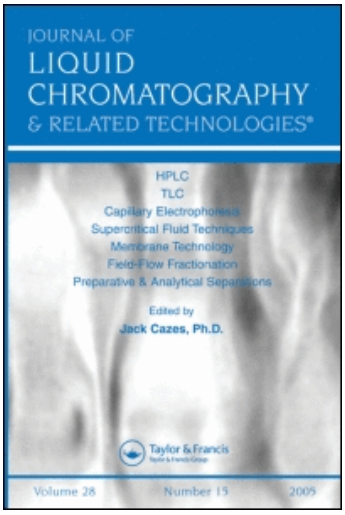

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OPTIMIZATION OF HPLC SEPARATION OF CARBAMATE INSECTICIDES (CARBOFURAN, HYDROXYCARBOFURAN AND ALDICARB) BY EXPERIMENTAL DESIGN METHODOLOGY

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ABSTRACT

The experimental design methodology has allowed to optimize the chromatographic separation of polar pesticide molecules (carbofuran, hydroxycarbofuran and aldicarb) by HPLC, owing to the determination of mathematical equations. Second degree equations have been elaborated, dependent upon injected volume, eluent flow and eluent composition. These equations have allowed us to plot Isoresponse Curves which are used to easily visualize and optimize separations. Interactions between eluent flow and polarity are emphasized.

INTRODUCTION

Generally, the analyst has to perform many experiments in order to find optimal practical conditions for the development of pesticide residue analytical methods.

The chromatographic analysis depends on many factors; their influences are not easily controlled. The practical conditions for optimal separation of similar molecules are usually determined by independant or step-by-step variations of factors. Owing to the purposed methodology, a cheaper and more direct strategy can be followed, which is an interesting alternative to a Simplex methodology. Until now, experiments planning methodology was not often applied to the optimization of residue analysis.[1]

An *a priori* experimental design methodology has been planned in order to determine mathematical equations applicable to the separations of organic residues by classic chromatographic techniques (High Performance Liquid Chromatography); separations of carbamate insecticide residues were then performed using the previous equations.

THE DESIGN OF EXPERIMENTAL METHODOLOGY

Table 1

Experimental Matrix of 2^3 Plan

	A	B	C	Y
Test 1	-1	-1	-1	y1
Test 2	+1	-1	-1	y2
Test 3	-1	+1	-1	y3
Test 4	+1	+1	-1	y4
Test 5	-1	-1	+1	y5
Test 6	+1	-1	+1	y6
Test 7	-1	+1	+1	y7
Test 8	+1	+1	+1	y8
Level -1	a	a'	a"	
Level +1	b	b'	b"	

For the A factor, the value a is set at level -1 and b at level +1; for B, the value a' is set at level -1 and b' is set at level +1; for C, the value a" is set at level -1 and b" at level +1.

The effects of factors which could modify the separation are measured, their interactions are displayed, and those which affect the results are pointed out. In a second time, analytical expressions of influences are set up and used to determine the best combination of experimental conditions.

We briefly describe the method, refering to cited literature for general justification.[2,3,4,5] The difference **y** between retention times of 2 molecules, distinguished by HPLC, is governed by **k** factors (A, B, C...), for instance eluent mixture polarity, injected volume, etc. Each factor has been brought to 2 values (levels a (-1) and b (+1)), corresponding to the limits of a reasonable variation interval. So, 2 experiments had to be performed (Table 1). The influences of the factors, and their interactions have been evaluated as the $\mathbf{h_n}$ coefficients. For instance, with 3 factors, the $\mathbf{h_n}$ coefficients are listed as follows:

$$h_A = 1/8(-y1 + y2 - y3 + y4 - y5 + y6 - y7 + y8)$$
$$h_B = 1/8(-y1 - y2 + y3 + y4 - y5 - y6 + y7 + y8)$$
$$h_C = 1/8(-y1 - y2 - y3 - y4 + y5 + y6 + y7 + y8)$$
$$h_{AB} = 1/8(+y1 - y2 - y3 + y4 + y5 - y6 - y7 + y8)$$
$$h_{AC} = 1/8(+y1 - y2 + y3 - y4 - y5 + y6 - y7 + y8)$$
$$h_{BC} = 1/8(+y1 + y2 - y3 - y4 - yS - y6 + y7 + y8)$$
$$h_{ABC} = 1/8(-y1 + y2 + y3 - y4 + y5 - y6 - y7 + y8)$$
$$H = 1/8(+y1 + y2 + y3 + y4 + y5 + y6 + y7 + y8)$$

h_A, h_B, h_C display effects of the A, B, C factors, respectively.
h_{AB}, h_{AC}, h_{BC} characterize first degree interactions between A and B, A and C, B and C, respectively.
h_{ABC} characterizes the second degree interaction between A, B and C.
y1, y2, ..., y8 are results for test 1, test 2, ..., test 8, respectively.
H is the average of the results.

There is an interaction between 2 factors, A and B, for instance, if the effect of A depends on the level of B. and *vice-versa.* The calculation of the global effect of A (h_A) results from 8 measured responses y_i. The variance analysis of the results allows us to determine if the observed effect can be explained by measurement uncertainties or if it is significant. The variance analysis is described in detail in the literature.[6,7] Then, the response is expressed as a linear mathematical equation, for instance, in the event of 3 significantly influential factors, without significant interactions:

$$Y = H + h_A * A + h_B * B + h_C * C$$

or, when interactions are significant:

$$Y = H + h_A * A + h_B * B + h_C * C + h_{AB} * AB + h_{AC} * AC + h_{BC} * BC + h_{ABC} * ABC$$

Table 2

Validation Assay

	A	B	C	Y
Level -1	a	a'	a"	y1
Level +1	b	b'	b"	y4
Level 0	d=(a+b)/2	d'=(a'+b')/2	d"=(a"+b")/2	y5
Level x	c	c'	c"	y6
-1<x<+1	a<c<b	a'<c'<b'	a"<c"<b"	

Four experiments were performed, setting a, b, c and d values to A, a', b' c' and d' to B, a", b", c" and d" to D. (a, b, c, d, a', b', c', d', a", b", c", d")∈[-1; +1].

To validate this mathematical equation, an additional experiment set has to be carried out, in which new values have been affected to factors, inside of the previous intervals, for example 0 and **c** (Table 2). If the value between the experimental response and the calculated one does not exceed 5%, the linear equation is confirmed, and can be used to choose the best values of the factors. In the opposite case, a complementary experiment set, also called *centered composite plan,* has to be performed. In Table 3, an experimental matrix for the study of 3 factors has been planned. It contains main tests (factors are fixed at levels of - 1 and + 1), six centered points (level 0), repaired for statistical results treatment, and α points (factors are fixed at levels - α and + α*):* the α value and the number of centered points depend on **k** (factors number). In a three factors plan, α = 1.6. From the effects matrix of the centered composite plan, a more complex mathematical equation (quadratic one) can be built up:

$$Y = a_o + a_1A + a_2B + a_3C + a_4AB + a_5AC + a_6BC + a_7A^2 + a_8B^2 + a_9C^2$$

Table 3

Centered Composite Plan: Experimental Matrix

	A	B	C	Y
Test 1	-1	-1	-1	y1
Test 2	+1	-1	-1	y2
Test 3	-1	+1	-1	y3
Test 4	+1	+1	-1	y4
Test 5	-1	-1	+1	y5
Test 6	+1	-1	+1	y6
Test 7	-1	+1	+1	y7
Test 8	+1	+1	+1	y8
Test 9	0	0	0	y9
Test 10	0	0	0	y10
Test 11	0	0	0	y11
Test 12	0	0	0	y12
Test 13	0	0	0	y13
Test 14	0	0	0	y14
Test 15	$-\alpha$	0	0	y15
Test 16	$+\alpha$	0	0	y16
Test 17	0	$-\alpha$	0	y17
Test 18	0	$+\alpha$	0	y18
Test 19	0	0	$-\alpha$	y19
Test 20	0	0	$+\alpha$	y20
Level $-\alpha$	d	d'	d"	
Level -1	a	a'	a"	
Level 0	(a+b)/2	(a'+b')/2	(a"+b")/2	
Level +1	b	b'	b"	
Level $+\alpha$	e	e'	e"	

A routine scientific calculator is enough to run matrix calculations,[8] as described below. a_i coefficients are calculated from the matrix with real values, as reported in Table 4.

Table 4

Effects Matrix of the Centered Composite Plan (X) (Y): Matrix of Measured Responses. y = (y9 +y10 +y11 + y12 + y13 + y14)/6

	-----(X)-----									(Y)
	A	**B**	**C**	**AB**	**AC**	**BC**	**A^2**	**B^2**	**C^2**	
T1	a	a'	a"	aa'	aa"	a'a"	aa	a'a'	a"a"	y1
T2	b	a'	a"	ba'	ba"	a'a"	bb	a'a'	a"a"	y2
T3	a	b'	a"	ab'	aa"	b'a"	aa	b'b'	a"a"	y3
T4	b	b'	a"	bb'	ba"	b'a"	bb	b'b'	a"a"	y4
T5	a	a'	b"	aa'	ab"	a'b"	aa	a'a'	b"b"	y5
T6	b	a'	b"	ba'	bb"	a'b"	bb	a'a'	b"b"	y6
T7	a	b'	b"	ab'	ab"	b'b"	aa	b'b'	b"b"	y7
T8	b	b'	b"	bb'	bb"	b'b"	bb	b'b'	b"b"	y8
T9	(a+b)/2	(a'+b')/2	(a"+b")/2	(a+b)/2 * (a'+b')/2	(a+b)/2 * (a"+b")/2	(a'+b')/2 * (a"+b")/2	(a+b)/2 * (a+b)/2	(a'+b')/2 * (a'+b')/2	(a"+b")/2 * (a"+b")/2	y9
T15	d	(a'+b')/2	(a"+b")/2	d * (a'+b')/2	d * (a"+b")/2	(a'+b')/2 * (a"+b")/2	dd	(a'+b')/2 * (a'+b')/2	(a"+b")/2 * (a"+b")/2	y15
T16	e	(a'+b')/2	(a"+b")/2	e * (a'+b')/2	e * (a"+b")/2	(a'+b')/2 * (a"+b")/2	ee	(a'+b')/2 * (a'+b')/2	(a"+b")/2 * (a"+b")/2	y16
T17	(a+b)/2	d'	(a"+b")/2	(a+b)/2 * d'	(a+b)/2 * (a"+b")/2	d' * (a"+b")/2	(a+b)/2 * (a+b)/2	d'd'	(a"+b")/2 * (a"+b")/2	y17
T18	(a+b)/2	e'	(a"+b")/2	(a+b)/2 * e'	(a+b)/2 * (a"+b")/2	e' * (a"+b")/2	(a+b)/2 * (a+b)/2	e'e'	(a"+b")/2 * (a"+b")/2	y18
T19	(a+b)/2	(a'+b')/2	d"	(a+b)/2 * (a'+b')/2	(a+b)/2 * d"	(a'+b')/2 * d"	(a+b)/2 * (a+b)/2	(a'+b')/2 * (a'+b')/2	d"d"	y19
T20	(a+b)/2	(a'+b')/2	e"	(a+b)/2 * (a'+b')/2	(a+b)/2 * e"	(a'+b')/2 * e"	(a+b)/2 * (a+b)/2	(a'+b')/2 * (a'+b')/2	e"e"	y20

$$[A] = \begin{bmatrix} a_0 \\ a_1 \\ a_2 \\ a_3 \\ a_n \end{bmatrix} \qquad A = (X'X)^{-1}X'Y$$

$A = a_i$ coefficients matrix — X = tests matrix
X' = transposed tests matrix — X^{-1} = inverted tests matrix
Y = responses matrix

The quadratic equation has to be validated before using it to choose the best values of the factors.

The process can require variable changes, new study intervals, or the split of the **k** factors study into 2 studies of **k'** and **k"** factors ($k' + k'' = k$).

dt being the difference of the retention times, and **t** the average analysis time, the optimal chromatographic separation of 2 molecules M and M' is reached fitting with the highest value of the $\frac{dt}{t}$ ratio (the highest separation associated with the lowest analysis time).

Applications to carbamate residue analysis are reported.

EXPERIMENTAL

Carbofuran (CAR), hydroxycarbofuran (OHC) and aldicarb (ALD) are polar pesticides belonging to the N-methylcarbamate chemical family (Figure 1).[9] Residue analysis was usually designed by HPLC.[10,11,12]

Products and Materials

- acetonitrile, methanol, HPLC grade, S.D.S. (Peypin, France),
- deionized water, Seral System (Grosseron, St Herblain, France),
- sodium hydroxide, analytical grade, Osi (Paris, France),

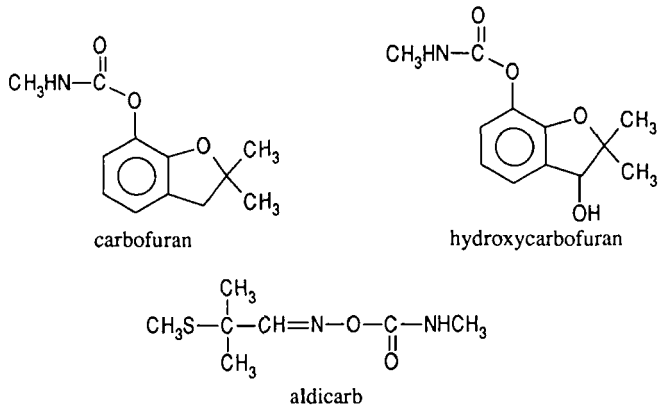

Figure 1. Carbofuran (CAR), Hydroxycarbofuran (OHC) and Aldicarb (ALD).

- hydrochloric acid, analytical grade, Osi,
- o-phthaldialdehyde (OPA), analytical grade, Fluka (Buchs, Switzerland),
- sodium tetraborate, decahydrate, analytical grade, Osi,
- helium N55,1'Air Liquide (Paris, France),
- standards: carbofuran, aldicarb, 99.8%, Cluzeau Info Labo (Ste Foy, France), hydroxycarbofuran, pure, Bayer (FRG).

Syringes, 25 and 1000 µL, Hamilton (Reno, NE, US).

HPLC

- injector, U6K, Waters (Milford, MA, US),
- chromatograph 600E Multisolvent Delivery System, Waters,
- detector, 470 Scanning Fluorescence Detector, Waters,
- integrator, 740 Data module, Waters,
- pumps, PCRS, Waters,
- temperature controller, TCM, Waters,
- stainless steel column, Lichrosorb RP-18 (5 µm), 250 mm x 4.6 mm I. D., Merck (Darmstadt, FRG),
- stainless steel pre-column, Lichrosorb RP- 18 (5 µm), 2.5 mm x 4.6 mm I. D., Merck.

Post-column derivatization was used to detect CAR, OHC and ALD. The eluate was continuously mixed with sodium hydroxide 0.1 N at 80°C, and

then OPA reagent (100 mg/L, in sodium tetraborate aqueous solution (20 g/L)) was added at 25°C (ref. 12, 13). The N-methylcarbamates derivatization reaction sequence is presented Figure 2. Fluorescent products resulted, which were detected

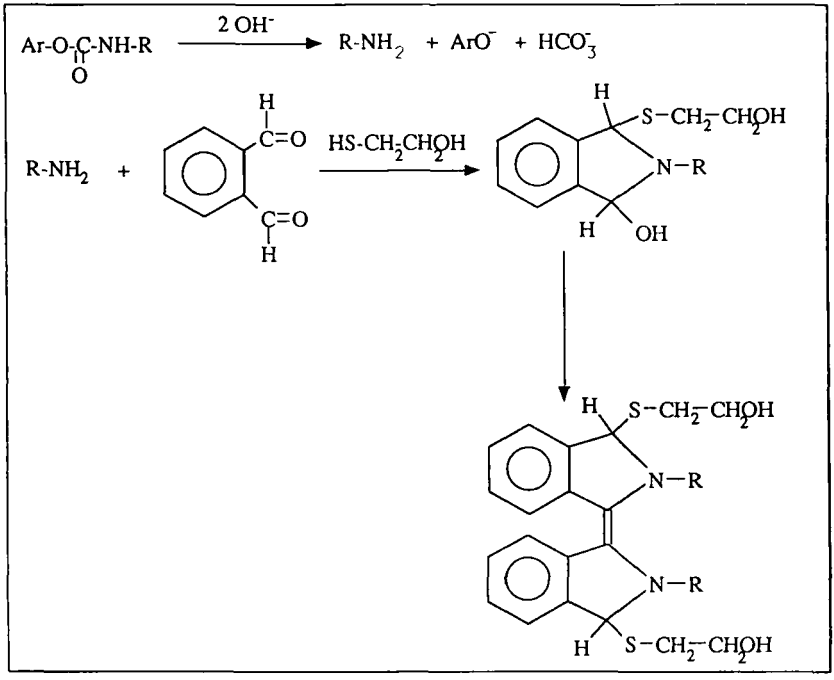

Figure 2. Post-column derivatization of N-methylcarbamates. The reaction yields a highly fluorescent compound which is used to detect low carbamate concentrations (1 μg/L).

The chromatographic parameters were:

- column temperature: 30°C,
- eluent sparge: He, 50 mL/min,
- sodium hydroxyde pump flow: 0.5 rnL/min,
- OPA reagent pump flow: 0.5 mL/min,
- excitation wavelength: 339 nm,
- emission wavelength: 445 nm,
- hydrolysis temperature: 80°C.

Mixture solutions of CAR, OHC and ALD [3 x 25 µg/L (injected volume: 400 µL), and 3 x 500 µg/L (injected volume: 20 RL)] were prepared in acidified water (pH 4) / methanol / acetonitrile mixture (45/10/45). Injections were spaced 1 hour apart, in order to reach the stationary rate; injections were repeated three times.

RESULTS

The studied factors were:

- injected volume (A)
- eluent flow (B)
- composition (polarity) of ternary eluent mixture (C)

The composition of ternary eluent mixtures (water / acetonitrile / methanol) has been expressed as a polarity coefficient, due to the following arbitrary **p** coefficients: $p_{water} = 1.0$, $p_{methanol} = 0\ 9$ and $p_{acetonitrile} = 0\ 5$. So, in this scale, the polarity of the mixture of water, methanol and acetonitrile (45/10/45 v/v/v) is 0.765.

The measured responses were:

$$\frac{dt}{t}(1) = \frac{\text{aldicarb retention time - hydroxycarbofuran retention time}}{0.5\ (\text{aldicarb retention time + hydroxycarbofuran retention time})}$$

$$\frac{dt}{t}(2) = \frac{\text{carbofuran retention time - aldicarb retention time}}{0.5\ (\text{aldicarb retention time + carbofuran retention time})}$$

A chromatogram is displayed Figure 3.

The effects matrix is recorded Table 5, chosen values of - 1 and + 1 levels are indicated. They agree with usual chromatographic parameter ranges. The effects values reported in Table 6 result from the calculation process of $\mathbf{h_n}$ coefficients discussed above; the following linear equations have been deduced:

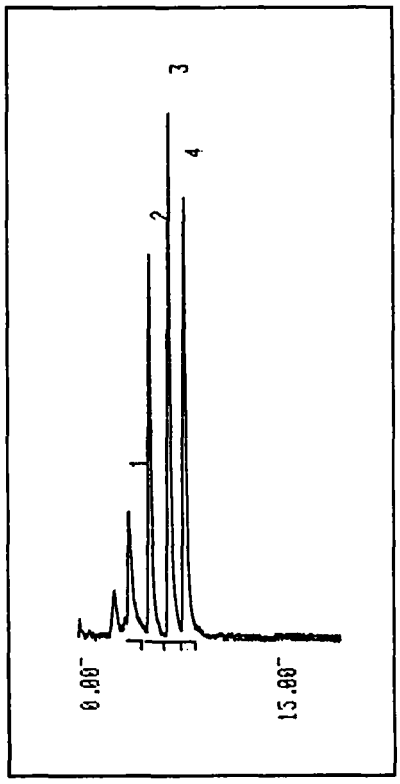

Figure 3. HPLC chromatogram; hydroxycarbofuran (2), aldicarb (3) and carbofuran (4). The factors A, B and C are, respectively, set at the following values: 210 μL, 0.75 mL/min and 0.765.

$$\frac{dt}{t}(1) = 0.324 - 0.012A + 0.015B + 0.181C - 0.018AC + 0.019BC \qquad (1)$$

$$\frac{dt}{t}(2) = 0.231 - 0.008A + 0.017B + 0.171C - 0.008AC + 0.018BC \qquad (2)$$

(A, B. and C in interval [- 1; + 1])

Table 5

Effects Matrix of the HPLC Separation Study

	A	B	C	AB	AC	BC	ABC	M	dt/t(1) ($x10^3$)	dt/t(2) ($x10^3$)
Test 1	-	-	-	+	+	+	-	+	141±0	63±2
Test 2	+	-	-	-	-	+	+	+	152±14	58±6
Test 3	-	+	-	-	+	-	+	+	133±1	57±3
Test 4	+	+	-	+	-	-	-	+	147±1	61±1
Test 5	-	-	+	+	-	-	+	+	504±5	380±6
Test 6	+	-	+	-	+	-	-	+	437±6	355±0
Test 7	-	+	+	-	-	+	-	+	566±7	456±11
Test 8	+	+	+	+	+	+	+	+	511±4	418±8
Level -1	20 µL	0.5mL/ min	0.665							
Level +1	400 µL	1.0mL/ min	0.865							

Table 6

Observed Effects

	dt/t(1)	dt/t(2)
h_A	**-0.012**	**-0.008**
h_B	**0.015**	**0.017**
h_C	**0.181**	**0.171**
h_{AB}	0.002	-0.001
h_{AC}	**-0.018**	**-0.008**
h_{BC}	**0.019**	**0.018**
h_{ABC}	0.001	0.002
H	0.324	0.231

The effects (h_n) which are influential at 5% risk are bold values; other effects are not significant at 40% risk.

Table 7

Validation Tests of the Linear Equation 1 (dt/t(1)): It is not Validated

	A	0	-0.5	0	-1
Experimental Conditions	**B**	0	1	0.2	-0.6
	C	0	0	0.5	-0.5
Calculated dt/t(1)		0.324	0.345	0.356	0.197
Experimental dt/t(1)		0.236	0.253	0.419	0.233
Deviation (%)		27	26	15	17

Table 8

Validation Tests of the Linear Equation 2 (dt/t(2)): It is not Validated

	A	0	-0.5	0	-1
Experimental Conditions	**B**	0	1	0.2	-0.6
	C	0	0	0.5	-0.5
Calculated dt/t(1)		0.231	0.252	0.322	0.144
Experimental dt/t(1)		0.153	0.171	0.270	0.122
Deviation (%)		34	32	16	22

Validation tests are reported Tables 7 and 8. They did not support the previous equations. So, a centered composite plan of experiments had to be performed in order to determine the quadratic equations.

The effects matrix of the centered composite plan is reported Table 9. From the matrix calculation, the following quadratic equations have been extracted:

$$10^3 \frac{dt}{t}(1) = 3.52 + 0.5A + 0.70B - 11.36C - 0.16AB - 0.61AC - 0.81BC + 0.05A^2 - 0.02B^2 + 9.17C^2 \quad (1')$$

$$10^3 \frac{dt}{t}(2) = 3.34 + 0.24A + 0.62B - 10.85C - 0.16AB - 0.24AC - 0.63BC + 0.08A^2 - 0.05B^2 + 8.64C^2 \quad (2')$$

A, B and C factors range, respectively, from 0.020 to 0.400 mL/min, from 0.5 to 1.0 mL/min and from 0.665 to 0.865 (arbitrary unit).

Results of the validation tests are recorded in Tables 10 and 11, in agreement with the above two equations.

DISCUSSION

The relationship between $\frac{dt}{t}(1)$ or $\frac{dt}{t}(2)$ and the three factors (injected volume (A), eluent flow (B) and eluent polarity (C)) yields quadratic equations. The eluent composition (C) is the main factor acting on the separation of the 3 molecules; the coefficients linked to this factor, its square value and interactions are higher than other coefficients. So, the separations of those molecules are thought to be mainly under control of the difference of solvation of the molecules. In a previous study,[14] concerning the HPLC separation of simazine and atrazine, the role of eluent polarity and eluent flow have already been emphasized.

According to these equations, the HPLC separations have been plotted, in the way of Isoresponse Curves: Figure 4 shows isoresponse curves illustrating Equation 1' and Figure 5 shows isoresponse curves illustrating Equation 2'. On those plots, the injected volume (A) is set at its central value (210 μL). Isoresponse curves, in every case, are parabolic portions or straight line segments, as would be generally expected.[2] They can be used to determine parameter combinations that lead to a desired separation. For instance, when the injected volume is 210 μL, the eluent flow is 0.75 mL/min and the eluent mixture composition is water / methanol / acetonitrile (42/10/48) (polarity 0.75), $\frac{dt}{t}(1)$ and $\frac{dt}{t}(2)$ are expected to be 0.205 and 0.160, respectively. The experimental results are 0.202 and 0.161, respectively, in good agreement with the expected ones.

Table 9

Effects Matrix of Centered Composite Plan

K	A	B	C	AB	AC	BC	A^2	B^2	C^2	dt/t (1)	dt/t (2)
1	0.097	0.60	0.705	0.06305	0.06839	0.45825	0.00941	0.4225	0.4970 3	0.155	0.079
1	0.323	0.60	0.705	0.20995	0.22772	0.45825	0.10433	0.4225	0.4970 3	0.157	0.086
1	0.097	0.90	0.705	0.0873	0.06839	0.6345	0.00941	0.81	0.4970 3	0.174	0.097
1	0.323	0.90	0.705	0.2907	0.22772	0.6345	0.10433	0.81	0.4970 3	0.168	0.091
1	0.097	0.60	0.825	0.0631	0.08003	0.53625	0.00941	0.4225	0.6806 3	0.399	0.305
1	0.323	0.60	0.825	0.2100	0.26648	0.53625	0.10433	0.4225	0.6806 3	0.387	0.304
1	0.097	0.90	0.825	0.0873	0.08003	0.7425	0.00941	0.81	0.6806 3	0.398	0.305
1	0.323	0.90	0.825	0.2907	0.26648	0.7425	0.10433	0.81	0.6806 3	0.373	0.294
1	0.210	0.75	0.765	0.1575	0.16065	0.57375	0.0441	0.5625	0.5852 3	0.243	0.163
1	0.020	0.75	0.765	0.015	0.0153	0.57375	0.0004	0.5625	0.5852 3	0.263	0.181
1	0.400	0.75	0.765	0.30	0.306	0.57375	0.16	0.5625	0.5852 3	0.230	0.155
1	0.210	0.5	0.765	0.105	0.16065	0.3825	0.0441	0.25	0.5852 3	0.236	0.152
1	0.210	1.0	0.765	0.210	0.16065	0.765	0.0441	1.00	0.5852 3	0.251	0.172
1	0.210	0.75	0.665	0.1575	0.13965	0.49875	0.0441	0.5625	0.4422 3	0.137	0.060
1	0.210	0.75	0.865	0.1575	0.18165	0.64875	0.0441	0.5625	0.7482 3	0.536	0.443

To run the matrix calculation, a constant value (K) is needed. A: injected volume (μL). B: eluent flow (mL/min). C: eluent mixture polarity.

According to the plots $\frac{dt}{t}(2) = 0.100$ when:

1 - (A = 210 μL; B = 0.6 cm^3/min; C = 0.72) or
2 - (A = 210 μL; B = 0.8 cm^3 /min; C = 0.65).

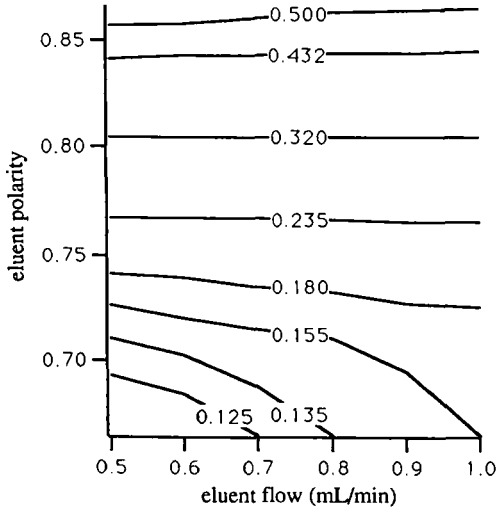

Figure 4. Isoresponse curves resulting from the quadratic Equation 1' ($\frac{dt}{t}(1)$). Injected volume is 210 μL. $\frac{dt}{t}(1)$ values are reported on the curves.

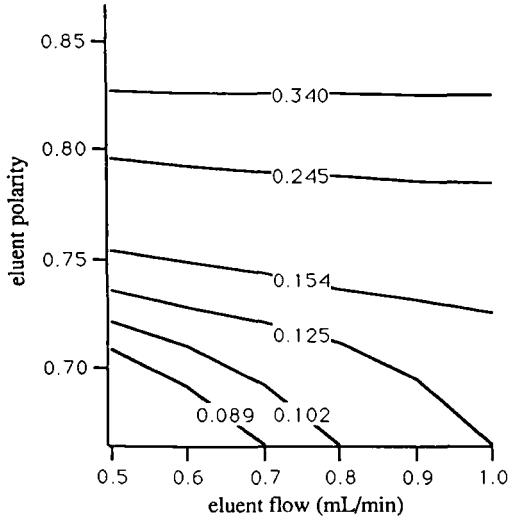

Figure 5. Isoresponse curves resulting from the quadratic Equation 2' ($\frac{dt}{t}(2)$). Injected volume is 210 μL. $\frac{dt}{t}(2)$ values are reported on the curves.

This example shows that a very polar eluent is not required to separate CAR and ALD well.

Table 10

Validation Tests of the Quadratic Equation 1' (dt/t(1)): The Equation is Validated

Experimental Conditions	**A (μL)**	210	115	210
	B (mL/min)	0.75	1.0	0.8
	C	0.765	0.765	0.815
Calculated dt/t(1)		0.243	0.258	0.362
Experimental dt/t(1)		0.243	0.253	0.356
Deviation (%)		0.0	2.0	1.7

Table 11

Validation Tests of the Quadratic Equation 2' (dt/t(2)): The Equation is Validated

Experimental Conditions	**A (μL)**	210	115	210
	B (mL/min)	0.75	1.0	0.8
	C	0.765	0.765	0.815
Calculated dt/t(2)		0.164	0.175	0.278
Experimental dt/t(2)		0.163	0.171	0.270
Deviation (%)		0.0	2.3	3.0

CONCLUSION

According to our study, mathematical equations which characterize separation of molecules could be established quickly; of course, other factors

(nature of the stationary phase, temperature of the column,...)[14] could be studied. Elaboration of mathematical equations and plot of isoresponse curves are easy to derive, with simple computers. A direct strategy for detecting significant chromatographic parameters can be defined, using experimental design.

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