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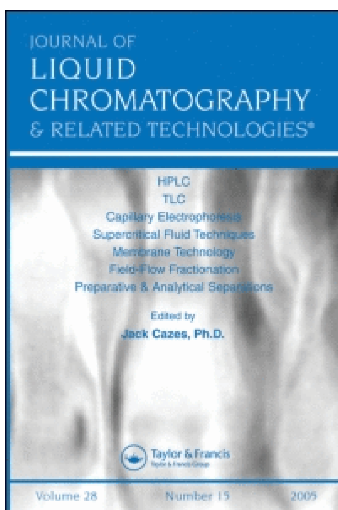

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DETERMINATION OF CHLOROPHENOXY ACID AND DICAMBA HERBICIDE RESIDUES BY CAPILLARY REVERSED-PHASE LIQUID CHROMATOGRAPHY

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ABSTRACT

Capillary liquid chromatography combined with solid phase extraction in a column packed with an anion exchanger based on MFE-Polymer (hydroxyethylmethacrylate, HEMA) containing quaternary ammonium functional groups, has been studied for multiresidual analysis of seven chlorophenoxy acid herbicides; 2,4-D, MCPA, 2,4-DP, 2,4-DB, MCPB, 2,4,5-TP, and the benzoic acid derivative Dicamba in green bean samples. The chromatographic analysis was carried out on a C_{18} packed capillary column with gradient elution at 20°C. The chlorophenoxy acid and Dicamba have been assayed in green bean samples spiked with amounts between 0.30 and 0.08 $mg.kg^{-1}$ for 2,4-D and MCPA, and between 0.15 and 0.04 $mg.kg^{-1}$ for all the other herbicides. The recoveries obtained were between 41 and 119% (n=4 for each spiked level).

INTRODUCTION

Chlorophenoxy acids are an important group of translocated herbicides widely used to control broad-leaved weeds. Their formulation is usually either

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esters emulsified in oil or water-soluble amine or alkaline salts, and they are frequently used in mixtures of two or more of them. Their persistence in the environment, which ranges between 5 and 25 weeks, depends on how they are formulated and on the biological activity. Toxicological aspects are in relationship with the presence of chlorine atoms that could lead to teratogenic effects, these being stronger when the number of chlorine atoms increases. Consequently, many countries have severely restricted the use of these herbicides as well as the maximum allowable concentration. Thus, the European Union has limited their presence to a maximum of 0.1 $\mu g\ L^{-1}$ for an individual pesticide noted as their acid form, in drinking water, and 0.05 $mg.kg^{-1}$ in vegetable foods.[1] Therefore, the availability of sensitive and selective methods of analysis is essential.

Thus, several procedures have been proposed to determine these residues in samples like water,[2,3] soil and cereals,[4,5,6] and other vegetable products,[7,8] using in all cases, chromatographic techniques (GC or LC). Usually, these require a previous step where the sample is extracted and preconcentrated and, particularly in the case of complex samples as vegetables, this step is followed by a laborious process of selective clean up, needed to minimize the disturbing effect of the organic matter co-extracted, thus permitting reaching the low limits required.

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At present, many of the Liquid Chromatography systems involved in chloropehonoxy acids determination use conventional columns (those with inner diameter between 2 and 4.6 mm and particle size bigger than 3 μm); but, in the last few years, efforts to improve separation in Liquid Chromatography have been made by developing columns with smaller inner diameters and packing material size. According to the criterion established by Vissers,[9] these LC modes are called *micro LC* (i.d. 1.0 to 0.5 mm), *capillary LC* (i.d. 500 to 100 μm) and *nanoscale LC* (i.d. smaller than 100 μm). The combination of capillary columns and nano-volume flow cells led to the development of capillary LC systems of high resolution and detection sensitivity. Regarding the pesticide analysis only a few studies have been reported for micro or capillary liquid chromatography.[10,11]

The aim of the present study is to explore the possibility of multiresidual analysis of seven chlorophenoxy acid herbicides in vegetable samples by capillary-LC with UV detection. Names and structural formulae of these herbicides are shown in Figure 1. Dicamba, a benzoic acid derivative, also a translocated herbicide frequently used together with chlorophenoxy acids, has been included in this study. The performance of the LC method, combined with SPE, was evaluated using fortified green bean samples.

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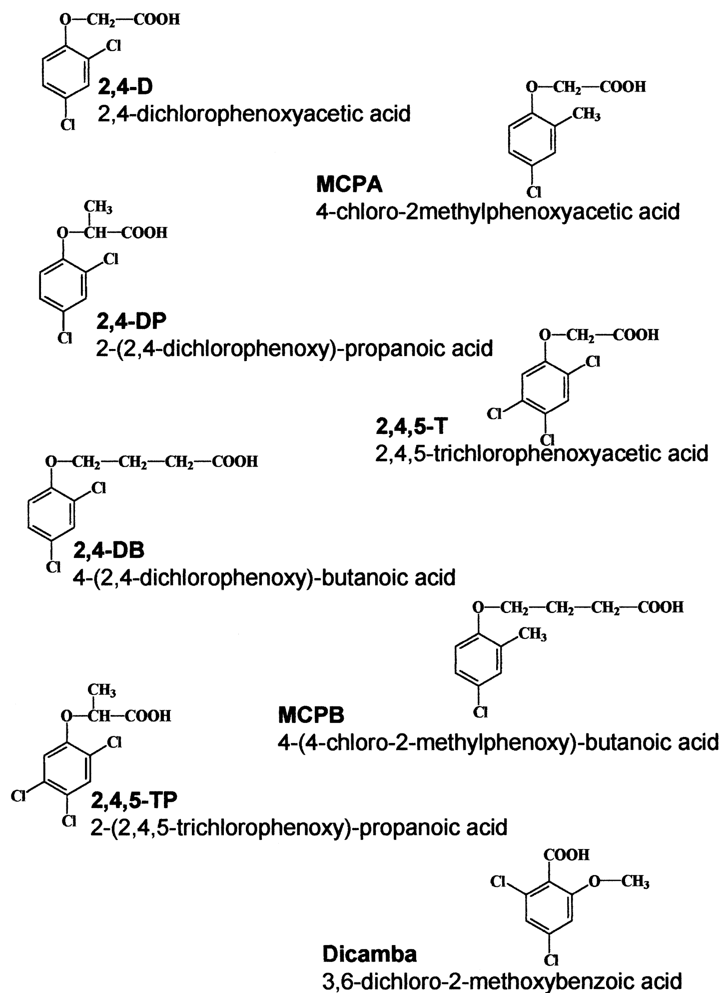

Figure 1. Names and structural formulae of the chlorophenoxy acid herbicides and Dicamba.

EXPERIMENTAL

Equipment

The chromatographic determination of the studied phenoxy acid herbicides was carried out in a HPLC system with a gradient Beckman 125S Solvent

Module SYSTEM GOLD. A microflow restrictor ACURATE supplied by LC Packing was placed between the pump and the injector. This is a four channel valve with a fixed volume of 100 nl. A Beckman UV programmable variable wavelength 166 Detector SYSTEM GOLD with a microcell (35 nl, 8 mm path-length) was used as the detector. All the components were interfaced to a DELL Computer equipped with a MMX Pentium Processor and containing a Gold Nouveau Chromatography Workstation Software for Windows. A 150 x 0.3 mm id LC Packing Analytical Column packed with 3 μm Spherisorb ODS(2) was used in the chromatographic separation of the herbicides studied. Temperature control was made with a column thermostat oven MISTRAL.

Preconcentration of the samples prior to analysis was made in solid phase with a preparative chromatographic column 150 x 4.6 mm id packed with about 3 g of 50 μm particle size MFE®-Polymer SAX (hydroxyethylmethacrylate, HEMA, containing quaternary ammonium functional groups), supplied by Análisis Vínicos, and a Series II Digital Pump, supplied by Konik-Tech, equipped with one pump head capable to dispense up to 10 $mL.min^{-1}$.

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Chemicals

2,4-D (99% pure), 2,4,5-T (98% pure), and 2,4,5-TP (97% pure) were obtained from Aldrich; 2,4-DB (97%), MCPA (95-97% pure), and 2,4-DP (95% minimum) from Sigma; MCPB (99% pure) from Riedel-de Haën and Dicamba (99%) from Chem Service. All Chemicals for sample preparation were analytical grade and methanol HPLC grade was used for chromatographic mobile phase and standard solution preparation. Purified water was obtained from a Milli-Q apparatus.

Stock solutions of the chlorophenoxy acids and Dicamba (200 $mg.L^{-1}$) were prepared in methanol and stored in the dark at not more than 4°C for 2 months maximum. Working standard solutions were prepared by suitable dilution of the stock solutions with methanol.

Capillary Chromatographic Determination of Chlorophenoxy Acid Herbicides and Dicamba in Standards

The mobile phase flow-rate was adjusted to a flow of 500 $\mu L.min^{-1}$ in the pump and splitted in a 1/100 relation by means of the ACURATE® splitter, so that a flow-rate of 5 $\mu L.min^{-1}$ was achieved through the microcolumn. Isocratic elution of the analytes was made by continuous mixing of an adequate proportion (53/47 v/v) of methanol and H_3PO_4 0.8% aqueous solution by programming the Beckman 125S Solvent Module. Gradient elution of the chlorophenoxy acids was made by the following program: it started with an isocratic step of methanol 50%/ H_3PO_4 0.8% aqueous solution 50% for 5 min, then a linear

increase to methanol 60% over 15 min, an additional increase to 80% methanol for 10 min, and then another isocratic step till the end of the chromatogram. The detection wavelength was 232 nm and the chromatographic column was thermostated at 20°C.

Detection and quantitation limits were estimated by decreasing the concentration of the chlorophenoxy acid down to the smallest detectable peak; this concentration was multiplied by three to obtain the detection limit and by ten for the quantitation limit. Calibration graphs of the chlorophenoxy acid herbicides in the optimum conditions for the separation were obtained in the range 0.5-4.0 $mg.L^{-1}$ for Dicamba, 2,4-DP, 2,4,5-T, 2,4-DB, MCPB, and 2,4,5-TP, and from 1.0 to 8.0 $mg.L^{-1}$ for 2,4-D and MCPA.

Green Bean Sample Preparation and LC Determination

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Hydrolysis of the sample pretreatment was made by following in part and adapting a previous method proposed by Hogendoorn and coworkers.[12] Green bean samples were spiked with each herbicide in the concentration range between 0.04 and 0.15 $mg.kg^{-1}$ for Dicamba, 2,4,5-T; 2,4,5-DB, 2,4-DP, MCPB, and 2,4,5-TP, and between 0.08 and 0.30 $mg.kg^{-1}$ for 2,4-D and MCPA. Amounts of 40 g of the spiked samples were thoroughly blended with 25 mL of 0.1M NaOH and 25 mL of water, and the mixture was hydrolyzed in a water bath at 95°C for about 30 min. After cooling, the mixture was acidified by adding 2 mL of 1:1 H_2SO_4 solution, the suspension was then filtered through a paper filter and the residue was carefully washed with three volumes of ca. 10 mL of pure water. The resulting solution was filtered through a Teflon filter with 1.5 µm pore and passed through the preparative ion exchanger column containing MFE-Polymer SAX at 8 $mL.min^{-1}$.[8] The retained herbicides were eluted with 10 mL of acetonitrile-60 mM hydrochloric acid solution (2 $mL.min^{-1}$); then volume was reduced down to 2 mL in a Kuderna-Danish apparatus and 100 nL were injected in the LC system and determined under the conditions described previously.

RESULTS AND DISCUSSION

Chromatographic Separation

Previous assays were carried out to study the main factors affecting the capillary chromatographic separation of the chlorophenoxy acid herbicides. The influence of the organic modifier and the pH of the aqueous component of the mobile phase were tested. Since the herbicides studied are all weak acids with pK_a below 4.9, only acidic buffers as acetic/acetate, formic/formate, and diluted phosphoric acid solution were studied, the latter being the one that gives better results (narrow and almost symmetrical chromatographic peaks).

Regarding the organic modifier, acetonitrile and methanol were tested, methanol giving the most satisfactory results; so, mixtures of methanol and aqueous phosphoric acid solutions below 1% were chosen as mobile phases for further studies. In order to set up the best chromatographic conditions for the separation of the seven chlorophenoxy acid herbicides and Dicamba and subsequent determination in vegetable samples, studies of isocratic and gradient elution were carried out.

Isocratic Capillary Chromatographic Separation

Different mixtures containing methanol/H_3PO_4 0.8% aqueous solution containing between 60 and 45% of methanol were first tested as mobile phases for isocratic elution. All the experiments were performed at 20°C. Mobile phase with 60% methanol/40% H_3PO_4 aqueous solution allowed chromatographic separation in a reasonable time of 35 min, with acceptable or total resolution of all the peaks except the pair corresponding to 2,4-DB and MCPB, which are strongly overlapped. Separation to the base line was achieved with a mobile phase containing 45% methanol, but the time of the analysis was nearly 90 min. Consequently, the percentage of the organic modifier should be that which leads to an acceptable resolution of the 2,4-DB - MCPB critical pair.

The average percentage of methanol/ H_3PO_4 0.8% aqueous solution (53/47 v/v) was also tested and it was the last in which acceptable resolution was obtained for the critical pair 2,4-DB - MCPB. The time for chromatographic analysis under these conditions was 45 min. Figure 2 shows the chromatogram obtained and Table 1 shows the limits of detection (LOD) and quantitation (LOQ) achieved when chromatographic analysis is performed in isocratic mode.

Gradient Capillary Chromatographic Separation

Gradient elution of the chlorophenoxy acid herbicides and Dicamba has also been studied due the possibilities of getting better analysis conditions. As could be seen in the study of the isocratic capillary chromatography, complete separation of 2,4-DB and MCPB is very difficult because both have quite similar structures. Therefore, all the experiments carried out to set up the appropriate chromatographic conditions started with a percentage of methanol/aqueous solution of 50/50 or lower, increasing the ratio of methanol linearly or in several steps.

Specifically, the methanol rate was maintained for a few minutes and/or increased slowly till around six minutes before the expected elution time for 2,4-DB and MCPB, time at which the percentage of methanol was increased quickly. After studying several elution gradients, the best conditions found were a MeOH/H_3PO_4 0.8% aqueous solution (50/50 v/v) for 5 minutes, then a linear increase to 60/40 v/v over 15 min, a second linear increase to 80% MeOH

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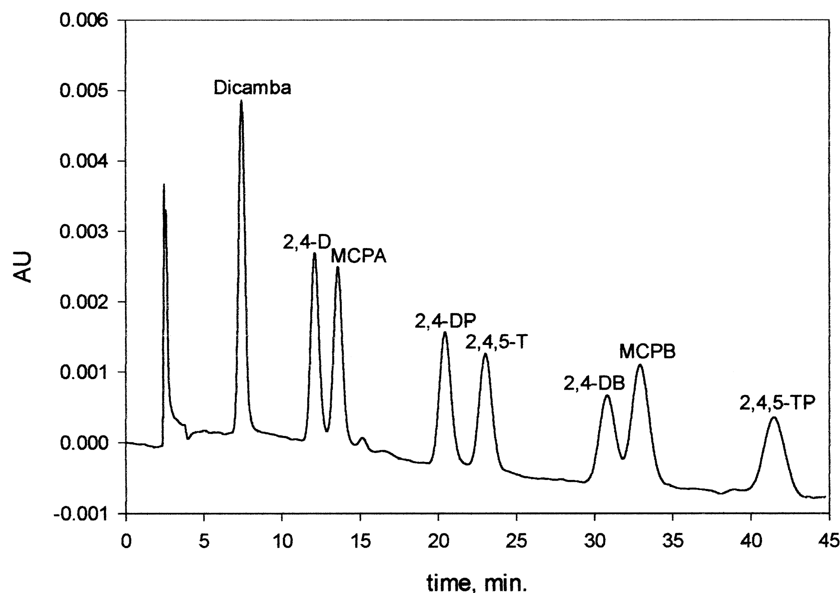

Figure 2. Liquid chromatogram of Dicamba and the chlorophenoxy acids obtained in isocratic elution mode. Column: LC Packing Spherisorb ODS(2), 3 μm, 150 x 0.3 mm id. Mobile phase: MeOH/ H_3PO_4 0.8% aqueous solution (53/47 v/v). Flow rate in column: 5 $\mu L.min^{-1}$.

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for another 10 min, and final isocratic step till the end of the chromatogram. This gradient elution program allowed a reasonable separation of the seven chlorophenoxy acid herbicides and Dicamba in 35 min (Figure 3). The analytical characteristics obtained for the different analytes studied when the capillary chromatographic elution is performed under the gradient elution program above commented are shown in Table 2.

Determination of Chlorophenoxy Acid Herbicides and Dicamba in Green Bean Samples

To evaluate the applicability of the capillary chromatography in the determination of these herbicides, green bean samples were spiked with different amounts of the studied chlorophenoxy acids and Dicamba and hydrolyzed according to the procedure referred above. After hydrolysis, the sample was treated as described in the Experimental section and preconcentrated in acidic medium using the column packed with the MFE-Polymer SAX.

Table 1

Analytical Characteristics for Isocratic Chromatographic Elution

Herbicide	Ret. Time (Min.)	LOD (pg)	Resolution
Dicamba	7.4	5	
			5.4
2,4-D	12.1	5	
			1.5
MCPA	13.6	5	
			5.6
2,4-DP	20.4	10	
			1.7
2,4,5-T	23.0	10	
			4.7
2,4-DB	30.8	20	
			1.1
MCPB	32.9	20	
			3.7
2,4,5-TP	41.5	20	

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Once the preconcentration process was carried out, the herbicides were eluted from the column and the elution volume reduced down to 2 mL as described previously. For capillary chromatographic determination, gradient elution was preferred, although very few important differences have been found between the analytical characteristics of the isocratic and gradient elution modes; only a slight time reduction of the chromatogram analysis. An important problem encountered in the preconcentration of acidic compounds from natural samples is that many matrix interferences are co-extracted, and often co-eluted, resulting in a broad peak at the beginning of the chromatogram often affecting some of the analyte peaks. This elution program shows the additional advantage that the chromatographic peak corresponding to the polar substance does not overlap with Dicamba peak, which is the first one.

The spiked samples were prepared with an amount of each herbicide in order to achieve concentrations below the maximum permitted by the Food and Agricultural Organization/World Health Organization (FAO/WHO), which ranges between 0.05-0.5 $mg.kg^{-1}$ for different types of food,[13] or by the Spanish and European regulations, which establish 0.10 $mg.kg^{-1}$ as the maximum level

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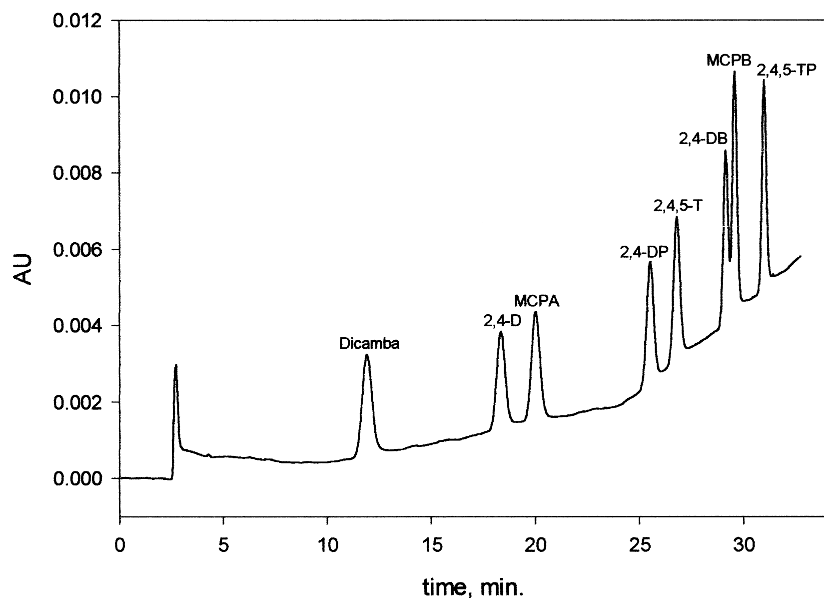

Figure 3. Liquid chromatogram of Dicamba and the chlorophenoxy acids obtained in gradient elution mode (for gradient program see text). Column: LC Packing Spherisorb ODS(2), 3 μm, 150 x 0.3 mm id. Flow rate in column: 5 μL. min^{-1}.

Table 2

Analytical Characteristics for Gradient Chromatographic Elution

Herbicide	Ret. Time (Min.)	LOD (pg)	Resolution
Dicamba	11.9	10	
			7.9
2,4-D	18.3	10	
			2.2
MCPA	20.0	10	
			7.6
2,4-DP	25.5	20	
			2.1
2,4,5-T	26.8	20	
			5.7
2,4-DB	29.1	10	
			1.2
MCPB	29.6	10	
			3.5
2,4,5-TP	31.0	10	

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Table 3

Recovery of Chlorophenoxy Acids and Dicamba Added to Green Bean Samples*

Herbicide	Added ($mg.kg^{-1}$)	Found (Mean ± S.D., %)
	0.15	107 ± 11
Dicamba	0.10	92 ± 24
	0.04	119 ± 31
	0.30	67 ± 24
2,4-D	0.15	74 ± 23
	0.08	75 ± 27
	0.30	63 ± 8
MCPA	0.15	67 ± 5
	0.08	57 ± 8
	0.15	64 ± 18
2,4-DP	0.10	63 ± 20
	0.04	81 ± 13
	0.15	42 ± 13
2,4,5-T	0.10	50 ± 8
	0.04	118 ± 13
	0.15	46 ± 15
2,4-DB	0.10	45 ± 6
	0.04	93 ± 33
	0.15	46 ± 15
MCPB	0.10	70 ± 23
	0.04	106 ± 31
	0.15	41 ± 15
2,4,5-TP	0.10	48 ± 15
	0.04	87 ± 39

* n = 4.

for 2,4-D and MCPA, and 0.05 $mg.kg^{-1}$ for Dicamba and all the other studied chlorophenoxy acid in vegetable samples.[1] For reproducibility studies, the concentrations of Dicamba and chlorophenoxy acid herbicides were kept constant throughout the four determinations made for each spiked amount of each herbicide, and the results, mean of four analyses, are listed on Table 3.

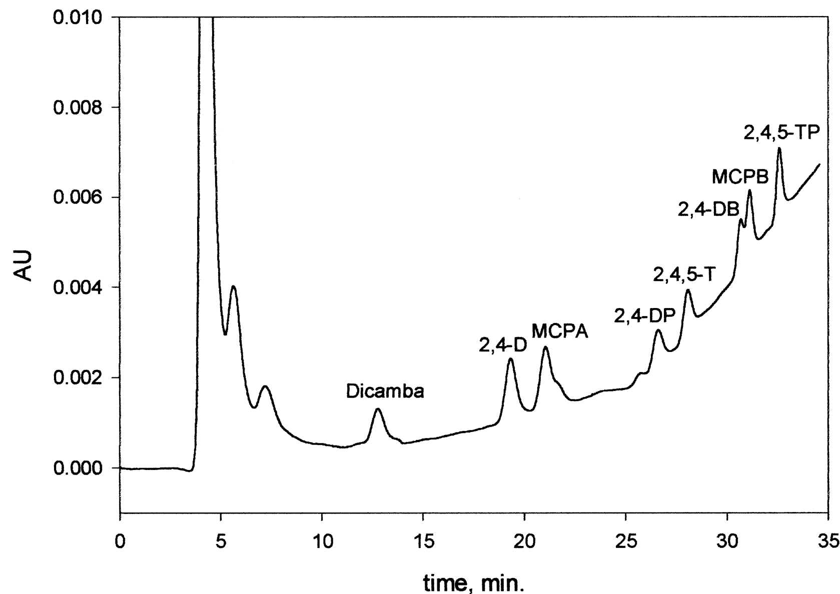

Figure 4. Liquid chromatogram of an extract of green bean spiked with 0.20 $mg.kg^{-1}$ of Dicamba and chlorophenoxy acids obtained in gradient elution mode.

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As can be seen, in general, acceptable recoveries, between 41 and 119%, were achieved. Since the studies of retention/elution in the preparative column packed with MFE-Polymer SAX for these analytes yielded recoveries around 100% when no matrix effect is present (samples of purified water with the analytes added),[8] it is reasonable to think that this decrease of the recovery values is due to the effect that the high content of non-polar compounds present in this type of samples has on the breakthrough volume of the chlorophenoxy acids in the preconcentration step. Figure 4 is a typical chromatogram obtained from a sample of green bean spiked with 0.20 $mg.kg^{-1}$ of each herbicide.

CONCLUSIONS

Combination of capillary liquid chromatography with solid phase extraction in columns packed with an anion exchanger based on MFE-Polymer (hidroxyethylmetacrylate, HEMA) yields an overall procedure capable of determining seven chlorophenoxy acid herbicides and Dicamba in green bean samples down to a level of 0.04 $mg.kg^{-1}$.

The use of adequate gradient elution programs in the chromatographic analysis can lead to avoiding or minimizing the problem of the overlapping peak of the polar matrix interference, co-extrated from the sample.

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