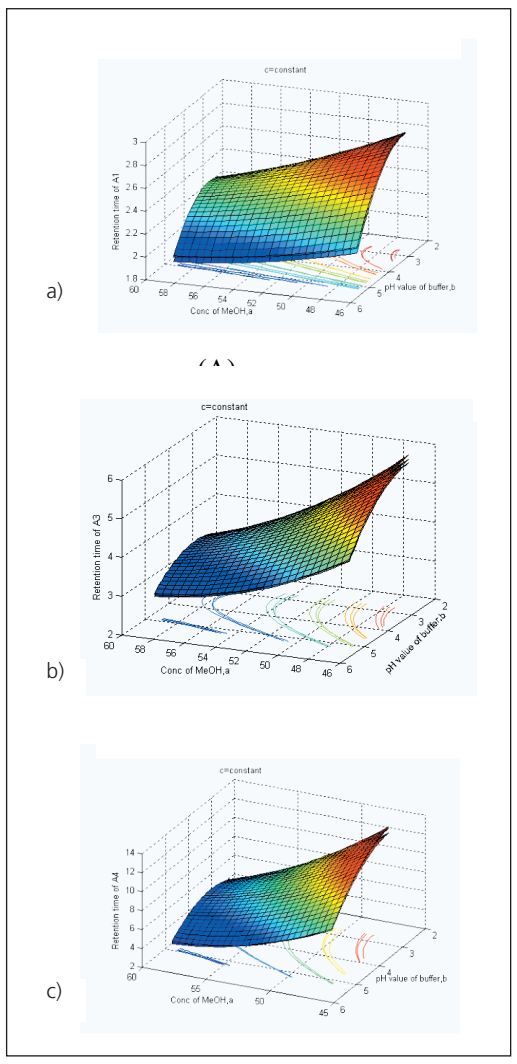

Figure 3. Response surface plots representing the retention time of (A) protocatechuic acid, (B) p-coumaric acid, and (C) chlorpropamide as a function of content of methanol and pH value of buffer by keeping x3 at 12mM, 25mM and 37mM.

> ***The data from the experiments was examined by multivariate regression analysis by an edited MATLAB program at each design point to build the second-order polynomial model***

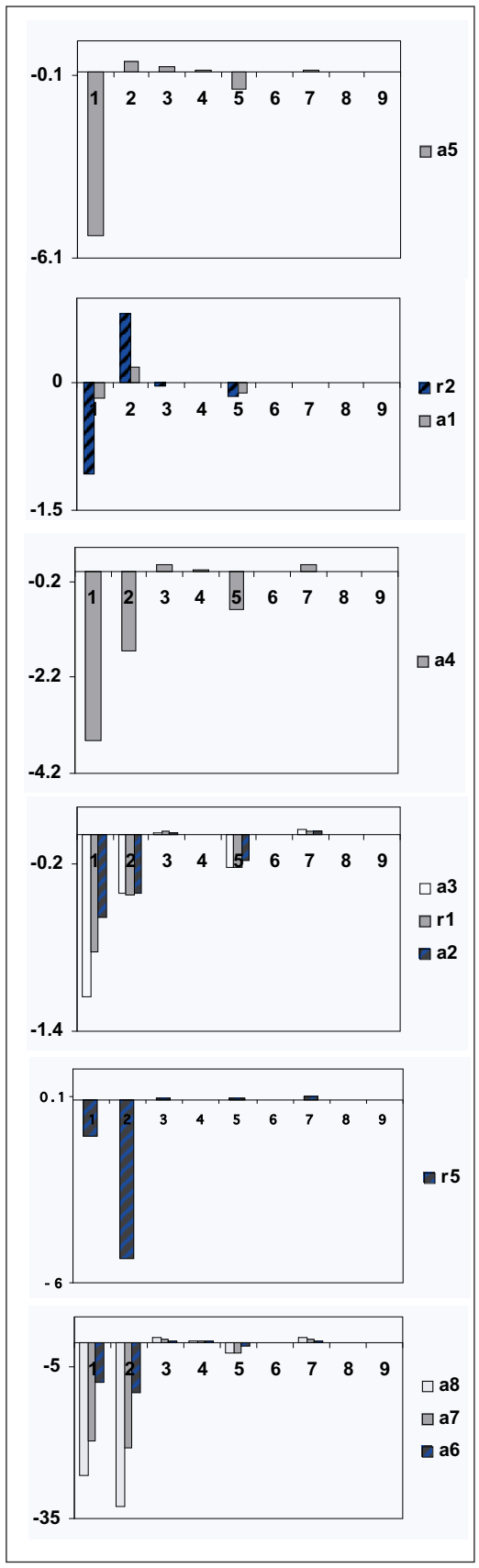

Figure 4. Coefficient plots for retention times and critical resolutions. The y-axis describes the size of coefficients in the polynomial equations. The x-axis presents the variables, where, 1:x1; 2: x2; 3: x3; 4: x1x1; 5: x2x2; 6:x3x3; 7: x1x2; 8: x1x3; 9: x2x3.

However, the retentions of a1 and a5 were the exception in this study. Because the opposite influence of x1, x22 and x2, a maximum retention time was observed for a1 when pH was between 3.5-4 (depending on the concentration of methanol). The same influence can also explain the retention order inversion of tolbutamide (a5) and indoprofen (a6). The retention of a5 was almost not influenced by the change of pH while the retention of a6 was reduced with the increase of pH, so the retention time of a6 was equal or less than that of a5 when the pH reaches to a specific value (depending on the concentration of MeOH). Thus, a reversed elution between tolbutamide (a5) and indoprofen (a6) was observed when pH value varied from 2.7 to 5.2.

For resolution R1, retention time of a2, a3 and a4 (group 2) and resolution R5 together with retention time of a6, a7, a8 (group 3), both x1 and x2 had negative effects on the responses. *Figure 2* shows that the responses in these two groups are increased by deceasing the percentage of methanol due to the lower polarity of mobile phase and the responses also decreased with increasing the pH value of the buffer.

For the responses in the group 2, the influence of x1 was more significant on the responses than the influence of parameter x2. Therefore, the polarity of the mobile phase has a dominant effect on chromatographic behaviour of solutes. So, retentions of analytes are determined predominantly by the methanol concentration in the mobile phase.

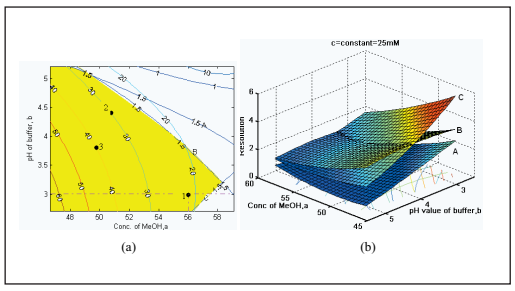

Figure 5. Superimposed contour maps (a) and response surface plot (b) representing the overlapping maps of critical resolutions as a function of content of methanol and pH value of buffer, where, protocatechuic acid/syringic acid (A), syringic acid/ p-coumaric acid (B), and tolbutamide/indoprofen (C).

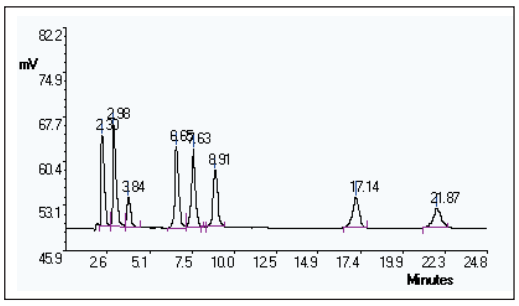

Figure 6. The chromatogram of the separation of the acidic mixture use optimal conditions from the simulation result. Column FluoroSep-RP Phenyl HS 15×4.6cm; Flow rate 1ml/min; Mobile phase was methanol-pH 3/25 mM buffer (56: 44, v/v). UV detection was at 220nm. Elutes: (according to sequence) protocatechuic acid, syringic acid, p-coumaric acid, chlorpropamide, tolbutamide, indoprofen, fenoprofen, and flubiprofen.

However, the influence of x2 was more significant than the effect of x1 on the responses in the group 3, which means the change of pH value influenced the retention of compounds in this group most.

The results from statistical analysis and diagram shows that the buffer concentration was not a critical factor for the responses, which can be proved from response surface plots. In *Figure 3*, the 3-D plot was constructed by superimposing three surfaces of the same response with different x3 value (from 12 to 37 mM). The overlapping of the three surfaces exhibits that the variation of the buffer concentration didn't change the retention time of analyte significantly.

Optimisation of separation with the aid the overlapping contour plot

The successfully modelling of the retention of test analytes on the PFP phase not only was applied to elucidate the retention behaviour of those compounds but also to provide an important predictive tool for optimising the separations.

Because R^2 for the models were more than 0.92 for all the responses, the good agreement between observed and calculated data makes it is possible to use the model as a predictive tool for the separation. The resolution between the peaks pair R3, R4, R6 and R7 were more than 1.5 in the most experimental space, so only the R1, R^2 and R_5 were chosen as objective responses for the optimisation of separation.

It is suggested that the contour plots could be used to help identify the different regions defining the experimental domain, and these were used here to assist in finding desired resolution for the compounds in the test mixture. The MATLAB program was applied to visualise the three-dimensional response surfaces and contour plots where the critical resolutions was used as a function of x1 and x2 whist keeping x3 constant at the centre level since x3 didn't show significant influence on the responses (*Figure 3*).

By overlapping the contour plots of resolution of R_1, R^2, R_5 and elution time of flubiprofen in one diagram and plotting the contour lines with the value of 1.5 amongst all the individual resolution maps could detect regions that define optimal conditions of mobile phase composition and buffer pH.

All the mobile phase conditions in the coloured area of *Figure 5a* could be applied to baseline separate all the eight acidic analytes. However, the analysis time is changed from 20-60 min by using the conditions at the different points. For example, the analysis time will be about 20, 30 and 40 min by using the conditions at point 1, 2 and 3, respectively.

On the other hand, the robustness of the method was also considered, which means the small variation of parameters should not be able to influence the separation significantly, so the conditions which are located at the corner of the diagram should be avoided during the method development process. Therefore, on the basis of the 3-D response surfaces and simulation results of MATLAB, the best separation conditions for the acidic test mixture were obtained by using a mobile phase of MeOH/25 mM di-potassium hydrogen phosphate buffer pH 3 (56:44, v/v) and 8 acidic solutes were baseline separated in 22 min (*Figure 6*). By using the final optimal conditions, a better separation result was obtained within a shorter analysis time. The prediction shows good agreement with the experimental retention times and resolutions, which demonstrates that the model is able to effectively predict the separation of the analytes for the optimisation purposes.

CONCLUSION

In this work, a flexible and accurate HPLC simulation method has been presented for the simultaneous separation of eight acidic analytes on a PFP phase.

Strategy for the optimisation was developed by combination of both the experimental designs and response surface methodology scheme.

The advantages of this model are that more than two variables can be optimised at the same time and the relations between responses and variables can be elucidated by a second-order equation.

This advantage will be useful for the new packing materials when the retention mechanism of analytes on the packing is not completely clear or the retention models cannot be described by linear relationship.

The results show that the chromatographic properties of the fluorinated phase cannot be explained by a simple hydrophobic mechanism and probably the specific properties of the fluorinated phase participate in the chromatographic process at the same time. The PFP phase gave a shorter retention time and a better separation for the test compounds used, compared to an ODS packing using the same elution conditions.

The method developed in the study proved to be an efficient tool for separation of acidic compounds with different properties on the PFP packing. The model has been shown, after a minimum number of experimental runs, to give accurate predictions for the separation of a broad range of acidic compounds. It has also been shown that time-consuming laboratory work can be done in a short simulation time. The simultaneous separation has been shown to be more efficient and flexible.

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