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Large injection volumes in capillary liquid chromatography: Study of the effect of focusing on chromatographic performance

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ABSTRACT

This paper describes a multivariate approach to study the effect on chromatographic conditions and to optimize such conditions in capillary liquid chromatography when high injection volumes are required. Several separations have been evaluated by using isocratic and gradient solvent elution, as well as isocratic elution combined with temperature programming. In this study, easily ionisable organic compounds with low log *P* have been used as representative analytes. Injection volume and nature of the injection solution have been evaluated in order to increase the sensitivity (peak area) and column performance (*N* values). The equations obtained by multiple linear regressions and response surfaces allow achieving the optimum on-column focusing conditions for chlorophenoxy acids, carbamates and heterocyclic amines.

1. Introduction

The most common method for the determination of traces and ultra traces of ionisable or thermally unstable organic compounds is liquid chromatography (LC) with different detectors in combination with pre-treatment methods. The determination of organic pollutants in soils, water or food has been described using a wide variety of techniques to preconcentrate and purify the analytes, such as liquid–liquid extraction, solid phase extraction (SPE), on-line SPE and solid phase microextraction.

Capillary LC (cLC) with packed 0.1–0.5 mm inner diameter columns has been established as an alternative technique to conventional sized LC [1]; cLC is environmentally friendly (low consumption of solvents, samples and reagents, and low waste), cost saving and, in general, better suited for screening and/or for coupling separation techniques to each other and with various detection techniques. In addition, the relatively low heat capacity and the small diameter of the columns allow using temperature programming [2,3]. Opposite to other miniaturized techniques like capillary electrophoresis, up- and down-scaling of capillary LC methods is possible by adjusting the separation to the actual analytical requirements, e.g., with respect to the available sample amount [1]. A general problem in cLC is the loss of sensitivity due to the small volumes or masses injected. This problem is associated with the need to adapt injection volume to the size of the column to prevent band broadening. In some cases, this problem can be overcome by the use of so-called on-column focusing techniques with large injection volumes [1–6]. In these techniques, the sample solvent has significantly lower elution strength compared to that of the mobile phase at the beginning of the chromatographic run [7–9] or is set at a lower temperature than that of the mobile phase [2]. For analytical separations, it is usually preferable that the sample is dissolved in the mobile phase. In this case, there is no difference in solvent strength (*k* values) between the sample solvent and the mobile phase. Regarding large sample volumes, they could be used when resolution is not a limiting factor or when the sample is dissolved in a solvent with elution strength lower than that of the mobile phase [10].

Two strategies could be used when preconcentration is needed in trace analysis before separation in cLC or micro liquid chromatography (microLC): either solid phase extraction or solid phase microextraction alone, or any of those combined with large volume injections of the sample. Extraction and preconcentration of polar and acidic organic compounds at trace levels have been carried out by employing several methods, mainly solid phase extraction (SPE), on-line SPE [5,7,11–17] and solid phase microextraction (SPME) [13]. All these preconcentration methods are even more critical in cLC or microLC that in conventional HPLC, due to the low elution strength required for focusing sample solutions, because of the high solubility of polar and acidic compounds in water. Therefore, preconcentration step and cLC determination with high injection volumes must be made compatible by using focusing method-

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Table 1
Characteristics of the analytes included in the study.

Compound	pK_a	log P
2-Amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx, CAS no: 77500-04-0)	5.95	1.08
9H-Pyrido[3,4-b]indole (norharman, CAS no: 244-63-3)	6.80	2.80
1-Methyl-9H-pyrido[3,4-b]indole (harman, CAS no: 486-84-0)	7.16	2.71
7-Methoxy-1-methyl-9H-pyrido[3,4-b]indole (harmine, CAS no: 442-51-3)	7.70	3.22
2,4-Dichlorophenoxyacetic acid (2,4-D, CAS no: 94-75-7)	2.73	2.80
2-methyl-4-chlorophenoxyacetic acid (MCPA, CAS no: 94-74-6)	3.05	1.77
2,4-Dichlorophenoxyacetic methyl ester (2,4-D-1-methyl ester, CAS no: 1928-38-7)	–	2.90
4-(2,4-Dichlorophenoxy)butyric acid (2,4-DB, CAS no: 94-82-6)	4.80	3.53
2,4-Dichlorophenoxyacetic butyl ester (2,4-D-1-butyl ester, CAS no: 94-80-4)	–	4.40
2-(2,4,5-Trichlorophenoxy)-propanoic acid (2,4,5-TP, CAS no: 93-72-1)	3.60	3.80
2-(2,4-Dichlorophenoxy)-propanoic acid (2,4-DP, CAS no: 120-36-5)	3.00	3.43
2-(4-Chloro-2-methyl)-phenoxypropanoic acid (MCPP, CAS no: 96-65-2)	3.75	3.13
4-(4-Chlroro-2-methylphenoxy)-butanoic acid (MCPB, CAS no: 94-81-5)	3.10	3.50
1-Naphthyl methylcarbamate (carbaryl, CAS no: 63-25-2)	–	2.34
4-(Dimethylamino)-3-methylphenyl methylcarbamate (aminocarb, CAS no: 2032-59-9)	–	1.73
2-(1-Methylethoxy)phenyl methylcarbamate (propoxur, CAS no: 114-26-1)	–	0.14
2,2-Dimethyl-2,3-dihydro-7-benzofuranyl N-methylcarbamate (carbofuran, CAS no: 1563-66-2)	–	2.32
4-Methylthio-3,5-xylyl methylcarbamate (methiocarb, CAS no: 2032-65-7)	–	3.18

ologies [7,14–17]. Capillary or microLC using focalization or peak compression has been used for the determination of triazines in water using in-tube solid phase microextraction coupled to capillary liquid chromatography [13]. Chlorophenoxy acids have been determined in apple juice [14] and urine [16] using high injection volumes and focusing on the head of the capillary column. Trace amounts of heterocyclic amines have also been determined in cooked ham [15], smoked salmon and soft cheese [17] by cLC, again with high injection volumes and on-column focusing. Most of these separations have been carried out using C_8 or C_{18} packed columns.

Peak dispersion deteriorates selectivity and sensitivity of separation methods. Peak volumes are directly related to the square of the column diameter; therefore, the effect of band broadening sources becomes more evident with smaller columns. Extracolumn band broadening can compromise separations and it is one of the major challenges in miniaturized LC. The dispersion caused by pre-column components, such as sample injectors, column selection valves, column inlet filters and connecting tubing can produce a wide initial sample zone with a large dispersion that severely degrades resolution. Benefits of on-column concentration are available only if dispersion is minimized without peak height reduction.

The dispersion caused by injection of high volumes of sample can be expected to be dependent on many parameters, including injection volume, composition of injection mixture, pH and nature of the analytes injected. Multivariate statistical techniques have been employed frequently for the optimization of chromatographic systems [18]. All methods require the user to supply minimum and maximum values for each factor that defines the experimental domain to be investigated during the optimization procedure. Multivariate optimization of chromatographic systems can be carried out using the following procedure:

(i) Choose a statistical design to investigate the experimental region of interest.
(ii) Perform the experiments in random chronological order.
(iii) Perform analysis of variance (ANOVA) on the regression results so that the most appropriate model can be used to represent the data.

This paper presents an optimization study of focusing conditions for several compounds at trace levels, including easily ionisable compounds and acidic compounds with low log P (Table 1). Elution conditions studied are: isocratic and isothermal elution, gradient and isothermal elution, and isocratic and thermal gradient. The effect of an increase in sample volume on peak width (in terms of values of N) and on peak area has been evaluated. The band-spreading effects associated with large sample injection volumes are most pronounced for early-eluting peaks, since they have the smallest volume (narrowest peaks); for this reason, these peaks have been used for optimization of sensitivity (expressed as peak area) and performance (values of N). In this study, nine chlorophenoxy acids in their acid and ester forms, five carbamates and four heterocyclic aromatic amines were used due to their low log P and their ionisable character (Table 1).

2. Materials and methods

2.1. Chemicals and reagents

All reagents and solvents were of analytical grade and purified water from a Milli-Q system was used in all procedures (Millipore, Bedford, MA, USA). Methanol and acetonitrile of gradient HPLC quality were supplied by Scharlau (Barcelona, Spain). Chemicals including ammonium acetate, sodium hydroxide, phosphoric acid (85% pure), sulphuric acid (96% pure) and hydrochloric acid (35% pure) were purchased from Panreac (Barcelona, Spain). Acetic acid (99%) was obtained from Sigma (Steinhem, Germany).

Pesticides studied were: 2,4-D (99% pure), MCPA (95% pure) and 2,4,5-TP (97% pure) supplied by Aldrich; 2,4-D-1-methyl ester (97% pure), 2,4-DP (95% pure) and 2,4-DB (97% pure), from Sigma; MCPB (99% pure), 2,4-D-1-butyl ester (98.3% pure), MCPP (99% pure) and carbaryl (99.7% pure), from Riedel-de-Häen; aminocarb (98% pure), propoxur (99% pure), carbofuran (99% pure) and methiocarb (98.5% pure), from Chem Service (Table 1).

Heterocyclic amines studied were 1-methyl-9H-pyrido[3,4-b]indole (harman), purchased from Fluka (Buchs, Switzerland); 9H-pyrido[3,4-b]indole (norharman) from Sigma (Steinhem, Germany); 7-methoxy-1-methyl-9H-pyrido[3,4-b]indole (harmine) purchased from Sigma–Aldrich (Schnelldorf, Germany); and 2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline (MeIQx), provided by Toronto Research Chemicals (Toronto, Canada) (Table 1). According to the manufacturers, the chemical purity of the synthetic reference compounds was higher than 98%.

2.2. Preparation of standard solutions

Stock solutions of chlorophenoxy acid herbicides were prepared by dissolving 20 mg of each one in 100 mL of methanol, while those of carbamates were prepared by dissolving 10 mg of each one in 10 mL of acetonitrile and then diluting to 50 mL with purified water.

Heterocyclic aromatic amines stock solutions were prepared by dissolving 2.5 mg of each amine in 25 mL of methanol.

All stock solutions were stored in the dark at 4 °C for a maximum of two months. In order to prevent the influence of the possible analyte degradation on the results, standard mixtures of analytes were prepared daily by diluting a few microliters of the stock solutions in an aqueous solution containing different percentages of methanol.

2.3. Instrumentation and software

Chromatographic analysis by cLC was performed by an Agilent cLC instrument Mod. 1100 Series (Agilent Technologies, Madrid, Spain) which was equipped with a G1376A binary capillary pump, a G1379A degasser and a G1315B diode array detector (500 nL, 10 mm pathlength). Several loops were placed into a Rheodyne® injector valve, model 7010, for the separation of the different groups of analytes studied. Standard external stainless steel loops with volumes of 5, 10 and 20 μL and an internal loop of 2 μL were tested (Cotati, CA, USA). All components were interfaced to a computer with an Agilent Chemstation Software for LC systems G2170AA and a spectral evaluation module G2180AA.

Reversed phase separations of heterocyclic amines were made on a 150 mm × 0.3 mm I.D. capillary column packed with 3 μm Inertsil C_8 (GL Sciences, Tokyo, Japan). A Hypersil® C_{18} BDS analytical column (150 mm × 0.3 mm I.D., 3 μm) from LC Packings (Amsterdam, The Netherlands) was used for the separation of chlorophenoxy acids and carbamates.

All capillary columns were thermostated during the chromatographic run by employing a MISTRAL programmable oven (Spark Holland, Emmem, The Netherlands).

The software package Statgraphics Plus version 5.0, running under Windows XP, was used for application of chemometric tools.

2.4. Reversed phase cLC separation

The cLC separation of nine chlorophenoxy acids in their acid and ester forms was performed using the Hypersil C_{18} analytical column at a flow rate of 8.0 μL min^{-1}. Gradient elution was made by a mobile phase composition methanol–0.8% phosphoric acid aqueous solution (40:60, v/v) for 25 min, then a linear increase to 70% methanol over 15 min, and final isocratic step till the end of the chromatogram. Column temperature was maintained at 25 ± 1 °C during the chromatographic run and wavelength for UV detection was fixed at 232 nm. The on-column focusing step of the studied herbicides was carried out by the injection of mixtures containing 300 μg L^{-1} of 2,4-D-1-butyl ester and 120 μg L^{-1} of the rest of herbicides, with methanol ratios in the range 5–20%.

Regarding carbamates, cLC separation was carried out using the Hypersil C_{18} analytical column at a flow rate of 10.0 μL min^{-1}, by isocratic elution mode with acetonitrile–acetic acid/sodium acetate 20 mM pH 5.5 buffer solution (28:72, v/v) as mobile phase. For separation by temperature program, an isothermal step at the initial temperature (20 °C) for 7 min was made, followed by a linear increase to 50 °C for 5 min, then another isothermal step at 50 °C for 5 min and, finally, return to the initial temperature in 5 min. The on-column focusing step of the studied carbamates was carried out by injection of mixtures containing 0.5 mg L^{-1} of carbaryl and 2 mg L^{-1} of the rest of insecticides, with methanol ratios between 5 and 20%. Wavelength for UV detection was fixed at 220 nm.

Separation of heterocyclic amines (HAs) was done on an Inertsil® C_8 analytical column with a mobile phase of ammonium acetate 50 mM pH 3.8 buffer (adjusted with acetic acid) as solvent A and acetonitrile as solvent B. An isocratic elution (82% A and 18% B) with a mobile phase flow rate of 15 μL min^{-1} was applied. Column temperature was maintained at 25 ± 1 °C during the chromatographic run, and the wavelength for the UV-diode array detection was fixed at 265 nm for MeIQx and at 250 nm for the other HAs studied. The on-column focusing was made using an aqueous or ammonium acetate 50 mM pH 3.8 buffer solution containing 0.5 mg L^{-1} of each analyte and methanol ratios ranging from 5 to 20%.

3. Results and discussion

3.1. Optimization of injection conditions for cLC separation

Experimental parameters such as peak area, width at half-height, resolution and number of plates were measured under all experimental conditions. A multifactor ANOVA study showed that all these parameters can be affected by the delay of the injection volume and the composition of the focusing solution. A total of 136 chromatograms, with a replicate, were evaluated to establish sensitivity (peak area), performance (N) and resolution using different injection volumes (between 2 and 20 μL), different pH and different percentages of methanol in the injection solution. The ANOVA study of the factors that affect the separation in several on-column focusing conditions shows that analyte pK_a could be taken into account for the separation of chlorophenoxy acids and heterocyclic amines. The same study shows that the first peak of the chromatographic run is the one most affected by focusing conditions in all separations tested (isocratic elution and gradient elution mode and separation by temperature program). In the case of heterocyclic amines, focusing solutions with elution strength similar to that of the mobile phase can affect resolution between several peaks.

3.1.1. Chlorophenoxy acid herbicides

For the experimental design, 64 chromatograms, divided into two blocks, have been used, with the objective to evaluate three factors (injection volume, amount of organic modifier and pH) and several levels: four for the injection volume, four for the methanol ratio, and two for the pH (absence and presence of 0.8% phosphoric acid). The design selected for the optimization process was determined by the loop size which is commercially available for the 6-port injection valve; thus, loops of 2, 5, 10 and 20 μL were employed. The injection solutions contain different percentages of methanol (5, 10, 20 and 30%), so that they have lower elution strength than has the mobile phase used for the separation (gradient elution starts by a mobile phase composition 40/60 methanol/0.8% phosphoric acid aqueous solution). In 32 of the chromatograms, the injection solution contained an aqueous solution acidified with 0.8% phosphoric acid, like in the mobile phase, while, in the other 32 chromatograms, no pH adjustment was performed.

Results obtained for 2,4-D and its esters showed that an increase in the peak area of the esters and a decrease in the peak area of the acid occurred when the focusing solution contained 0.8% phosphoric acid and 20–30% methanol. For this reason, it was decided to go on with the study using only aqueous solutions for the injection. The injection conditions have been optimized for the first compound eluted, 2,4-D, since it is the one most affected by such conditions. Table 2 shows the mean values obtained for experimental areas and N at different injection conditions. Eq. (1) shows the regression which fitted for peak area of 2,4-D:

$$\begin{aligned}\text{Area} = {} & -19.1304 + 28.0648 \times \text{injection volume} - 2.93734 \\ & \times \text{methanol} - 0.560359 \\ & \times \text{injection volume}^2 + 0.03819 \times \text{injection volume} \times \text{methanol} \\ & + 0.0845252 \times \text{methanol}^2\end{aligned} \quad (1)$$

The R-squared statistic of Eq. (1) indicates that the model explains 99.1% of the variability in peak area. Analysis of variance for peak area shows that two effects have P-values lower than 0.05,

Table 2
Experimental peak area and calculated *N* of 2,4-D-chlorophenoxy acid herbicide at different injection volumes and different amounts of methanol in the injection solution.

Injection volume (μL)	Methanol (%)	Area[a] (u.a.)	*N*[a]
2	5	23	1260
2	10	21	1444
2	20	16	1311
2	30	16	942
5	5	99	2274
5	10	83	2415
5	20	91	2080
5	30	93	2026
10	5	186	2999
10	10	170	2343
10	20	209	2845
10	30	214	3205
20	5	327	3187
20	10	298	3527
20	20	294	3984
20	30	332	2091

[a] Mean of two experiments.

injection volume and its quadratic factor, which indicates that they are significantly different from zero at the 95% confidence level.

Regression equation (2) for the plate number of 2,4-D explains 81.6% of the *N* variability:

$$N = 298.832 + 339.696 \times \text{injection volume} + 68.2969 \times \text{methanol} - 9.76104 \times \text{injection volume}^2 - 1.27744 \times \text{injection volume} \times \text{methanol} - 1.95682 \times \text{methanol}^2 \quad (2)$$

In this case, the ANOVA study shows that two effects have *P*-values lower than 0.05 injection volume and its quadratic factor. Similarly to the equation for the peak area, the first term is positive while the second one is negative.

Generally, responses are usually transformed into an appropriate desirability scale for balance [19]. In that process, different weight variables are to be assigned for each of the response and after obtaining its individual desirabilities, they are combined to get a measure of the composite desirability of the multi-response system [20]. The chromatographic separation efficiency is optimized by maximizing peak area and *N* values which maximized the desirability function over the indicated region. The combination of factor levels which maximizes the desirability function is 19.6 μL for injection volume and 9.9% for methanol ratio, the optimum value of the desirability being 0.863174.

Fig. 1a and b shows the response surfaces estimated and the experimental results for the peak area and for the plate number of 2,4-D. The figures show a linear increase of peak area and *N* for injection volumes up to 10 μL, and then a slow increase of peak area and nearly a plateau for *N*, probably because chromatographic dispersion affects more than retention in the head of the chromatographic column under the focusing conditions. As can be seen, an optimal area with the maximum efficiency was achieved for injection volumes between 10 μL and 20 μL, with methanol ratios lower than or equal to 20%. In both Eqs. (2) and (3), the quadratic term of the injection volume is negative, and therefore, other commercially available injection volumes, such as 50 μL, have not been tested because chromatographic dispersion due to higher volumes decreases separation efficiency and/or peak area. Fig. 2 shows two chromatograms obtained for a mixture of phenoxy acids under the same injection conditions with different injection volumes. The increase in sensitivity is significant in all cases, but is especially important for the first compounds eluted. As can also be observed, retention time for the first eluted compounds was higher when a 20 μL injection volume was used. Capillary LC using focalization has been used for determination of chlorophenoxy acids in apple

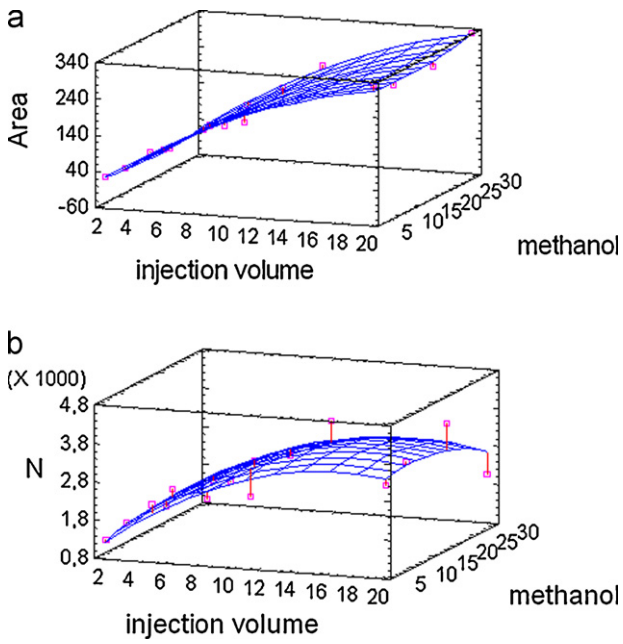

Fig. 1. Estimated response surfaces for peak area (a) and for *N* (b) of 2,4-D chlorophenoxy acid herbicide. Symbol: □, experimental results.

juice [14] and urine [16]. Clean-up and preconcentration of acid and esters were carried out in an Oasis MCX polymer in apple juice samples spiked with amounts permitted by the European Regulations. In order to make SPE preconcentration and chromatographic determination of chlorophenoxy acids compatible, a total volume of 20 μL containing 15% of methanol was injected into the LC system. As can be seen in Fig. 2, peak area in such conditions is maximum for 2,4-D and *N* is acceptable. Analysis of herbicides in low volumes of untreated human urine [16] was possible by using a restricted-access material (RAM) Lichrospher RP-18-ADS packed in a steel column. The human urine extracts eluted from the RAM column were diluted in order to obtain a low-elution-strength solution containing 10% methanol for focusing purposes. A 20 μL injection volume of this solution allows the determination of chlorophenoxy acid herbicides at levels expected for occupationally exposed workers without solvent changes or evaporation steps for the final urine extracts.

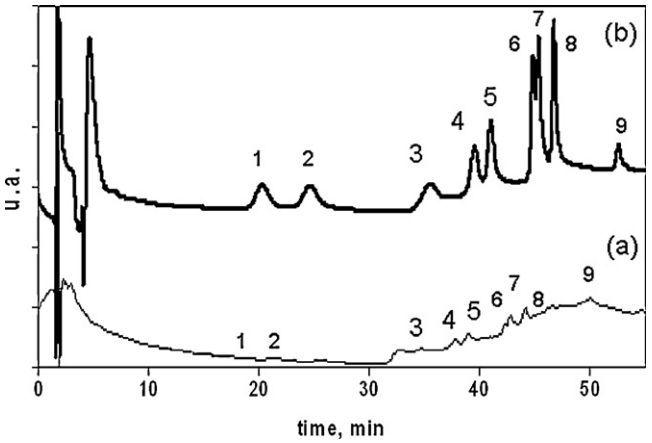

Fig. 2. Chromatograms of a mixture of chlorophenoxy acids corresponding to 2 μL (a) and 20 μL (b) injection volumes with 5% of methanol in water solution. Peaks: (1) 2,4-D; (2) MCPA; (3) 2,4-D-1-methyl ester; (4) 2,4-DP; (5) MCPP; (6) 2,4-DB; (7) MCPB; (8) 2,4,5-TP; (9) 2,4-D-1-butyl ester. Amount injected 125 μg L^{-1} of each herbicide and 300 μg L^{-1} of 2,4-D-1-butyl ester. Flow rate 8 μL min^{-1}.

Table 3
Experimental peak area and calculated *N* of aminocarb insecticide at different injection volumes and different amounts of methanol in the injection solution.

Injection volume (μL)	Methanol (%)	Area[a] (u.a.)	*N*[a]
2	5	265	1029
2	10	150	871
2	20	135	982
5	5	604	1300
5	10	669	1430
5	20	603	1305
10	5	1120	1956
10	10	1164	2056
10	20	1227	1564
20	5	2062	2191
20	10	1991	1981
20	20	1927	1739

[a] Mean of two experiments.

3.1.2. Carbamate insecticides

For the experimental design of carbamate insecticides, 24 chromatograms, also divided into two blocks (with a replicate), were employed, with the objective to evaluate two factors (injection volume and amount of organic modifier). While, in the previous study with phenoxy acids, injection conditions were optimized for a separation that uses an elution gradient, in the present case, isocratic elution with thermal gradient has been employed for the separation of a series of carbamates and a degradation product. Again, the design is conditioned by the injection loop volumes commercially available. Although acetonitrile is used as the eluent for the separation, this solvent does not have the low elution strength required for the on-column focusing; therefore, methanol has been used instead, in ratios between 5% and 20%. Optimization was carried out using the data from 24 chromatograms (a replicate) related to the first peak eluted, as it is the one most affected by the on-column focusing. Table 3 shows the mean values obtained for experimental areas and *N* at different injection conditions for aminocarb. Eqs. (3) and (4) respectively show the regression for the area and for the plate number of aminocarb.

$$\begin{aligned}\text{Area} = {} & -9.3354 + 153.831 \times \text{injection volume} - 5.65393 \\ & \times \text{methanol} - 2.55902 \\ & \times \text{injection volume}^2 - 0.13471 \times \text{injection volume} \times \text{methanol} \\ & + 0.20333 \times \text{methanol}^2\end{aligned} \quad (3)$$

The above equation explains 99.4% of the peak area variability. Similarly to the case of the chlorophenoxy acids, the main effects are injection volume and its quadratic term.

$$\begin{aligned}N = {} & 508.015 + 198.6 \times \text{injection volume} + 20.876 \times \\ & \text{methanol} - 5.52662 \\ & \times \text{injection volume}^2 - 1.72714 \times \text{injection volume} \times \text{methanol} \\ & - 0.786667 \times \text{methanol}^2\end{aligned} \quad (4)$$

Eq. (4) explains 96.2% of the *N* variability. Once more, the main effects are injection volume and its quadratic term, but in this case it also necessary to consider the cross factor methanol × injection volume.

The chromatographic separation efficiency was optimized by maximizing peak area and *N* values, which maximized the desirability function over the selected region. The combination of factor levels which maximize the desirability function is 20 μL for injection volume and 5% for methanol ratio, the optimum value of the desirability being 0.984642.

Fig. 3a and b shows the response surfaces estimated for the peak area and for the plate number of the aminocarb. As expected on seeing the figures and Eq. (3), the optimum sensitivity is achieved for an injection volume of 20 μL, although, with this volume, no further efficiency is noticed in the system. In both Eqs. (3) and (4), the quadratic term of the injection volume and the cross factor methanol × injection volume are negative. For this reason other higher injection volumes, such as 50 μL, have not been tested because chromatographic dispersion due to higher volumes decreases separation efficiency and/or peak area as in the case of chlorophenoxy acids. Fig. 4 shows two chromatograms at different injection volumes. An improvement in the sensitivity is again observed, but a slight delay is noticed in the chromatographic peaks as the injection volume is increased. Therefore, an increase in sensitivity could be obtained for the high volumes studied by keeping *N* nearly constant.

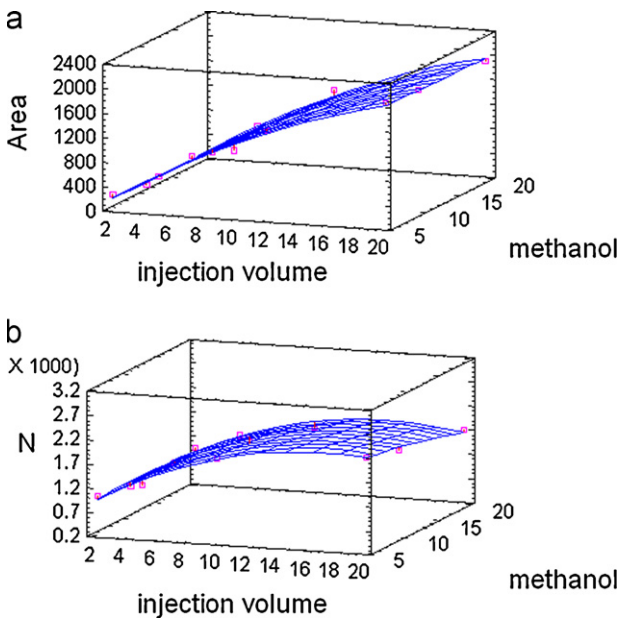

Fig. 3. Estimated response surfaces for peak area (a) and for *N* (b) of aminocarb insecticide. Symbol: □, experimental results.

3.1.3. Heterocyclic aromatic amines

In the case of HAs, 48 chromatograms, divided into two blocks (with a replicate), have been used for the experimental design, with the aim to evaluate three factors (injection volume, pH and amount of organic modifier). The injection solutions contained different ratios of methanol (5, 10 and 20%), so that they have a lower elution strength than has the mobile phase used for the separation (acetonitrile–ammonium acetate 50 mM pH 3.8 buffer aqueous solution 18:72, v/v). In 24 of the chromatograms, the injection solution contained an ammonium acetate 50 mM pH 3.8 buffer aqueous solution, like in the mobile phase, while, in the other 24 chromatograms, no pH adjustment was performed. Although ace-

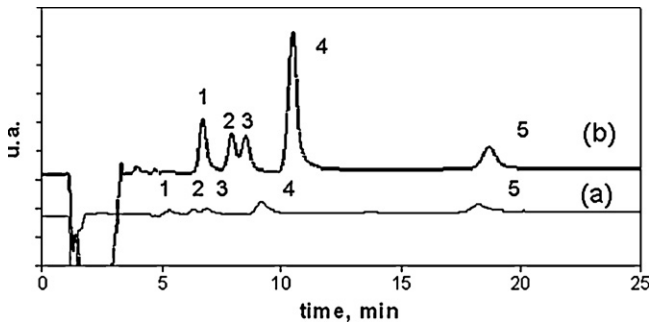

Fig. 4. Chromatograms of a mixture of carbamates obtained at 2 μL (a) and 20 μL (b) injection volumes with 5% of methanol in water solution. Peaks: (1) aminocarb; (2) propoxur; (3) carbofuran; (4) carbaryl; (5) methiocarb. Amount injected 2 mg L^{-1} of each insecticide and 0.5 mg L^{-1} of carbaryl. Flow rate 10 μL min^{-1}.

Table 4
Experimental peak area and calculated *N* of MeIQx heterocyclic amine at different injection volumes and different amounts of methanol in the injection solution.

Injection volume (μL)	Methanol (%)	Area[a] (u.a.)	*N*[a]
2	5	1084	14,341
2	10	1060	9029
2	20	1020	3274
5	5	3913	7547
5	10	3599	1364
5	20	4242	906
10	5	6502	13,875
10	10	6333	7989
10	20	6713	2254
20	5	12,342	13,875
20	10	12,404	3321
20	20	Peak not detected	Peak not detected

[a] Mean of two experiments.

Table 5
Resolution between the peaks of norharman and harman with different injection solutions at an injection volume of 20 μL.

Buffer solution containing ammonium acetate 50 mM at pH 3.8	Methanol (%)	Rs[a]
Yes	15	2.5
No	15	1.6
Yes	10	2.9
No	10	1.7
Yes	5	3.1
No	5	1.7

[a] Mean of two experiments.

tonitrile was used as the eluent for the separation, this solvent does not have the adequate elution strength for the on-column focusing required; therefore, methanol has been used again, like in the carbamate study.

As response variables, peak area and number of plates for the first compound eluted (MeIQx) were considered (Table 4); but in this case, resolution between the worst resolved pair of peaks (norharman–harman) (Table 5) should be taken into account, since the focusing solutions have an elution strength similar to that of the mobile phase, and hence affect resolution of these peaks.

The presence of buffer solution in the injection solution produced a negligible effect on peak area of MeIQx, but the decrease of peak width produced an increase of *N*.

Eqs. (5) and (6) show the regression for the peak area and for the plate number, *N*, when the injection solution contained an ammonium acetate 50 mM pH 3.8 as buffer solution:

$$\begin{aligned}\text{Area} = {} & -301.016 + 809.381 \times \text{injection volume} - 19.9647 \\ & \times \text{methanol} - 8.01252 \\ & \times \text{injection volume}^2 - 1.10722 \times \text{injection volume} \times \text{methanol} \\ & + 1.347 \times \text{methanol}^2\end{aligned} \quad (5)$$

The above equation explains 99.4% of the area variability, but the main effect is the injection volume, with a *P*-value of 0.0018. Eq. (6) explains 83.4% of the *N* variability:

$$\begin{aligned}N = {} & 13454.7 + 816.299 \times \text{injection volume} - 1273.66 \\ & \times \text{methanol} - 5.80977 \\ & \times \text{injection volume}^2 - 51.5106 \times \text{injection volume} \times \text{methanol} \\ & + 40.052 \times \text{methanol}^2\end{aligned} \quad (6)$$

In this case, the optimum value of the desirability function is 0.617929 and the optimum sensitivity is achieved for an injection volume of 20 μL and 7.7% for methanol ratio when the injection solution contained ammonium acetate 50 mM pH 3.8 buffer solution. Fig. 5a and b shows the response surfaces estimated for the peak area and for the plate number of MeIQx, the first eluted

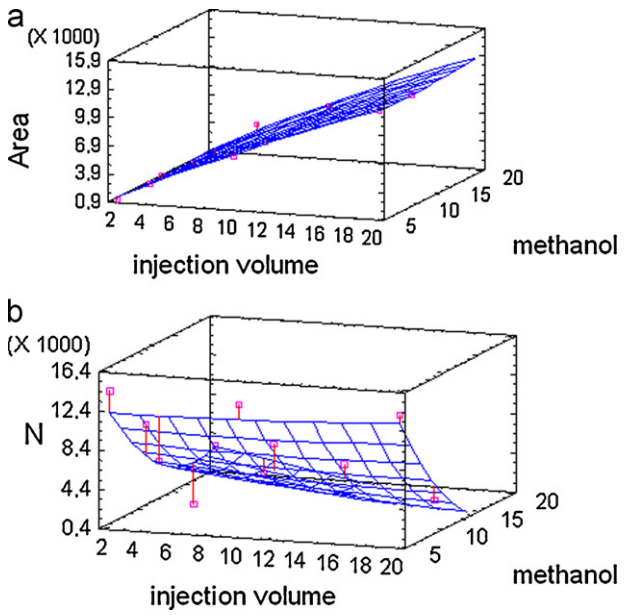

Fig. 5. Estimated response surfaces for peak area (a) and for *N* (b) of MeIQx heterocyclic amine. Symbol: □, experimental results.

compound. As can be observed, an optimal area was achieved for injection volumes between 10 μL and 20 μL, with methanol ratios around 10%, but a loss of efficiency was observed at the higher injection volumes and the higher methanol ratios because the effect of chromatographic dispersion is not compensated in such focusing conditions. This is mainly due to an increase of peak width when the injection solution does not contain the buffer solution, as can be seen in the chromatograms shown in Fig. 6. In addition to such peak broadening, a loss of resolution happens between the peaks of norharman and harman at different injection conditions with the higher volume studied, as can be observed in Table 5. Determination of heterocyclic aromatic amines in ready-to-eat cooked ham [15], smoked salmon and soft cheese [17] treated with electron-beam irradiation, using solid-phase extraction and purification procedure, and making use of focusing techniques, was possible at ng g^{-1} levels. Sample preparation procedure included a previous treatment with 1 M NaOH, followed by two solid phase extraction steps; firstly on diatomaceous earth and then on mixed-mode SPE cartridges. Several cartridges were tested for preconcentration of the heterocyclic aromatic amines; the retained analytes were eluted and the extract evaporated to dryness. Dry extracts were reconstituted in 1 mL of a mixture of MeOH-buffer solution (5:95, v/v). The resulting solutions, with low elution strength, have led

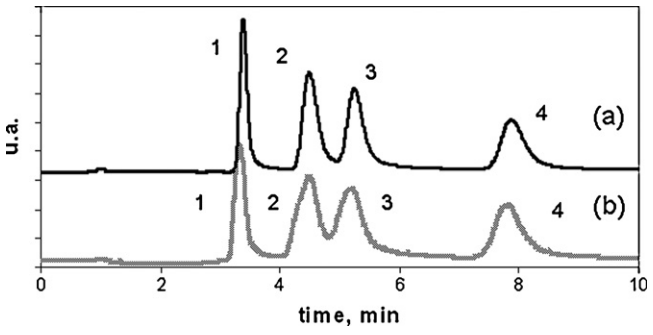

Fig. 6. Chromatograms obtained for HAs mixtures at 20 μL of injection volume with 5% of methanol in different injection solutions: (a) with buffer solution at pH 3.8 and (b) without buffer solution. Peaks: (1) MeIQx; (2) norharman; (3) harman; (4) harmine. Amount injected 0.5 mg L^{-1} of each amine. Flow rate 15 μL min^{-1}.

to an increase of the peak area of MeIQx while maintaining the efficiency (see Fig. 5), and have allowed an acceptable resolution between norharman and harman (see Table 5).

4. Conclusions

Experimental design can provide information about the influence of several parameters on the injection conditions as well as about desirability functions to optimize sensitivity and efficiency (N), and therefore, the separation performance. This way, the present global study has allowed evaluating the on-column focusing conditions with capillary columns.

Combination of large injection volumes and focusing techniques based on the injection of solutions with low elution strength has provided an increase of sensitivity while maintaining adequate separation conditions for analytes with different acidic and ionisable characteristics. Therefore, these methodologies could be applied in capillary liquid chromatography to the analysis of substances with acid–base properties and medium polarity at lower concentration levels.

The on-column focusing study of chlorophenoxy acid herbicides, heterocyclic aromatic amines and carbamate insecticides proposed in this paper offers the possibility to evaluate the experimental injection conditions to obtain an adequate sensitivity, efficiency and resolution with capillary columns, especially when preconcentration or clean-up of the sample is needed.

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