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Optimization of the separation of mono- and dichloroanilines in ion interaction high-performance liquid chromatography

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Abstract

To optimize the ion-interaction chromatographic separation of nine chloroaniline isomers, the effect on retention of six experimental parameters is investigated by means of multivariate analysis. The factors considered are the organic modifier concentration in the mobile phase; the length of the alkyl chain of the alkylammoniumion salts used as the ion-interaction reagents (IIRs); the concentration of IIRs; the pH of the mobile phase, the flow-rate and the ionic strength. The use of fractional factorial and star designs allowed one to draw out useful information on the retention mechanism involved and to build a model characterized by both descriptive and predictive ability. Concerning descriptions, the results suggest a retention mechanism mainly based on reversed-phase partition, while the main role of the alkylamine (used as IIR) seems to mask the activity of the residual silanol groups on the stationary phase. As a result efficiency is improved. For prediction purposes, the regression models allow the optimization of the chromatographic separation, as regards both resolution and total analysis time. The study allowed one to develop a method able to separate the nine mono- and dichloroanilines in a total analysis time within 66 min and with detection limits ranging from 4.0 to 21.0 μg/l.

Keywords: Optimization; Chloroanilines

1. Introduction

According to the most well-established theory, retention in ion-interaction high-performance liquid chromatography (HPLC) is due to the dynamic formation of an electrical double layer on the reversed stationary phase [1,2]. The double layer is formed when a suitable ion-interaction reagent (IIR), comprising a lipophilic moiety with a polar head, is added to the mobile phase and adsorbed onto the stationary phase surface. The modification makes it possible the separation on the new stationary phase of anionic and cationic species and also, since not all the original reversed-phase (RP) sites are modified, also neutral species can be retained, so that ion-interaction and conventional RP mechanisms can coexist and alternatively predominate, as a function of the experimental conditions.

When working in IIR mode many variables are involved in the retention, such as the chemical properties and the concentration of the ion-interaction reagent, the amount of organic modifier, the

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pH and the ionic strength of the mobile phase. Variations of the experimental conditions affect both the retention of the analytes and the extent and the properties of the moiety adsorbed onto the stationary phase. As a result the effect of the these factors on retention can be non linear and interdependent [3–5].

Due to the dependence on so many factors, the technique is very versatile and can be made suitable to solve different separation problems, but the optimization of the experimental conditions is complex, due to the large number of the variables which must be simultaneously treated. For this purpose, methods of multivariate analysis are usually used.

Aromatic amines are a widespread class of environmental water pollutants [6], that can be released into the environment both as industrial effluents and as breakdown products (from phenylcarbamates and phenylurea herbicides, pharmaceuticals dyestuffs [7]). They can be present at trace levels in drinking water and soft drink beverages, in food [8], and in antibacterial products [9,10]. Toxicological data indicate that some of aromatic amines are suspected to induce cancer [11,12] and in particular aniline and mono-, di- and trichloroanilines have been included by the European Union in the list of priority pollutants.

For the determination of chloroanilines some chromatographic procedures have been published, gas chromatographic methods after derivatization [13–15], cation-exchange chromatography [8], RP-HPLC with UV or fluorescence [16–24] detection. Only one method is reported for the separation of all the isomers of mono- and dichloroanilines [20].

Based on the protonation equilibrium undergone by some of these analytes as a function of mobile phase pH, the use of ion interaction technique for the separation of the nine isomers was tried: 2-chloroaniline (2-CA), 3-chloroaniline (3-CA), 4-chloroaniline (4-CA), 2,3-dichloroaniline (2,3-dCA), 2,4-dichloroaniline (2,4-dCA), 2,5-dichloroaniline (2,5-dCA), 2,6-dichloroaniline (2,6-dCA), 3,4-dichloroaniline (3,4-dCA), 3,5-dichloroaniline (3,5-dCA). By using alkylammonium salts as the IIRs six experimental variables were optimized, namely, the ion-interaction reagent concentration C_{IIR}, the alkyl chain length N, the organic modifier concentration C_M, the mobile phase pH, the ionic strength I and the flow-rate F. For this purpose chemometric methods of experimental design and multivariate analysis are employed.

2. Theory

2.1. Factorial design

In factorial design, firstly introduced by Fisher [25] each factor is investigated at fixed levels. Full factorial design contains all the possible combinations of the selected settings of the experimental factors and allows a straightforward calculation of the effects of the factors and of all the possible multi-factor interactions. A two-level full factorial design requires 2^p experiments (where p is the number of the factors). To perform a lower number of experiments a fractional factorial design can be used, but this leads to a partial loss of information. The theory of two-level factorial design has been widely described elsewhere [26] and we shall not enter into further details in the present paper.

The star design in turn is an experimental plan in which the experiments are performed at three levels along the factor axes and in the center of the dominion. This design allows the calculation of a regression model that contains first-order and quadratic terms, but no interaction term. The addition of the experiments of the star design to the experiments of a factorial design provides enough information for the calculation of a model containing the main factors plus interaction and quadratic terms and leads to the central composite design [27,28].

All statistical analysis were performed on range scaled factor values ($[-1, 1]$ with respect to the factorial design ranges).

3. Experimental

3.1. Apparatus

The analyses were carried out with a Merck–Hitachi Model L-6200 Lichrograph chromatograph (Tokyo, Japan) equipped with a two-channel Merck–Hitachi Model D-2500 chromato-integrator and interfaced with a Model L-4200 UV–Vis detector.

A Metrohom 654 pH meter (Herisau, Switzer-

land), equipped with a combined glass-calomel electrode, was employed for pH measurements.

Potentiometric measurements were performed at $T=298$ K and ionic strength $I=0.1$ mol/l (KCl) with a Metrohm E-605 potentiometer equipped with combined glass electrode. The couple was calibrated in $-\log [H^+]$ units (pH) employing alkalimetric titrations of hydrochloric acid with standard, carbonate-free, potassium hydroxide. The ionic strength (KCl) of the calibrating solutions was the same as the solutions being examined. The alkalimetric titrations were carried out in a stream of purified nitrogen. Temperature control was achieved by means of a liquid circulation from a thermocryostat (Model D1-G Haake).

3.2. Reagents

Ultrapure water from a Millipore Milli-Q system (Milford, MA, USA) was used for the preparation of all the solutions. Alkylamines (methyl-, butyl-, pentyl-, hexyl- and octylamine) and orthophosphoric acid were Fluka (Buchs, Switzerland) analytical grade chemicals. Analytes [(2-chloroaniline (2-CA), 3-chloroaniline (3-CA), 4-chloroaniline (4-CA), 2,3-dichloroaniline (2,3-dCA), 2,4-dichloroaniline (2,4-dCA), 2,5-dichloroaniline (2,5-dCA), 2,6-dichloroaniline (2,6-dCA), 3,4-dichloroaniline (3,4-dCA), 3,5-dichloroaniline (3,5-dCA)] and sodium perchlorate were purchased from Aldrich (Milwakee, WI, USA). Acetonitrile (ACN) was a Merck (Darmstadt, Germany) analytical grade chemical. Hydrochloric acid, potassium hydroxide and potassium chloride were Carlo Erba (Milan, Italy) chemicals.

3.3. Chromatographic conditions

An end-capped Superspher 100 RP-18 column (250×4.6 mm, 4 μm) (Merck, Darmstadt, Germany) and a (15.0×4.6 mm) Lichrospher RP-18 (5 μm) guard precolumn were used for all the separations.

Reversed-phase separations were performed using a mobile phase of water–ACN (65:35, v/v) flowing at 1.0 ml/min.

The experiments in the ion-interaction mode planned by the experimental design required a number of eluents prepared with different combinations of the values of the six variables considered. The sequence of experiments was randomized.

The chromatographic system was conditioned by passing, under isocratic conditions, the eluent through the column until a stable baseline signal was reached and when reproducible retention times were obtained for three subsequent injections (a minimum of an hour, at flow-rate 1.0 ml/min, was usually necessary).

Spectrophotometric detection at 240 nm was employed.

3.4. Determination of protonation constant values

The protonation constant values (K^H) were evaluated by means of the STACO program [29], which minimizes the error squares sum on electromotive force values and takes into account eventual variations of ionic strength among and/or during titrations. Monochloroaniline log K^H values are present in the literature [30] while those related to dichloroanilines were experimentally determined in this work by means of alkalimetric titrations. The solutions of the analytes were acidified with HCl in order to induce protonation and to improve solubilization. The concentration range examined was from 1.0 to 4.0 mol/l for C_L (the analytical concentration of the analyte L).

4. Results

4.1. Preliminary experiments

As reported in literature, chloroanilines can be retained in reversed-phase conditions: the best chromatogram that can be obtained in RP mode, with a water–ACN (65:35, v/v) mobile phase, for a mixture of mono- and dichloroanilines shows the coelution of the three monochloroanilines, the coelution of two pairs of dichloroanilines and the separation of just two dichloroanilines (Fig. 1a). The peak identification is: (a) coeluted mono-CAs, (b) 3,4-dCA, (c) 2,3-dCA, (d) coeluted 2,4-dCA and 2,5-dCA, (e) coeluted 2,6-dCA and 3,5-dCA. Any adjustment of the experimental conditions, including pH variations in the range of 3.0 to 8.0, did not increase the resolution of the components of the mixture.

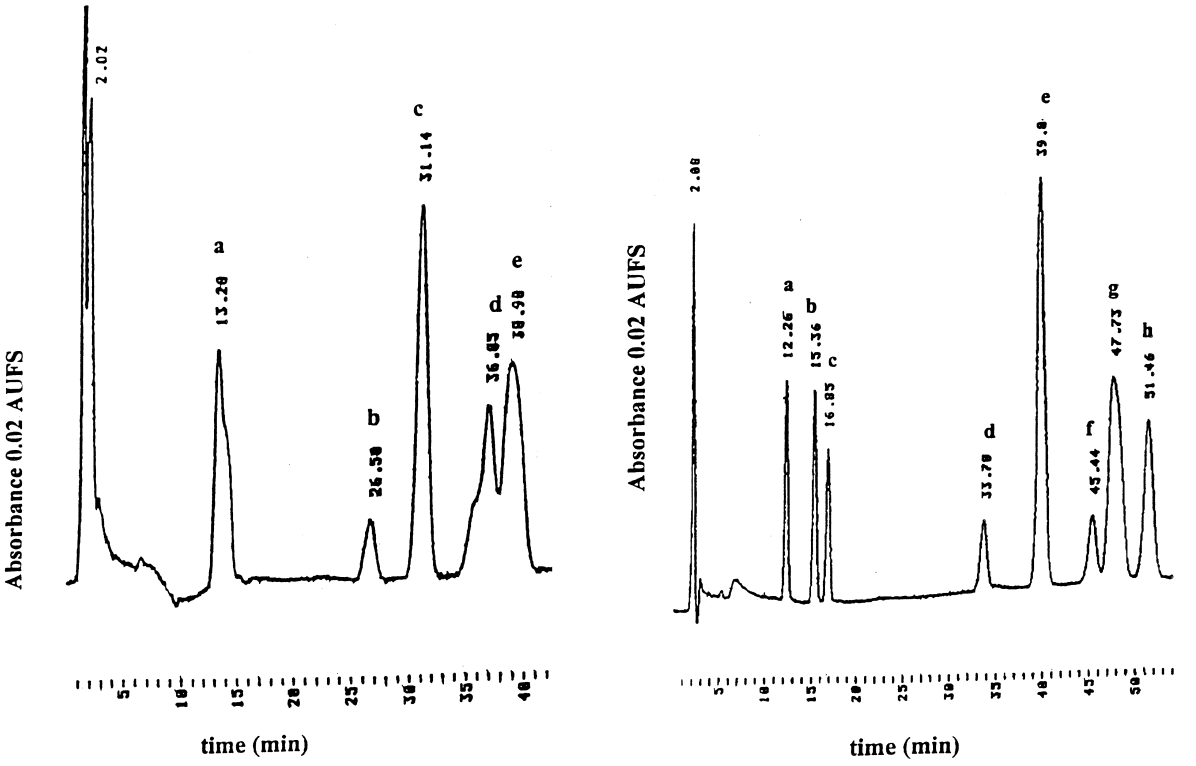

Fig. 1. (a) Chromatogram obtained in reversed-phase mode. Stationary phase: end-capped Superspher 100 RP-18 column (250×4.6 mm, 4 μm). Mobile phase: water–ACN (65:35, v/v), flow-rate 1.0 ml/min. UV detection at 240 nm. Peak identification: (a) 2-CA, 3-CA, 4-CA, (b) 3,4-dCA, (c) 2,3-dCA, (d) 2,4-dCA + 2,5-dCA, (e) 2,6-dCA + 3,5-dCA. (b) Chromatogram obtained in ion-interaction mode. Mobile phase: water–ACN (65:35, v/v), octylamine 1.0 m*M*, pH 4.0, flow-rate 1.0 ml/min. Other conditions as in (a). Peak identification: (a) 4-CA, (b) 3-CA, (c) 2-CA, (d) 3,4-dCA, (e) 2,3-dCA, (f) 2,4-dCA (g) 2,5-dCA + 2,6-dCA, (h) 3,5-dCA.

On the contrary, the addition of 1.0 m*M* octylamine to the same mobile phase and the adjustment by orthophosphoric acid at pH 4.0, greatly improved the resolution. Even if retention times are very similar to those obtained under RP conditions, the three mono- and four of the dichloroanilines are now resolved (Fig. 1b). Still unresolved is the coelution of 2,5- and 2,6-dichloroaniline. Thus verified the significant effect of the addition of alkylamine to the eluent, the study and the optimization of the IIR conditions was performed using experimental design.

4.2. Fractional factorial design

Six variables with potential effect on the retention in IIR mode were taken into account: the number of carbon atoms of the IIR reagent (N), the concentration of the IIR reagent (C_{IIR}), the concentration of the organic modifier (C_M), the ionic strength (I), the mobile phase pH and the mobile phase flow-rate (F). It could be observed that the effect of the elution flow-rate is predictable, but its effect is considered in the chemometric treatment in order to simultaneously optimize all the experimental variables that affect retention.

A full factorial design for six variables and two levels would require 64 experiments. To reduce the number of experiments, a two-level fractional experimental design consisting of 2^{6-3} experiments was used. This reduced design allows the first estimation of the principal factor effects confounded with the second-order interactions. Table 1 reports the experiments performed (experiments 3–10): the experiment in the central point was replicated (experiments 1–2) to estimate the pure experimental

Table 1
Experimental conditions for: central experiment replications (1–2, 11–12, 21, 34); fractional factorial design (3–10); fold–over (13–20); star design (22–33)[a]

Exp.	Design	N	C_{IIR}	C_M	pH	F	I
1	Center repl.	5	$2.75\cdot10^{-3}$	37	5.5	0.75	0.25
2		5	$2.75\cdot10^{-3}$	37	5.5	0.75	0.25
3	Fractional factorial design	1	$0.50\cdot10^{-3}$	34	8.0	1.00	0.50
4		8	$0.50\cdot10^{-3}$	34	3.0	0.50	0.50
5		1	$5.00\cdot10^{-3}$	34	3.0	1.00	0.00
6		8	$5.00\cdot10^{-3}$	34	8.0	0.50	0.00
7		1	$0.50\cdot10^{-3}$	40	8.0	0.50	0.00
8		8	$0.50\cdot10^{-3}$	40	3.0	1.00	0.00
9		1	$5.00\cdot10^{-3}$	40	3.0	0.50	0.50
10		8	$5.00\cdot10^{-3}$	40	8.0	1.00	0.50
11	Center repl.	5	$2.75\cdot10^{-3}$	37	5.5	0.75	0.25
12		5	$2.75\cdot10^{-3}$	37	5.5	0.75	0.25
13	Fold-over	1	$0.50\cdot10^{-3}$	34	3.0	0.50	0.00
14		8	$0.50\cdot10^{-3}$	34	8.0	1.00	0.00
15		1	$5.00\cdot10^{-3}$	34	8.0	0.50	0.50
16		8	$5.00\cdot10^{-3}$	34	3.0	1.00	0.50
17		1	$0.50\cdot10^{-3}$	40	3.0	1.00	0.50
18		8	$0.50\cdot10^{-3}$	40	8.0	0.50	0.50
19		1	$5.00\cdot10^{-3}$	40	8.0	1.00	0.00
20		8	$5.00\cdot10^{-3}$	40	3.0	0.50	0.00
21	Center repl.	5	$2.75\cdot10^{-3}$	37	5.5	0.75	0.25
22	Star design	1	$2.75\cdot10^{-3}$	37	5.5	0.75	0.25
23		8	$2.75\cdot10^{-3}$	37	5.5	0.75	0.25
24		5	$0.50\cdot10^{-3}$	37	5.5	0.75	0.25
25		5	$5.00\cdot10^{-3}$	34	5.5	0.75	0.25
26		5	$2.75\cdot10^{-3}$	40	5.5	0.75	0.25
27		5	$2.75\cdot10^{-3}$	37	5.5	0.75	0.25
28		5	$2.75\cdot10^{-3}$	37	3.0	0.75	0.25
29		5	$2.75\cdot10^{-3}$	37	8.0	0.75	0.25
30		5	$2.75\cdot10^{-3}$	37	5.5	0.50	0.25
31		5	$2.75\cdot10^{-3}$	37	5.5	1.00	0.25
32		5	$2.75\cdot10^{-3}$	37	5.5	0.75	0.00
33		5	$2.75\cdot10^{-3}$	37	5.5	0.75	0.50
34	Center repl.	5	$2.75\cdot10^{-3}$	37	5.5	0.75	0.25

[a] N = Alkyl chain length, C_{IIR} = ion-interaction reagent concentration (M), C_M = organic modifier concentration (%), pH of the mobile phase, F = eluent flow-rate (ml/min), I = ionic strength (for $NaClO_4$) (mM).

error and was replicated four time along the whole experimentation (experiments 11, 12, 21, 34 in Table 1) to check system reproducibility.

It must be noted that as the alkyl chain length is not a continuous variable, there is the possibility to take as the center point either the butyl- or the pentylamine: the latter was chosen. Of consequence, in the values of N, the range scaled value in the central point was not 0, as for the other factors, but 0.12857.

The experimental value of retention times for the experiments are reported in Table 2.

The analysis of these results shows that retention of all the analytes is largely affected by C_M and F and that the retention of 4-CA, 3-CA and 3,4-dCA is also influenced by the pH of the eluent. All the other factors and factor interactions seem to play a smaller role. In particular the small effects played by the alkyl chain length and (mostly) by the concentration of IIR are surprising, since usually these factors are

Table 2
Experimental results (retention times, min) obtained by direct (experiments 3–10), fold-over experimental design (experiments 13–20) and a star design (experiments 22–33)

Experiment	4-CA	3-CA	2-CA	3,4-DCA	2,3-DCA	2,4-DCA	2,5-DCA	2,6-DCA	3,5-DCA
1	15.37	17.36	18.42	35.37	41.51	48.83	49.17	49.92	53.28
2	15.04	16.92	17.97	34.09	40.09	46.72	47.11	48.41	51.23
3	14.93	16.77	17.41	38.56	44.82	51.91	52.48	52.45	59.86
4	11.77	17.44	27.46	51.02	72.34	84.42	84.76	85.70	91.04
5	6.50	10.48	15.35	29.38	39.02	44.28	47.04	47.27	49.53
6	25.70	29.11	30.57	59.59	70.18	80.02	84.64	84.71	90.34
7	17.76	19.61	21.10	34.45	40.41	46.91	47.43	49.76	49.58
8	5.10	7.57	10.08	15.87	19.91	22.79	23.39	24.25	24.19
9	9.36	13.13	20.30	32.62	45.99	53.29	53.72	56.10	55.78
10	8.70	9.61	10.36	17.72	20.88	24.04	24.05	25.65	25.84
11	13.19	14.66	15.71	27.91	32.83	38.22	38.27	39.95	41.33
12	14.50	16.24	17.28	32.19	37.77	43.91	44.26	45.61	48.05
13	17.44	23.91	31.48	60.92	77.75	88.26	93.51	93.65	99.52
14	13.10	14.74	15.52	30.75	35.80	40.94	42.50	43.09	46.27
15	26.29	29.21	30.81	63.35	74.95	86.52	86.77	89.20	97.08
16	5.49	8.32	13.76	25.81	37.60	43.37	43.59	43.91	47.11
17	4.76	6.66	10.20	16.53	23.16	26.71	26.87	28.17	28.07
18	18.96	21.03	22.52	40.03	47.21	54.63	54.84	57.17	59.20
19	9.10	10.08	10.80	17.93	21.05	23.87	24.67	25.84	25.84
20	9.36	15.02	20.64	32.48	41.19	46.59	48.55	50.24	49.85
21	13.17	14.72	15.73	28.05	32.90	37.81	38.45	40.10	41.22
22	13.49	15.04	16.10	28.96	34.02	39.04	39.78	41.54	42.72
23	14.24	15.94	17.06	31.25	36.80	42.13	42.93	44.32	46.61
24	13.65	15.20	16.26	29.33	34.56	39.52	40.37	42.08	43.36
25	13.38	14.88	15.94	28.53	33.54	38.61	39.20	40.90	42.13
26	17.54	19.68	20.58	42.13	48.74	56.64	57.01	59.25	62.93
27	11.36	12.53	13.49	22.40	26.29	30.13	30.50	32.21	32.16
28	6.88	10.02	14.98	24.85	33.92	38.40	39.78	41.33	41.38
29	13.33	14.72	15.84	28.05	33.17	37.86	38.72	40.37	41.49
30	19.68	21.76	23.46	41.28	49.01	55.78	57.38	59.57	61.38
31	10.08	11.20	12.00	21.60	25.33	29.17	29.54	30.93	31.73
32	13.76	15.25	16.37	28.53	33.65	38.08	39.73	41.01	42.02
33	13.28	14.66	15.68	28.48	33.54	38.72	38.82	40.80	42.08
34	13.28	14.66	15.84	28.00	33.28	37.76	38.88	40.26	41.70

important in ion-interaction retention. For this separation we found that: (i) the main effects are given by the traditional reversed-phase factors; and (ii) the addition of alkylammonium is of fundamental importance to improve the resolution (see the comparison of the chromatograms in Fig. 1a and b).

4.3. Fold-over fractional factorial design

To go deeper in the study of the retention mechanism, in the same range of experimental factors, a complementary set of experiments, was performed (Table 1, experiments 13–20). This represents a fold-over of the previous design and is obtained by changing the sign of the three last columns of the fractional design. A fold-over design is suggested to separate the principal effects from the confounded second-order interactions.

The combined results (fold-over+fractional factorial design) confirm the results previously observed. For all the analytes the largest effects are due to C_M and F, which are the typical factors that govern the retention in the reversed-phase mode. No significant effect is due to N and C_{IIR}.

Since pH seems to affect the retention of only three anilines, (4-CA, 3-CA and 3,4-dCA) the protonation constants of the analytes (not available in literature) were determined.

Table 3
Values of the log of the protonation constants at 298 K and $I=0.1$ mol/l for the analytes considered

Analyte	log $K^H \pm 3s$	Ref.
Aniline	4.65	[27]
2-CA	2.69	[27][a]
	2.74±0.01	This work
3-CA	3.52	[27][a]
4-CA	3.97	[27][a]
2,3-dCA	1.69±0.02	This work
2,4-dCA	1.98±0.03	This work
2,5-dCA	1.56±0.06	This work
2,6-dCA	0.6±0.1	This work
3,4-dCA	2.88±0.02	This work
3,5-dCA	2.45±0.02	This work

[a] $I=0$ mol/l.

4.4. Protonation constants

Table 3 reports the results obtained for the protonation constants of the dichloroanilines and of 2-chloroaniline to check the whole of the procedure: the 2-chloroaniline log $K^H=2.74\pm0.01$ ($3s$) obtained is in good agreement with the literature value. It can be observed that, owing to the presence of the electron-drawing effect of the –Cl substituent, chloroanilines show acidic properties when compared to aniline (whose log K^H is 4.65 at 298 K and $I=0.1$ mol/l) and that the position along the aromatic ring affects the value of the protonation constants.

4.5. Star design and separation optimization

The scatter plot representing the experimental versus the predicted retention times shows a satisfactory fitting ability of the regression models (R^2 always≥0.90). For 2,5-, 2,6-, 3,5-dCA the central experiments show large differences between experimental and calculated results: the deviation could be due to the regression model inadequacy and in particular to a non-linear behavior of the system. The existence of quadratic significant effects was tested by means of an F-test that compares the difference between the experimental and the estimated retention time in the central point with the purely experimental variance:

$$F_{(1,\nu,\alpha)} = \frac{(\bar{y}_0 - \bar{y}_F)^2}{s_{pe}^2 \cdot \left(\frac{1}{n_0} + \frac{1}{n_F}\right)}$$

where $\bar{y}_0$ is the average response of the replicated central experiment and $\bar{y}_F$ the average response from the factorial design experiments, which represents the estimated value in the center.

Since several values lay close to the critical F value (10.13), which is exceeded for 4-CA, it was concluded to further analyze the quadratic effects.

For this purpose 13 experiments of a star design (reported in Table 1 as experiments 22–35) were added to the factorial experimental design to provide a composite design. We could have tested only the quadratic effects of F and C_M (four experiments), the most important factors, but for completeness all the experiments were performed and the central point was again replicated (experiment 34 in Table 1) in order to be able to perform the calculation of a complete second-order regression model.

The best regression models were obtained by a variable selection algorithm (forward search) and the final models and their R^2 values are reported in

Table 4
Regression models and multiple determination coefficient (R^2) obtained for each analyte

Analyte	Regression model	R^2
4-CA	$y=14.09-4.36F+3.96\text{pH}-2.46C_M-1.62\text{pH}^2-1.05\text{pH}\cdot F$	0.9233
3-CA	$y=15.53-5.27F+2.91\text{pH}-3.03C_M+0.90N\cdot C_M+0.91\text{pH}\cdot F$	0.9270
2-CA	$y=16.45-6.27F-3.52C_M+2.65F^2+0.95C_M\cdot F$	0.9652
3,4-dCA	$y=29.74-11.20F-9.55C_M+5.34C_M^2+2.43C_{IIR}\cdot\text{pH}+2.28\text{pH}+1.84N\cdot C_M$	0.9593
2,3-dCA	$y=34.72-13.97F-11.95C_M+4.93C_M^2+3.01C_M\cdot F+4.59F^2$	0.9660
2.4-dCA	$y=39.88-16.07F-13.74C_M+5.94C_M^2+3.42C_M\cdot F+5.03F^2$	0.9614
2,5-dCA	$y=40.62-16.53F-14.35C_M+5.87C_M^2+3.66C_M\cdot F+5.58F^2$	0.9653
2,6-dCA	$y=42.21-16.92F-13.88C_M+5.78C_M^2+3.57C_M\cdot F+5.30F^2$	0.9686
3,5-dCA	$y=44.45-17.52F-16.29C_M+10.78C_M^2+4.05C_M\cdot F$	0.9504

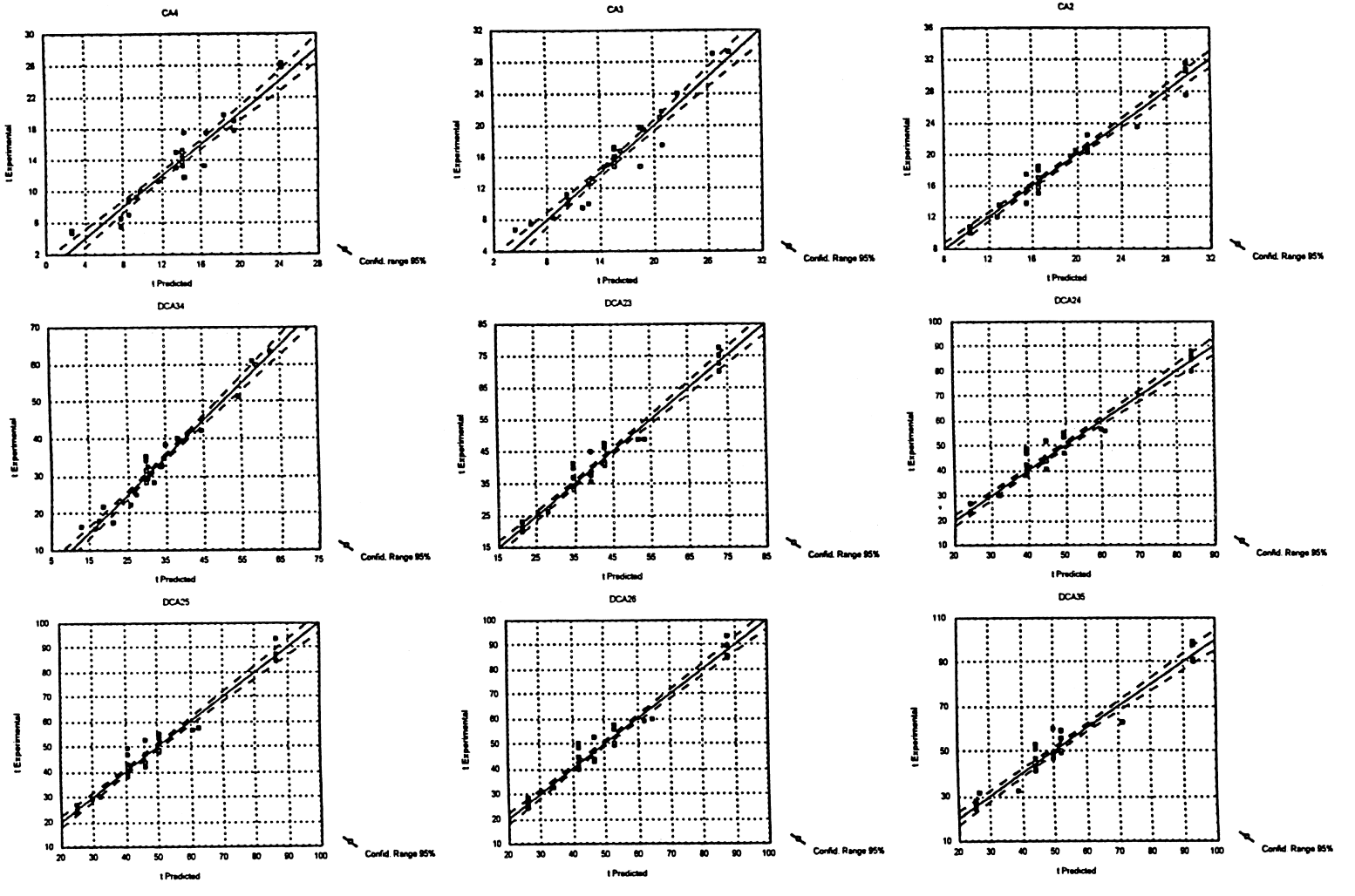

Fig. 2. Plot of the experimental vs. cross-validated predicted retention times according to the regression models reported in Table 4 for the nine chloroanilines.

Table 4 while Fig. 2 shows the experimental versus the calculated retention times: the satisfactory fitting of the models can be appreciated.

The final models confirm that, in retention, the reversed-phase mechanism predominates, since F and C_M have the most significant effects. Anyway, for 4-CA, 3-CA and 3,4-dCA some role is also played by the eluent pH and by its interaction with C_{IIR}. Retention increases with increasing pH and seems to be strictly related to the log K^H of the chloroanilines (Table 1), with the largest effect observed for chloroanilines with higher log K^H values (3-CA, 4-CA and 3,4-dCA).

Ionic strength does not seem to affect retention and this result seems advantageous since it suggests the easy application of the method for surface waters.

In order to optimize the separation of the nine chloroanilines a grid search algorithm was employed, by imposing conditions for a maximum t_R difference between the nearest peaks. For the optimization the further condition of a maximum analysis time of 1 h

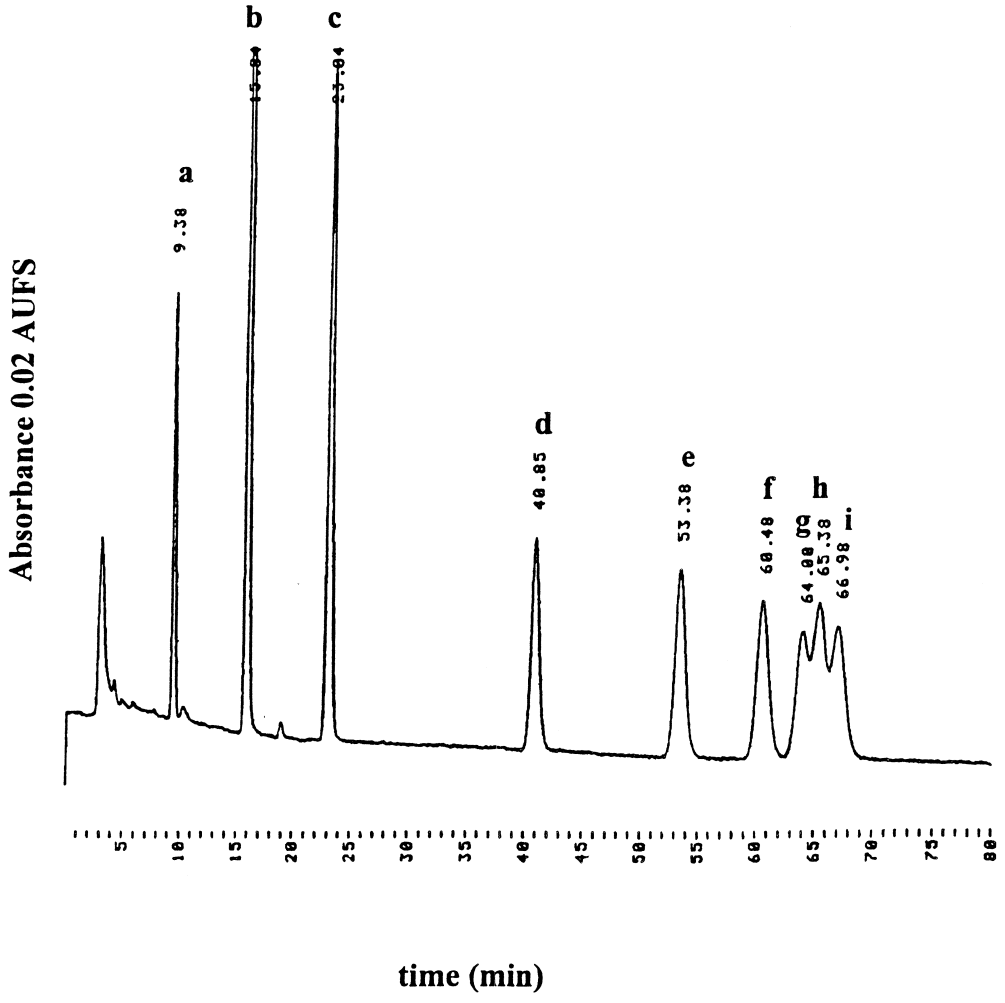

Fig. 3. Chromatogram obtained in the ion-interaction unconstrained optimized conditions: water–ACN (64:36, v/v), pH 3.0, flow-rate 0.6 ml/min, 2.75 m*M* butylamine orthophospate. Peak identification: (a) 4-CA, (b) 3-CA, (c) 2-CA, (d) 3,4-dCA, (e) 2,3-dCA, (f) 2,4-dCA, (g) 2,5-dCA, (h) 2,6-dCA, (i) 3,5-dCA.

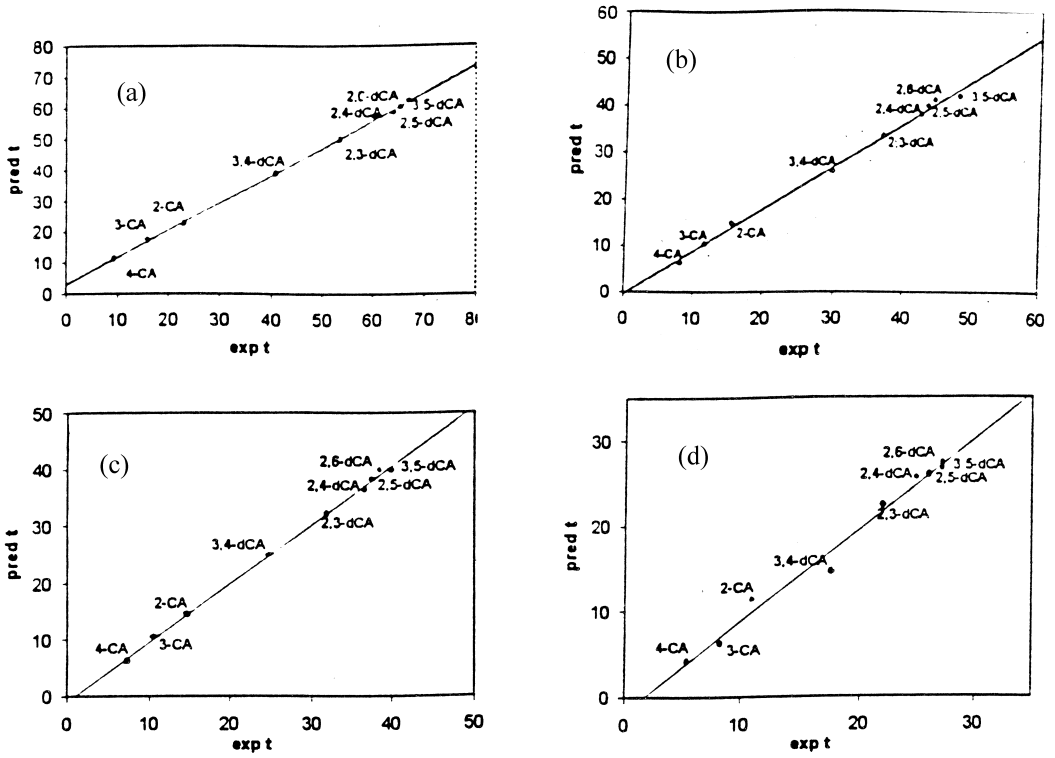

Fig. 4. Predicted versus experimental retention times obtained in the (a) unconstrained and (b) constrained $t_{max}<50$ min; (c) $t_{max}<40$ min; (d) $t_{max}<30$ min. Conditions: (a) flow-rate=0.60 ml/min, pH=3.0, $C_M=36\%$, $N=4$ ($2.75\cdot10^{-3}$ M); (b) flow-rate=0.90 ml/min, pH=3.0, $C_M=35\%$, $N=4$ ($2.75\cdot10^{-3}$ M); (c) flow-rate=0.90 ml/min, pH=3.0, $C_M=36\%$, $N=4$ ($2.75\cdot10^{-3}$ M); (d) flow-rate=0.95 ml/min, pH=3.0, $C_M=39\%$, $N=4$ ($2.75\cdot10^{-3}$ M).

was imposed. The grid search algorithm used takes into account these constraints and allows the achievement of the best conditions by iterating the optimization procedure with progressively shorter search steps.

The chromatogram obtained under the predicted conditions (Fig. 3) shows the resolution of all the analytes, even if three dichloroanilines are not baseline separated. Fig. 4a–d, in turn, show the good agreement between predicted and experimental retention times under the optimized conditions, both constrained and unconstrained: the maximum percent error is 7.5.

Under the optimized conditions linearity between peak area and concentration was verified by building calibration plots. Regression coefficients were always greater than 0.99 in the concentration range between the quantitation limits and 2.0 mg/l.

From sensitivity (peak area for 1 mg/l concentration) and a signal-to-noise ratio of 3 the following detection limits were evaluated: less than 6.0 μg/l for 3-CA, below 10.0 μg/l for 2,3- and 2,4-dCA; under 14.0 μg/l for 4-CA, 3,4-dCA and 2,5-dCA.

5. Conclusions

Notwithstanding the retention models here obtained for the separation of nine chloroanilines show that both IIR alkyl-chain length and IIR concentration have small effects on the retention, the presence of alkylamine in the mobile phase was proved to be necessary for analyte resolution.

This behavior may be perhaps explained by considering the participation on the retention mechanism

of free silanols, that are always present also on ODS surfaces treated by end-capping processes. Since silanol groups are known to affect the amine peak shapes and to give tailing phenomena, it can be assumed in the separation of chloroanilines the addition of alkylamine (as IIR) to the eluent may in some cases mask the silanol action leading to a better separation efficiency.

In conclusion by the chemometric analysis of the experimental data, it can be said that:

1. Retention of chloroanilines mainly occurs by hydrophobic interactions and the most important variables are C_M and F.
2. Mobile phase pH, that also intervenes in interaction with other factors, has an effect related to the log K^H of the chloroanilines and can help in achieving resolution.
3. The presence of some alkylamine is necessary to obtain the well-shaped and sharp peaks that allow the resolution.
4. It is possible to build regression models with descriptive and predictive ability, which allows one to optimize the resolution of the mixture of the nine chloroanilines.
5. A separation method has been developed able to separate the nine mono- and dichloroanilines, with detection limits always lower than 14.0 μg/l.

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