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Short communication

Simultaneous reversed-phase high-performance liquid chromatographic separation of mono-, di-and trichloroanilines through a gradient elution optimised by experimental design

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Abstract

A new RP-HPLC method for the simultaneous determination of the 13 mono-, di- and trichloroanilines has been developed. In order to obtain the analyte resolution within an acceptable analysis time, a gradient elution program has been optimised through the use of an experimental design and a grid search algorithm. The optimized conditions provided the resolution of all the analytes in less than 80 min. The primary validation of the analytical method gave limit of detection values ranging between 0.02 and 0.06 mg/l and very good linearity of the calibration curves.

Keywords: Gradient elution; Experimental design; Chloranilines; Anilines; Amines

1. Introduction

Chloroanilines are widely used in industry as intermediate reagents in the synthesis of pigments, pharmaceutical, paints, etc. and are widely emitted into the environment through industrial waste waters. Some chloroanilines are reported to form in soils and waters through the degradation of largely used phenylurea pesticides and of some antibacterial agents, as chlorhexidine [1] and trichlocarban [2], added in hygiene products. Before the 1990s little attention has been devoted to the possible toxicity of chloroanilines, essentially because of their average good solubility in waters and their relatively general low concentrations. In turn, their large use associated to the low biodegradability increases their ubiquitous diffusion into the environment. The evaluation of the toxicity levels of chloroanilines has brought the US Environmental Protection Agency (EPA) to include these analytes in the list of so-called ''priority pollutants'' that must be quantified to evaluate the health of waters and soils, for the safeguard of the ground and drinkable waters.

In particular, the effect concentration (EC_{50}) for 2-chloroaniline was evaluated between 0.46 and 160 mg/l for fresh water species and 15 mg/l for sea water species; for 3-chloroaniline the ranges are 0.4–64.0 mg/l for fresh waters species and 0.46–160.00 for sea waters ones. Among monochloroanilines, the most toxic is 4-choloraniline with EC_{50}

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values of 0.10–43.0 mg/l (fresh water species) and 5.1 mg/l (sea water species). Similar data are reported for dichloroanilines, whose EC_{50} values lay between 0.14 and 18.00 mg/l for fresh waters species. Different toxicity levels characterise trichloroanilines: EC_{50} for 2,3,4- and 3,4,5- is around 1.4 mg/l, for 2,4,5- it is between 22.0 and 24.0 and for -2,4,6 between 1 and 10 mg/l [3].

Due to the different toxicity, suitable methods are required for the identification and determination of individual chloroanilines in waters and soils. In the literature, HPLC and GC methods are reported, with the use of different detectors. The pre-treatment of the samples is generally performed by an ion-exchange procedure or by liquid–liquid extraction methods. Most of the methods concern the determination of 4-chloroaniline, likely because of its highest toxicity; since it can also form in waters through degradation processes, methods were developed to separate 4-chloroaniline from parent compounds, as for example chlorhexidine [1,1′-hexamethylenebis-5-(*p*-chlorophenyl)biguanide] that is largely used as antibacterial agent in hygiene products and in cosmetics and that can naturally degrade in aqueous solution to 4-Chloroaniline: an ion-pair RP-HPLC [4] and a RP-HPLC method associated to a proper choice of the stationary phase [5] were employed. 4-chloroaniline was separated from chlorhexidine breakdown products in surgical scrubs by RP-HPLC [1] and from 3,4-dichloroaniline in antibacterial soaps containing trichlocarban by means of cation-exchange chromatography and UV detection [2]. HPLC methods with pre column fluorescamine derivatisation were employed to separate 4-chloroaniline, aniline, p-toluidine and 4-bromoaniline in environmental waters [6]. 4-Chloroaniline was also separated from 2-chloroaniline in sulfanilamides and sulfamicide industrial waste waters by RP-HPLC in the presence of ammonium carbamate [7], from phenols and polycyclic aromatic hydrocarbons with ion-interaction HPLC (sulfonates as ion-interaction reagents) [8] and from 4-chlorophenylurea in honey by HPLC and electrochemical detection [9]. 2-Chloroaniline was determined by HPLC in soils in the presence of aromatic amines and nitro-compounds [10].

Also dichloroanilines are supposed to form through degradation processes. 3,5-Dichloroaniline was determined by RP-HPLC in the presence of some pesticides in white must and wine extracts [11]. 3,4-Dichloroaniline was separated from bromacil and diuron (suspected to degrade to chloroanilines) in contaminated well waters by an HPLC column-switching procedure [12] and from diuron [*N′*-(3,4-dichlorophenyl)-*N,N*-dimethylurea] and its metabolites in surface and ground waters [13]. An HPLC method with amperometric detection was employed for the determination of traces of 3,4-dichloroaniline in pesticides [14]. With GC–MS methods monochloroanilines, together with 2,4-, 2,5- and 2,6-dichloroanilines, were determined in the water of the river Elbe near Hamburg, in the presence of some hundreds of other pollutants like hydrocarbons, haloethers, chlorobenzenes, nitro compounds, chloronitrobenzenes [15]. A mixture of 14 chloroanilines and chloromethylanilines was separated by RP-HPLC with UV detection at 240 nm after extraction performed by graphitic carbon black and ion-exchange columns [16].

Apart from the last two mentioned papers [15,16], the others concern the separation and determination of only some of the existing mono-, di- and trichloroanilines.

The simultaneous separation of mono- and dichloroanilines was recently achieved in our laboratory [17]. An ion-interaction method (alkylamine orthophosphate as the ion-interaction reagent) was initially used since, as observed by other authors, resolution greatly improves in the presence of an ion-interaction reagent in the mobile phase. An experimental design was used to optimise the conditions of pH, acetonitrile percentage, chain length and concentration of alkylamine. It was shown that, while pH and acetonitrile concentration greatly affect retention, the presence of the ion-interaction reagent is important to improve resolution, but its effect seems to be independent on both the alkyl chain and the concentration. It was concluded that the ion-interaction reagent, generally an alkylamine, plays its role on the residual silanol groups present on the stationary phase surface so avoiding the broadening of the peaks due to the interactions between silanol groups and the analytes so improving the resolution.

In this paper we present the simultaneous separation of the 13 (three mono-, six di- and four tri-) chloroanilines. A conventional reversed-phase HPLC

method is used in which the pH of the mobile phase is controlled and the addition of ion-interaction reagent is not required.

2. Experimental

2.1. Reagents

The 13 chloroanilines were purchased by Sigma-Aldrich (Milan, Italy). The HPLC-grade acetonitrile was purchased by Merck (Darmstadt, Germany). Ultrapure water was obtained by a Millipore Milli-Q system (Bedford, MA, USA). All the other reagents are ultra-pure-grade chemicals.

2.2. Apparatus

Chromatographic measurements were obtained by a Merck–Hitachi (Tokyo, Japan) chromatograph equipped with a L-7100 pump, a L-7400 UV detector, a L-7450 A diode array detector and a D-7000 interface.

pH Measurements were obtained by a Metrohom 654 pH meter (Herisau, Switzerland) equipped with a combined glass-calomel electrode.

2.3. Chromatographic conditions

Merck LiChrospher 100 RP-18 5 μm (250×4 mm) endcapped was the stationary phase. The experiments of the factorial design required the preparation of different mobile phases characterised by different combinations of the variables involved (pH and acetonitrile concentration). The detection has been performed at 240 nm where all the species show significant absorbtivity values.

2.4. Results

To optimise the two experimental variables considered (the pH and the concentration of acetonitrile in the mobile phase), a factorial experimental design was planned [18–20]. On the basis of preliminary experiments, the range of acetonitrile was chosen between 30 and 40% and the range of pH between 3.0 and 8.0. In addition to the four experiments required by the 2-factor-2-level factorial experimental design, in order to evaluate the pure experimental error three replicates have been performed in the centre of the experimental domain (pH 5.5 and acetonitrile percentage 35%).

The results obtained permitted to build mathematical models correlating the retention times to the two variables and their interactions. The greater effect is, as expected, played by the acetonitrile concentration. The effect of pH is mainly observed for monochloroanilines.

Through a grid-search algorithm [21] the following conditions for the best separation of the 13 chloroanilines were obtained: pH 3.0 and acetonitrile percentage of 30%.

In the chromatogram recorded under these conditions three well-separated groups of peaks can be individuated, that respectively correspond to the mono-, di- and trichloroanilines. It follows that the method allows the separation between chloroanilines containing different numbers of –Cl groups, but is not able to separate the isomers. In particular, the separation between 2,5- and 2,6-dichloroaniline is not achieved and only three out of the four trichloroanilines are resolved.

The use of greater concentrations of acetonitrile or of a gradient elution could shorten the total analysis time and make closer the retention times of the three groups, but could not favour the within-group resolution. An elution program should therefore be planned in which the acetonitrile concentration is kept constant at a suitable value within the window of the single group of analytes, while it is increased in the two time windows between the three groups of isomers. This strategy has been investigated by the help of a factorial design in which the five variables considered are, as represented in the plot of Fig. 1:

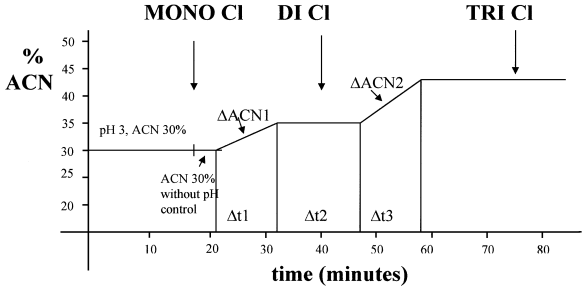

Fig. 1. Graphic representation of the gradient elution program.

Table 1
Range of variables used in the 2^{5-1} fractional factorial design

Value	Δ*t1*	Δ*t2*	Δ*t3*	ΔACN1	ΔACN2
+	15	25	10	10	10
−	10	15	5	5	6
0	12.5	20	7.5	7.5	8

Table 2
The experiments of the fractional 2^{5-1} factorial design

Experiment	1, Δ*t1*	2, Δ*t2*	3, Δ*t3*	4, ΔACN1	1·2·3·4 ΔACN2
1	−	−	−	−	+
2	+	−	−	−	−
3	−	+	−	−	−
4	+	+	−	−	+
5	−	−	+	−	−
6	+	−	+	−	+
7	−	+	+	−	+
8	+	+	+	−	−
9	−	−	−	+	−
10	+	−	−	+	+
11	−	+	−	+	+
12	+	+	−	+	−
13	−	−	+	+	+
14	+	−	+	+	−
15	−	+	+	+	−
16	+	+	+	+	+

1. Δt1: the time required to pass from a percentage of acetonitrile (ACN) ACN1=30% to a percentage ACN2
2. Δt2: the time during which the acetonitrile percentage is kept constant at the value ACN2
3. Δt3: the time required to pass from the acetonitrile percentage ACN2 to the acetonitrile percentage ACN3
4. ΔACN1: the variation of acetonitrile percentage from ACN1 to ACN2 (ΔACN1=ACN2−ACN1)
5. ΔACN2: the variation of acetonitrile percentage from ACN2 to ACN3 (ΔACN2=ACN3−ACN2)

To our knowledge, no example is present in literature in which experimental design is used to optimise gradient elution programs. Since a complete 2-level factorial design for five factors would require 32 experiments, a fractional 2-level design 2^{5-1} was employed (16 experiments). In all the experiments from $t=0$ to $t=18$ min the conditions of pH and acetonitrile are kept constant at the values (pH 3.0 and ACN1=30%) that correspond to the best conditions for the separation of monochloroanilines.

Table 1 reports the range chosen for the variables in the factorial design. As usual + and − indicate respectively the greater and the lower value of each experimental factor, while 0 indicates the central point; Table 2 reports the experimental design.

Table 3
The effect of the factors of the fractional 2^{5-1} factorial design. In bold the significant effects are reported

Factor	Positions of chloro substitution in chloroanilines									
	3,4	2,3	2,4	2,5	2,6	3,5	3,4,5	2,4,5	2,3,4	2,4,5
Mean	**36.7131**	**40.0793**	**43.0843**	**44.2725**	**44.5656**	**46.4068**	**61.5087**	**67.3575**	**67.2300**	**73.6131**
Δ*t1*	**0.9737**	**1.2587**	**1.2387**	**1.5000**	**1.3312**	**1.1712**	**2.3950**	**3.0650**	**3.0825**	**2.9487**
Δ*t2*	0.2412	0.1937	0.18625	**0.4950**	0.4762	**0.5162**	**3.3625**	**4.9875**	**4.9625**	**5.3262**
Δ*t1*·Δ*t2*	0.0887	0.1437	0.1162	**0.2250**	0.4112	0.2487	−0.3300	−0.1525	**−0.3300**	−0.1312
Δ*t3*	0.0812	0.0412	0.0087	**−0.5525**	−0.0062	−0.1112	**0.5875**	**1.2725**	**0.6925**	**1.2137**
Δ*t1*·Δ*t3*	−0.2912	**−0.3987**	**−0.4162**	**−0.3875**	−0.7362	**−0.4587**	**−0.9550**	**−0.8125**	**−0.5050**	**−0.5187**
Δ*t2*·Δ*t3*	−0.1787	−0.2187	**−0.2537**	**−0.1925**	−0.1962	−0.2037	−0.3475	−0.1150	−0.2100	**−0.4212**
ΔACN1·ΔACN2	0.0287	0.1312	0.1212	**0.3175**	0.0487	**0.3437**	**0.9100**	**1.7600**	**1.8075**	**2.3262**
ΔACN1	**−0.8687**	−1.9712	**−3.2637**	**−4.2500**	**−4.1062**	**−4.9337**	**−9.7950**	**−9.7875**	**−10.0075**	**−11.3230**
ΔACN1·Δ*t1*	0.2537	**0.3637**	**0.3962**	**0.4500**	0.3187	**0.5387**	−0.2525	−0.2575	−0.0950	0.2737
ΔACN1·Δ*t2*	0.3012	**0.3487**	**0.3487**	**0.2850**	0.3037	0.2637	**−1.8300**	**−0.4150**	**−0.4500**	0.1712
ΔACN2·Δ*t3*	0.1537	0.0837	0.0887	**−0.3700**	−0.3462	−0.1987	0.0725	−0.2050	−0.1825	0.3887
ΔACN1·Δ*t3*	0.1212	0.2162	**0.2462**	**0.6725**	0.6762	0.2662	−0.1250	0.3350	**0.2800**	0.2587
ΔACN2·Δ*t2*	0.2237	0.1712	0.1712	**0.3825**	0.5112	0.2437	**0.6075**	**1.0200**	**0.9625**	**0.9712**
ΔACN2·Δ*t1*	**0.9162**	**0.8712**	**0.9187**	**0.9075**	**1.1312**	**0.9487**	**1.2800**	**1.0775**	**0.8025**	**1.1987**
ΔACN2	0.0487	0.0262	0.0037	−0.0475	−0.1287	−0.1487	**−0.6975**	**−2.1875**	**−1.9100**	**−3.9887**

The effects of the factors have been calculated and reported in Table 3. In bold are represented the significant effects, i.e. the effects that are greater than the critical value b calculated as:

$$b_{\text{critical}} = S_b t_{a,\nu}$$

where:

$$S_b = \sqrt{\frac{4S_{\text{pe}}^2}{N}}$$

where S_{pe}^2 is the pure experimental variance, N is the number of experiments of the factorial design and $t_{\alpha,\nu}$ is the t-value for ν = the freedom degrees of S_{pe}^2 and α the significance level chosen ($\alpha = 0.05$). The values of b_{critical} are reported in Table 4.

The models obtained for the di- and trichloroanilines have been used for the optimisation of the resolution of the mixture. A grid-search algorithm has been applied, searching for the conditions that maximise the resolution between adjacent peaks. The best conditions were found for the following values:

$\Delta t1 = 10.9$, $\Delta t2 = 24.9$, $\Delta t3 = 5$, $\Delta\text{ACN1} = 5$,

$\Delta\text{ACN2} = 10$

We remind that the eluent has been kept constant between 0 and 18 min (pH 3.0 and ACN = 30.0%), i.e. during the monochloroanilines elution.

The experiment performed under the optimised conditions gives the chromatogram of Fig. 2, in which, within 80 min, all the 13 analytes are separated.

In the optimised conditions the calibration models have been calculated, in a concentration range between 0.25 and 2.00 mg/l. All the calibration models exhibit a linear behaviour, with R^2 values always greater than 0.9925. From sensitivity (peak area for 1 mg/l concentration) evaluated for each analyte and for a generic signal-to-noise ratio of 3, limits of detection (LODs) (Table 5) lower than 30 μg/l for 3-chloro-, 2-chloro-, 3,4-dichloro-, 2,4-dichloroanilines and lower than 60 μg/l for all the other analytes were calculated.

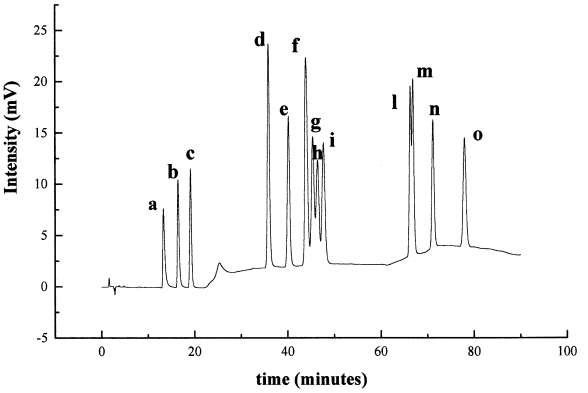

Fig. 2. Chromatogram obtained under the optimised conditions of the 2^{5-1} fractional experimental design. Stationary phase as in Fig. 1. Gradient elution: 0.0–18.0 min: 100% optimised mobile phase (water–ACN, 70:30; pH 3.0); 18.0–22.0 min: from 100% optimised mobile phase water–ACN (70:30); 22.0–32.9 min: from water–ACN (70:30) to water–ACN (65:35); 33.0–57.9 min: water–ACN (65:35) kept constant; 58.0–63.0 min: from water–ACN (65:35) to water–ACN (55:45); 63.0 min to the end: water–ACN (55:45) kept constant. Flow rate: 1 ml/min. Peak identification: (a) 4-chloroaniline, (b) 3-chloroaniline, (c) 2-chloroaniline, (d) 3,4 dichloroaniline, (e) 2,3-dichloroaniline, (f) 2,4-dichloroaniline, (g) 2,6-dichloroaniline, (h) 2,5-dichloroaniline, (i) 3,5-dichloroaniline, (l) 3,4,5-trichloroaniline, (m) 2,4,5-trichloroaniline, (n) 2,3,4-trichloroaniline, (o) 2,4,6-trichloroaniline. Analytes concentrations: mono-chloroanilines: 1.0 mg/l, di-and trichloroanilines: 2 mg/l. Injected volume: 100 μl. UV detection at 240 nm.

3. Conclusions

A new method for the RP-HPLC simultaneous determination of all mono-, di- and trichloroanilines has been developed. The isocratic method, optimised

Table 4
The values of b_{critical} calculated as $b_{\text{critical}} = s_b \cdot t_{\alpha,\nu}$

	Positions of chloro substitution in chloroanilines									
	3,4	2,3	2,4	2,5	2,6	3,5	3,4,5	2,4,5	2,3,4	2,4,6
S_{pe}^2	0.146	0.025	0.011	0.003	0.169	0.019	0.030	0.025	0.012	0.036
s_b	0.191	0.079	0.052	0.028	0.205	0.070	0.086	0.080	0.056	0.095
b_{crit}	0.823	0.341	0.228	0.122	0.885	0.301	0.373	0.345	0.244	0.412

Table 5
values (mg/l) for all the analytes considered

Analyte	LOD (mg/l)	Analyte	LOD (mg/l)
4-Chloroaniline	0.05	2,6-Dichloroaniline	0.06
3-Chloroaniline	0.02	3,5-Dichloroaniline	0.05
2-Chloroaniline	0.02	3,4,5-Trichloroaniline	0.05
3,4-Dichloroaniline	0.03	2,4,5-Trichloroaniline	0.05
2,3-Dichloroaniline	0.05	2,3,4-Trichloroaniline	0.04
2,4-Dichloroaniline	0.03	2,4,6-Trichloroaniline	0.05
2,5-Dichloroaniline	0.04		

by experimental design and grid search with respect to pH and acetonitrile concentration provided a too long analysis time and an incomplete resolution of all the analytes. Since under the optimised conditions the chromatogram contains three different groups of peaks corresponding to the three families of chloroanilines (mono-, di- and tri-), a gradient elution program was investigated by a factorial experimental design and a grid search. The optimised conditions allowed a good resolution of all the analytes with LOD values between 0.02 and 0.06 mg/l.

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