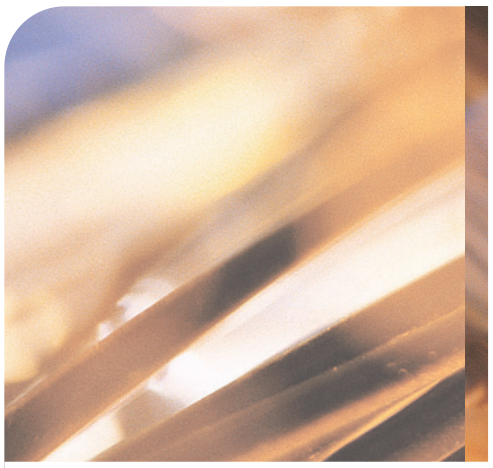

Chromatography Focus

COMPUTER-ASSISTED OPTIMISATION METHOD FOR MODELLING AND PREDICTION OF THE RETENTION BEHAVIOUR OF ACIDIC COMPOUNDS ON A FLUORINATED PACKING (PART 1)

Y. Wang, M. Harrison, B. J. Clark

The chromatographic behaviour of a pentafluoropheyl (PFP) HPLC phase has been evaluated using an optimisation strategy and standard test compounds. Eight acidic analytes with a variety of properties were used as model solutes. This study was aimed at elucidating the retention behaviour of test analytes on the PFP phase and finding optimal separation conditions in a reasonable duration time with the aid of response surface methodology. A central composite design was applied to scrutinise the influence of significant parameters and to derive the polynomial equations describing retention models after the screening experimental design was conducted. The quadratic terms in the model improve the description of chromatographic retention in a statistically significant manner. This rapid, isocratic methodology is suitable for the optimisation of the separation of acidic compounds on the PFP phases. By using the strategy, it was possible to separate an acidic test mixture in 22 min. Optimal conditions were obtained at 38 °C with the mobile phase consisting of MeOH/25mM pH 3.0 buffer (56:44, v/v). The work also demonstrates that buffer concentration has a completely different influence on the retention of acidic and basic compounds on the PFP phase.

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INTRODUCTION

Octadecylated silica gels (ODS) are the traditional packing materials used in the HPLC. However, for several reasons there has been a continuous drive to evolve stationary phases with more efficient and different selectivity. For example, peak tailing, broadening and excessive retention times are caused by undesirable electrostatic interactions between the analytes and packing materials. The presence of residual ionised silanol groups on the silica gel surface greatly complicates the retention process on the ODS phase. In addition, ODS is not suitable for isocratic separation of solutes which cover a wide range hydrophobic difference.

The recent marketed perfluorinated and fluorinated stationary phases are suggested as an alternative to the traditional C18 and C8 phases because the materials exhibit unique selectivity for the separation of complex compound classes in many cases [1-4]. They were used effectively for separations of fluorine-containing compounds [5, 6]; they showed good performance towards pharmaceuticals containing basic groups [7, 8]. In addition, pentafluoropheyl (PFP) phases presented good selectivity towards the compounds with conjugated double bond and hydroxyl groups, so this packing material is suggested as a suitable stationary phase for the separation of steroids [9, 10].

Because pentafluoropheyl phases (PFP) have very low surface energy and lack of ionic groups, the interaction between hydrophobic compounds with the packing materials is reduced [5, 11]. This character reduces excessive retention of compounds with strongly hydrophobic nature, and therefore the phase showed better peak shape than common C18 phases [12, 13]. The unique C-F bonds provide a large dipole character, which increase the phase's interaction with polar compounds. Therefore, the PFP packing has been shown to be suitable for the isocratic separation of compounds with a wide range hydrophobic character in reduced analysis time, which can only be realised by ODS packing with gradient conditions [14].

Different workers have studied the retention mechanism of the PFP. A study from Przybyciel and Santagelo [15] showed that nitro-naphthalenes were able to be baseline resolved on a PFP phase and retention time for every component of the mixture were longer than either a C18 or a phenyl phase. The chromatographic behaviour of nitronaphthalenes on the PFP phase may indicate the existence of several mechanisms for separation including some π-π interactions as well as other electrostatic or charge-transfer modes. By using a computer simulation method, Yamamoto and Rokushike studied retention properties of the fluorinated alkyl phase. The results indicated the methyl molecules reduced the retention of larger aromatics by hindering the retention of the aromatic test probes at higher methanol concentrations and the existence of exclusive interactions between the aromatic molecules and fluorinated phases [16]. However, the retention characteristics of fluorinated phases are complicated and remain largely unsolved, so the final mechanism requires future investigation [8, 15].

We have reported previous that a new developed optimisation strategy was successfully applied for elucidating the retention and predicting the separation of basic compounds on a PFP phase [14]. In order to investigate whether one general model can describe the chromatographic behaviour of different analytes on the PFP phase and whether the influence of independent parameters on the retention and separation of the acidic compounds are similar to the basic analytes, the same optimisation process was performed in this work. The optimisation scheme used a two-level orthogonal array design to screen the significant factors. Then, a central composite design was applied to optimise the parameters and the second-order models related the responses (the resolution and retention time) with significant independent parameters were obtained. On the basis of the mathematical models, the simulated responses were fitted to derive the response surface plots and contour maps. Then, the visualisation plots gave the indications of the optimal conditions for the separation within the shortest analysis time. At the same time, the work also investigated the difference of retention mechanism between acidic and basic compounds on the PFP phase.

EXPERIMENTAL

Apparatus

The experiments were carried out using a Hewlett Packard 1050 system consisting of a HP 1050 pump, a HP 1050 UV-Vis detector. The chromatographic data was collected using Kontron DS 450-MT2 data system (Kontron instruments Co. UK). The column used for the separation was a FluoroSep-RP Phenyl HS 15×4.6 cm from ES Industry (West Berlin, NJ, USA). UV detection was performed at 220 nm.

Reagent

All compounds were from Sigma Chemical Company (St. Louis, Mo, USA). The stock solutions were prepared in methanol: water (80:20) at 1.0 mg/ml. A mixture solution contained 50 µg/ml of each acidic compound in methanol-water (80:20) was prepared from the stock solution before analysis and was used as the working solution.

HPLC-grade acetonitrile and methanol were purchased from Fisher Scientific (Loughborough, Leicestershire, UK). The water used for the preparation of sample and mobile phase was from an Elga Water System Purelab option-R 175 (High Wycombe, Bucks, UK).

The test compounds were:

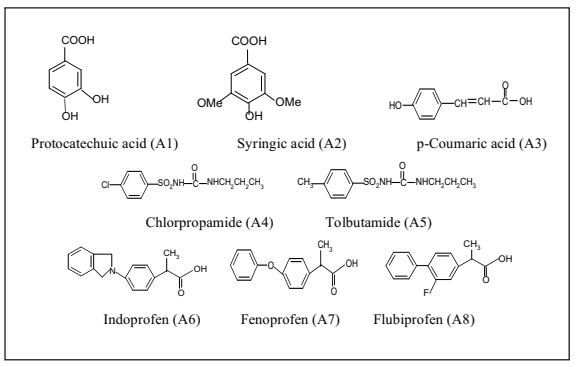

Figure 1. Structures of the acidic test compounds studied in the optimisation

Statistical methods and software

ANOVA analysis, correlation studies and second-order polynomial equations were generated by SPSS for Windows version 11.0 (SPSS Inc, Chicago, Illinois, USA). MATLAB 6.0 program and optimisation toolbox (Mathworks, Natick, MA, USA) was applied for making the response curve, contour plot and calculating optimum conditions.

RESULTS AND DISCUSSION

Screening significant factors by using the orthogonal array (OA) design

A two-level orthogonal array design (OAD) was used as screening experiments to extract the significant factors for separation optimisation. The type (A), percentage of organic modifier in the mobile phase (B), pH (C) and concentration of buffer (D) can affect the separation selectivity on the conventional ODS phase significantly. In addition, the column temperature (E) and flow rate (F) affect the duration time and the efficiency of separation. Therefore, the above six parameters were chosen as independent variables and an OA design was applied to investigate the influence of parameters on the retention of analytes on the PFP phase. The two-variable interaction was allocated to a column in the orthogonal array matrix as dependent variable [17]. The assignment of the parameters, interactions and levels are given in *Table 1*. The minimum and maximum values of the variables were chosen from the results of pilot experiments.

In the optimisation studies, the responses are the chromatographic functions that relate to the independent variables and describe the character of retentions or define the quality of the separation.

Three types of response were selected in this study:

(i) Resolution between the worst separated peaks, which were R1 (protocatechuic acid /syringic acid) and R2 (syringic acid / p-coumaric acid)

(ii) Retention time of the each analyte

The resolution (Rs) were calculated according to Eq. (1) and (2)

$$R_s = \frac{2 \times (t_j - t_i)}{(w_j + w_i)}$$

Where t_i and t_j and w_i and w_j are the retention time and the peak widths of two consecutive peaks, peak i being the first one and peak j being the next one adjacent to it.

Table 2. The estimation of p-value of the significant factors and two-level interaction

Independent parameters

Response	A		B		C		D	
	F	Sig.	F	Sig.	F	Sig.	F	Sig.
R1	55.0	0.02	3526.7	0.00	89.0	0.01	131.0	0.01
R2	0.0	0.89	35.5	0.03	10.0	0.09	0.7	0.49
t_f	33.6	0.03	370.9	0.00	16.4	0.06	1.1	0.41

Two-level interactions

	AB		BC		BD		CD	
	F	Sig.	F	Sig.	F	Sig.	F	Sig.
R1	81.5	0.01	24.6	0.04	120.8	0.01	42.5	0.02
R2	0.0	0.90	3.3	0.21	0.8	0.46	1.4	0.37
t_f	26.4	0.04	9.9	0.09	0.7	0.50	3.2	0.22

From the result of the screening experiments, protocatechuic acid /syringic acid (R1) and syringic acid / p-coumaric acid (R2) were the most difficult peak pairs to be separated. R1 and R2 together with the retention time of flubiprofen, the longest in the test mixture, were chosen as the response functions for the analysis of variance (ANOVA), and regression analysis to extract the most significant factors for the study. The fraction of the total variation of the response that can be explained by this model, R2 (n=16) was 0.993, 0.995, and 0.998 for R1, R2, and tf, respectively. R2 gives an indication about the regression of the model and the value close to 1 means a perfect fit to the experimental data.

The results from the OAD experiments are presented in *Table 2*. It indicate that the influence of factor B (the percentage of organic modifier) was most significant on the responses at the $p<0.01$ level. The next most important factors were the buffer pH and the type of organic modifier, which had a p-value at the $p<0.05$ level for R1 and tf. The Factor D and interactions AB, BC, BD and CD also had an important influence on resolution R1. The type of organic modifier influenced the selectivity and peak shape. A better separation result was obtained by using methanol in the mobile phase than acetonitrile.

On the basis of statistical analysis, temperature (E), flow rate (F) and the other two-factor interactions, which are not included in *Table 2*, did not show significant influence on the responses. So the factor E and F were fixed to a constant level which showed better separation results in the screening study. In the following experiments, the temperature was kept constant at 38ºC and flow rate at 1.1 ml/min.Because the two-level OA design only indicates the influence of parameters on response qualitatively, a higher-level experiment design was required to help establish the relationship quantitatively. Therefore, the significant factors were further optimised using a central composite design (CCD) with the aid of response surface methodology (RSM) to establish a mathematical model to describe the relationships between the responses and independent parameters.

Content of methanol, pH and concentration of buffer were set as independent variables in the CCD design. Although the influence of buffer concentration was not significant for most responses, it was included in the next CCD because buffer concentration demonstrated most significant influence on the retention of basic analytes on the same packing materials [14].

Table 1. The setting of parameters and interactions for the two-level Orthogonal Array Design

Exp. No.	A[a]	B[a]	AB/DE[b]	C[a]	AC/DF[b]	BC/EF[b]	DM[c]	D[a]	AD /BE/CF[b]	BD/AE	E[a]	CD/AF[b]	F[a]	DM2[c]	CE/BF[b]
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+
3	-	-	-	+	+	+	+	-	-	-	-	+	+	+	+
4	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-
5	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+
6	-	+	+	-	-	+	+	+	+	-	-	+	+	-	-
7	-	+	+	+	+	-	-	-	-	+	+	+	+	-	-
8	-	+	+	+	+	-	-	+	+	-	-	-	-	+	+
9	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
10	+	-	+	-	+	-	+	+	-	+	-	+	-	+	-
11	+	-	+	+	-	+	-	-	+	-	+	+	-	+	-
12	+	-	+	+	-	+	-	+	-	+	-	-	+	-	+
13	+	+	-	-	+	+	-	-	+	+	-	-	+	+	-
14	+	+	-	-	+	+	-	+	-	-	+	+	-	-	+
15	+	+	-	+	-	-	+	-	+	+	-	+	-	-	+
16	+	+	-	+	-	-	+	+	-	-	+	-	+	+	-

A: type of organic modifier: MeOH and MeCN;
B: concentration of A: 48% and 58%;
C: type of buffer (pH 3 and pH 7 phosphate buffer);
D: buffer concentration (5 mM, 25 mM);
E: temperature (32°C and 38°C);
F: flow rate (0.9 ml/min and 1.1 ml/min).
a factor column
b two-factor interactions
c DM and DM2 are dummy factors. They were used to estimate the importance of random error and the lack of fit (LOF) of the model.

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Part 2 of this Article can be seen in our February/March Issue