Large volume injection of acidic pesticides by reversed-phase micro high-performance liquid chromatography

Achille Cappiello*, Giorgio Famiglini, Arnaldo Berloni

Istituto di Scienze Chimiche, Università di Urbino, Piazza Rinascimento 6, 61029 Urbino, Italy

Abstract

Modern liquid chromatography–mass spectrometry interfaces often require a limited intake of mobile phase to work properly. Packed capillary columns combine high chromatographic efficiency, reduced flow-rate and an appreciable reliability and can be successfully employed in such applications. Acidic pesticides, a group of important compounds widely used in agriculture and often encountered as environmental pollutants, are positively analyzed by reversed-phase HPLC. The combination of micro-HPLC and particle beam mass spectrometry offer a powerful tool for their determination at very low concentrations. However, any detection limit obtained by this instrumentation is impaired by the constraint of a very small injection volume compatible with a narrow-bore column. In this paper, a procedure based on large volume injection is presented. This procedure is capable of increasing up to 800 times the volume of sample that can be injected into a packed capillary column. Seventeen acidic pesticides were selected for this study representing most of the compound classes commonly found in several environmental matrices. This method differs from previous attempts because of the acidic dissociation in water which may interfere with a correct solute focusing. The larger sample availability consistently improved the method sensitivity based on a LC–MS technique.

Keywords: Large volume injections; Pesticides; Phenoxy acid herbicides

1. Introduction

The use of scaled-down high-performance liquid chromatography techniques (micro-HPLC) are particularly advantageous when a reduced mobile phase flow-rate is required. The term micro-HPLC always refers to very narrow-bore packed columns instead of open-tubular columns so popular in gas chromatography (GC). They are usually classified on the basis of their internal diameter. Optimum flow-rate varies accordingly from over 100 μl/min for a 2 mm I.D.

microbore column to less than 1 μl/min for a 180 μm I.D. packed capillary column. Moreover, micro-HPLC outclasses conventional columns for some important aspects: increased efficiency in shorter time, significantly lower solvent consumption and enhanced signal-to-noise ratio of chromatographic peaks using concentration-sensitive detectors [1–4]. For different reasons, several current mass spectrometry soft ionization techniques and almost all the new LC–MS interfaces perform considerably better if most of the eluate solvents are removed before the ionization process takes place. Electrospray (ESI), fast atom bombardment (FAB) and matrix assisted laser desorption ionization (MALDI) when coupled

*Corresponding author.

to HPLC usually can only tolerate mobile phase flow-rates lower than 10 µl/min. A recently designed particle beam (PB) interface for capillary LC–MS [5,6] shows greater sensitivity, reduced chances for thermal decomposition phenomena and a much simpler operation procedure, only because of a drastic cut in the liquid intake. Our research group, who developed the new device, have successfully employed it for the analysis of explosives [7], pesticides [8,9] and other chemically labile compounds [10,11]. Micro-HPLC delivers sharper and narrower solute bands to the interface nebulizer using a minimal amount of an appropriate solvent mixture. Consequently, smaller droplets are generated carrying less solvent to evaporate. The solute, which is distributed among a larger number of lower mass particles, is rapidly vaporized into the ion source minimizing thermal decomposition. Acidic pesticides and in particular phenoxy acids herbicides take great advantage from the use of a micro-flow-rate nebulizer and show several improvements in their mass spectral results. Just to mention a few of them: peak tailing in the mass chromatographic profile is greatly reduced, decomposition products and their relative ion signals are eliminated from the mass spectra and more sample is available for ionization, thus improving sensitivity. Unfortunately, the great benefit in terms of reliability and instrument sensitivity achieved with a reduced flow-rate is often limited, in real world applications, by the inherent constraint of the typical injection volume compatible with a micro-column. This volume, ranging between 50 and 100 nl for a packed capillary column, impairs any method sensitivity relative to the extraction of very dilute analytes at trace-level concentrations. A recent paper [9] reported a new method for the analysis of acidic pesticides at very low concentrations (0.1–1 µg/l) in water, obtained by injecting 0.5 µl of sample into a micro-HPLC–PB–MS system. Although the response was unevenly distributed among the pesticides considered and some of them were poorly detected, it represented a considerable improvement over previous attempts. The slight increase of injection volume was clearly noticeable in terms of sensitivity and did not affect significantly the overall chromatographic performance but, unfortunately, larger injection volumes would not be tolerated without major modifications. Several au-

thors have contributed to overcome the injection volume limitation. Lin Ling et al. [12] presented an interesting comparison of the effects observed with conventional and micro-LC when large injection volumes are employed. Most of this scientific effort regarded micro-bore columns [13–19] with fewer applications involving capillary columns [20–24]. Very recently [25], a new method for the injection of large volumes of sample (up to 50 µl) into a packed capillary column was presented. This method differed from all previous attempts by the attention devoted to effects of a micro-flow-rate on chromatographic performance when different loops, in size and shape, are employed.

It was demonstrated that non-polar or slightly polar samples were easily focused at the head of the column when the analytes were dissolved in a weak, non-eluting solvent. Also a relationship was highlighted between loop shape and solute focusing. In reversed-phase liquid chromatography, water is the weakest solvent. When it is used as sample solvent, during the displacement of a large volume into the column from a long capillary injection loop, water plays the role of a weak, non-eluting mobile phase promoting focusing of the solute. This method has proven its validity for the analysis of several basic–neutral pesticides, allowing the injection of 800 times larger volumes (from 60 nL to 50 µl) and achieving a detection limit in aqueous samples of 20 ng/l.

However, some polar and water-soluble compounds are poorly retained by a reversed-phase column under such conditions and require specific modifications of the sample solution. Acidic pesticides are of great environmental interest and fall in this category. A similar topic, regarding the analysis of chemical warfare agents, was the matter of two papers published by Kientz et al. in which solute focusing was promoted by modifications in sample solution [20,21]. In this study, we reported the conditions required to inject up to 50 µl of an aqueous solution of selected acidic pesticides into a 250 µm I.D. reversed-phase packed capillary column [26]. The aim was to lower the current detection limits for this group of pollutants through the improvement of the injection procedure and to promote the use of capillary columns when they are coupled to other analytical techniques.

2. Experimental

2.1. Liquid chromatography

Liquid chromatography was carried out with a Kontron Instrument 420 dual-pump, binary-gradient, conventional HPLC system (Kontron Instrument, Milan, Italy). Microliter flow-rates were obtained with a laboratory-made splitter that was placed between the pumping system and the injector [27]. For sample injection, two Valco injectors were employed (Valco, Houston, TX, USA). One was equipped with an internal loop of 0.06 μl; the other was a six-port valve connected to laboratory-made external loops of different volume (Table 1). Fused-silica tubings of different internal and external diameter were used for the loop preparation and were purchased from Polymicro Technologies. The final loop volume was calculated from the length and the internal diameter value supplied by the manufacturer. The loops were connected using PTFE 1/16 in. adaptors and polyether ether ketone (PEEK) nuts and ferrules (1 in.=2.54 cm). A laboratory-made packed capillary column was used for the chromatographic separations [26]. These columns are routinely made in our laboratory from 1/16 in. O.D.×250 μm I.D. PEEK tubing (Alltech Associates, Deerfield, IL, USA) and are packed with C_{18} reversed-phase 5 μm particle size purchased from Phase Sep (Queensferry, UK). A 25 cm long column has a mean of 20 000 theoretical plates at 1 μl/min flow-rate. Acetonitrile or methanol were used as organic solvents in the mobile phase. Relative solvent concentration in the mobile phase, gradient programs and buffer additions are described in detail in Section 3.

Peak detection was performed using a Spectra-Physics, Spectra 100 UV–Vis detector equipped with a micro-cell. Unless otherwise specified, the detection of the analytes considered was performed at 225 nm.

2.2. Mass spectrometry

All the experiments were carried out with a Hewlett-Packard 59980B particle beam unit, coupled with a Hewlett-Packard 5989A mass spectrometer. The original nebulizer was replaced by a laboratory-made micro-flow nebulizer [5,6]. This device generates a mobile phase aerosol using flow-rates as low as 1 μl/min. A 50 μm I.D.×180 μm O.D. fused-silica capillary tubing (Polymicro Technologies, Phoenix, AZ, USA) was used as the nebulizer tip and to connect the liquid chromatograph. The nebulizing gas was helium 5.6 purity grade (>99.9996%) and was purchased from SOL (Milan, Italy). The helium pressure needed was 70 to 90 p.s.i. to supply 0.1 l/min (1 p.s.i.=6894.76 Pa). The desolvation chamber temperature was kept at 40°C. The operating pressure were 0.5 Torr in the desolvation chamber, 0.3 Torr in the second stage of the momentum separator and 8 to $10 \cdot 10^{-5}$ Torr in the manifold of the ion source (1 Torr=133.322 Pa). Mass spectrometer tuning and calibration were performed automatically using perfluorotributylamine (PFTBA) as a reference compound and monitoring m/z 69, 219, and 502. The repeller potential was adjusted manually. Mobile phase was allowed into the ion source during calibration. The dwell times during selected ion monitoring (SIM) analyses were adjusted in order to obtain 0.5 cycles/s and a mean of 10 acquisition samples for each HPLC peak. The ions used in the SIM mode were selected on the basis of their intensity and were collected in a single ion program. The final transfer tube, prior to the ion source, was shifted to a fully retracted position after the tuning procedure. The regular stainless steel target surface inside the ion source was replaced with a PTFE FEP insert prepared and adapted in our laboratory [28]. Because of the improved vaporization capability of this surface the ion source temperature was reduced at 190°C.

2.3. Reagents

All solvents were HPLC grade from Farmitalia

Table 1
External loop dimensions

I.D. (mm)	Length (mm)	Volume (μl)
0.15	282	5
0.15	565	10
0.25	407	20
0.32	622	50

Carlo Erba (Milan, Italy) and were filtered and degassed before use.

Pesticides were purchased from Riedel-De Haen (Hannover, Germany). TFA was purchased from Sigma Scientific (St. Louis, MO, USA). Reagent water was obtained from a Milli-Q water purification system (Millipore, Bedford, MA, USA).

3. Results and discussion

Acidic pesticides are advantageously analyzed by reversed-phase HPLC. Seventeen commonly used and easily encountered herbicides were chosen for this study [29] (Table 2). The selected analytes belong to various chemical classes such as phenols and phenoxy acids, are easily degradable by sunlight or heat and often show hard-to-solve drawbacks during the analytical procedure. When they are dissolved in water an acid–base equilibrium takes place with various degrees of proton dissociation. The typical injection volumes accepted by a 250 μm I.D. packed capillary column (50–100 nl) are usually delivered by a specific injector valve equipped with an internal loop. Larger volumes must be delivered through external loops assembled with capillary tubings of different lengths and diameters connected

to a conventional six-port injector. Because of the extremely reduced mobile phase flow-rate (1 μl/min), the solvent stream, which flows throughout the injection equipment, is influenced by the shape of the loop and all the internal connections encountered before the column head. Such parameters must be checked carefully in order to avoid turbulence and zones of stagnant liquid which may concur to a solute transfer delay.

Larger sample volumes are stored in external loops which are momentarily inserted into the mobile phase stream and transferred to the column. In this case the solute displacement is not instantaneous but depends upon the time required by a 1 μl/min mobile phase flow-rate to empty the loop and to transfer its whole content. The concentration profile is assumed to be a square wave function with a limited axial dispersion. During the displacement procedure the mobile phase delivered by the pumps surrounds the sample plug only at both sides while most of the solute is dispersed in the sample solution which acts as a mobile phase. As previously stated [25], if the solute is dissolved in an organic solvent it is not retained by the column and it is almost immediately eluted. If the solute is dissolved in pure water only the undissociated aliquot, based on a specific K_a, is conveniently retained and focused at

Table 2
Characteristics of the selected pesticides

No.	Pesticide	Class[a]	CAS-RN[b]
1	Picloram	Pyridinecarboxylic acid (H)	1918-02-1
2	4-Nitrophenol	Phenol (H)	100-02-7
3	Chloramben	Benzoic acid (H)	133-90-4
4	2,4-Dinitrophenol	Phenol (H)	51-28-5
5	Dicamba	Methoxybenzoic (H)	1918-00-9
6	Bentazone	Thiadiazinone (H)	25057-89-0
7	2,4,-D	Phenoxy acid (H)	94-75-7
8	MCPA	Phenoxy acid (H)	94-76-4
9	Mecoprop	Phenoxy acid (H)	7085-19-0
10	Dichlorprop	Phenoxy acid (H)	120-36-5
11	2,4,5-T	Phenoxy acid (H)	93-76-5
12	2,4-DB	Phenoxy acid (H)	94-82-6
13	MCPB	Phenoxy acid (H)	94-81-5
14	2,4,5-TP	Phenoxy acid (H)	93-72-1
15	Dinoseb	Phenol (H)	88-85-7
16	Dinoterb	Phenol (H)	1420-07-1
17	Pentachlorophenol	Phenol (H)	608-93-5

[a] Key: H; herbicide.
[b] Chemical Abstract Service registry number.

the column head while the rest of the solute is pushed forward by the water in the sample solution and poorly retained by the stationary phase. The addition of a convenient amount of an acidic buffer into the sample solution should suppress the acidic dissociation in the sample plug and enhancing solute focusing. Fig. 1 shows a direct comparison between the analysis of four acidic pesticides (picloram, chloramben, dicamba and bentazone) in gradient conditions using a 800 times larger external loop (50 μl) for the sample injection, without (a) and with (b) the addition of 0.05% of trifluoroacetic acid (TFA) in
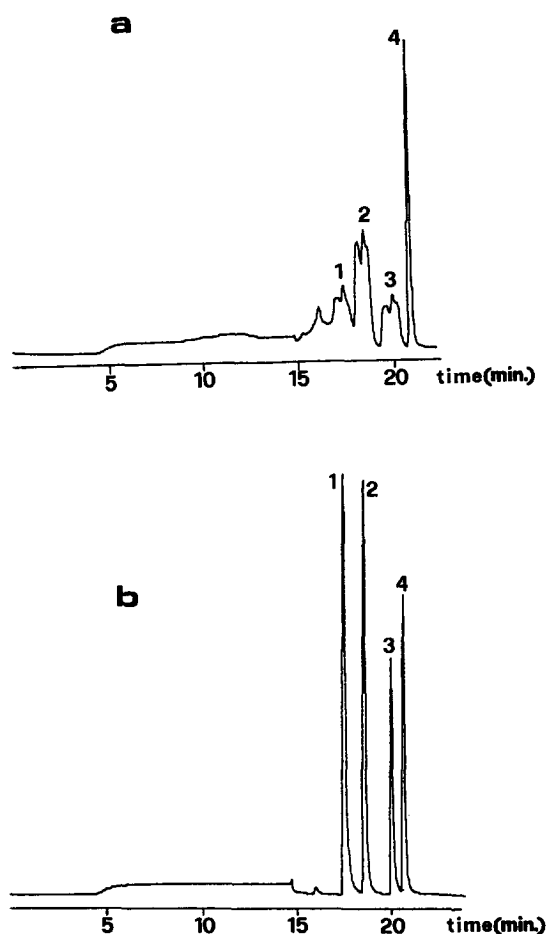

Fig. 1. Chromatographic profiles obtained injecting 50 μl of the pesticides aqueous standard solution: (a) without TFA in the sample matrix; (b) with the addition of 0.05% of TFA in the sample matrix. Chromatographic conditions: (water+0.05% TFA)–(acetonitrile+0.025% TFA), 80:20 to 20:80 in 40 min; flow-rate, 2 μl/min; sample concentration, 0.1 ng/μl.

the sample vial. The pesticides concentration was 0.1 mg/l, the flow-rate 2 μl/min, and the mobile phase, composed of water–acetonitrile modified with 0.05% and 0.025% of TFA, respectively, was varied from 80:20 to 20:80 in 40 min. Considering the extremely large gap between the volume injected and mobile phase flow-rate, the improvement shown by the chromatographic profiles is clearly evident. Picloram, chloramben and dicamba show a very similar molecular structure, are relatively strong acids ($1 < pK_a < 3$) and are almost completely dissociated in water. Consequently, when they are delivered under large volume conditions, they cannot be retained adequately without the addition of a pH modifier. Bentazone, the last eluted compound, is a very weak acid and it is scarcely influenced by the pH conditions. Once the analytes are converted into a non-ionic form they are focused at the column head and the band width is no longer influenced by the passage of 50 μl of sample solvent. The concentration of the acidic modifier depends upon the strongest acidic species in the sample and it should be sufficient to completely suppress its dissociation.

In order to have complete confidence in the large volume injection of acidic pesticides a calibration plot was obtained injecting increasing volumes of a 15 mg/l solution of the same four test pesticides. The separation of the analytes was obtained by gradient conditions at 2 μl/min as previously reported. The solution was injected five times using a 5 μl, 10 μl, 20 μl, and 50 μl external loop (Table 1). The experiment was required to evaluate the effectiveness of the focusing process by varying the volume injected, since an uncompleted focusing and loss of analytes is often observed when a too large volume is injected. Four regression plots were obtained from the collected data. The linearity was very good in the entire volume range considered as also indicated by the correlation coefficient very close to the unit picloram: 0.99956 ($y = 0.75x - 0.22$); chloramben: 0.99921 ($y = 0.67x - 0.4$); dicamba: 0.99978 ($y = 0.43x - 0.29$); bentazone: 0.99939 ($y = 0.45x - 0.11$). The new injection method is also surprisingly consistent for its reproducibility, calculated on the basis of the peak areas recorded for the calibration experiment, with a mean standard deviation of 1.4 for picloram; 0.7 for chloramben; 1.1 for dicamba; 0.8 for bentazone.

The incoming mobile phase, delivered by the pumps and continuously modified in gradient conditions, is diverted into the loop at the injection moment. From this moment, the stream of solvents is delayed by the volume of the loop and reaches the column head only after the loop has been emptied. To complete this transfer at 2 μl/min, the mobile phase requires 25 min with a 50 μl loop. This means that retention times are delayed approximately of 25 min compared to the values obtained by an internal loop injection. In addition, the increased resident time of the mobile phase inside the external loop may promote the axial dispersion of solvents with a change in the expected programmed composition of the mobile phase during a gradient analysis. In order to minimize any alteration in the solvents programmed composition, the isocratic solvent mixture found at the start of the program can be prolonged for the initial 25 min, sufficient to deliver the loop content at the column head. Once completed, the

mobile phase stream is re-diverted directly to the column by-passing the loop and the solvent program can be started. In this way the programmed mobile phase is addressed straightly toward the micro-column avoiding unwanted dead volumes. Fig. 2 shows two chromatographic separations of a 1 mg/l mixture of 17 acidic pesticides dissolved in acidified water (0.05% TFA) obtained in large volume injection conditions (50 μl) excluding (a) and not excluding (b) the loop by the mobile phase stream path after completion of the injection process. The mobile phase flow-rate was set at 2 μl/min and a mixture of water–methanol was linearly changed from 100:0 to 20:80 in 40 min. The solid line, superimposed on the chromatographic profile, represents the absorbance recorded at 190 nm during a blank injection in the same experimental conditions. Because of a different absorbance between methanol and water at 190 nm, this plot can easily visualize any modification in the mobile phase composition at
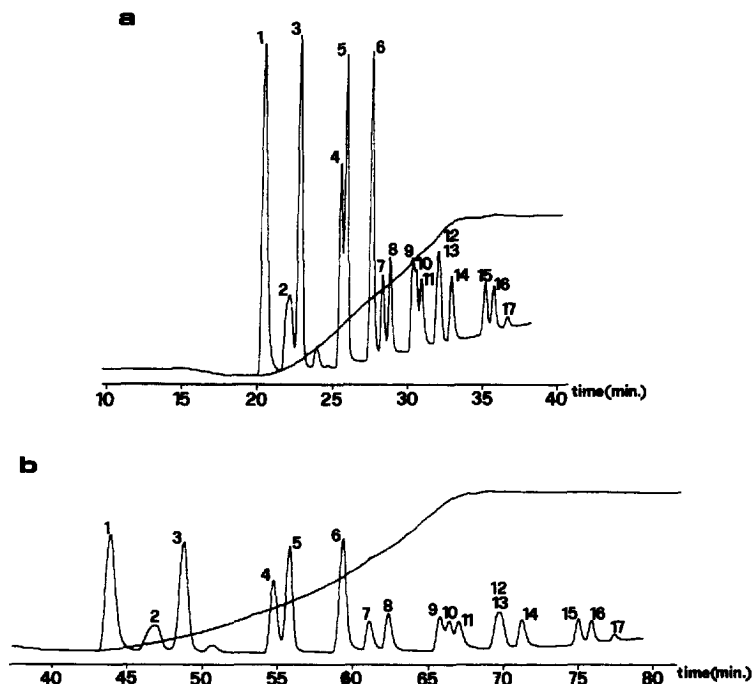

Fig. 2. Chromatographic separation of 17 acidic pesticides obtained injecting 50 μl of aqueous sample in different injection mode: (a) excluding and (b) not excluding the loop by the mobile phase path after completion of the injection process. Chromatographic conditions: (water+0.05% TFA)–(methanol+0.025% TFA), 100:0 to 20:80 in 40 min; flow-rate, 2 μl/min; sample concentration, 1 ng/μl. UV detection of pesticides was performed at 225 nm. UV detection of mobile phase concentration (solid line) was performed at 190 nm. See Table 2 for compounds list.

the end of the column. It appears clearly that the longer solvent path affects significantly the expected profile widening the peaks and heavily delaying their retention times, thus the by-pass injection mode should be preferred.

The determination of these pollutants in various matrices at trace-level concentrations often necessitate the use of specific detectors. The availability of reliable liquid chromatography–mass spectrometry techniques has extended the use of the mass spectrometer toward compounds once excluded because heat-sensitive and scarcely volatile. Particle beam interfacing benefited from several recent upgrading and showed very encouraging results for the analysis of acidic pesticides. Large volume injection in micro-HPLC supplies more sample at a very advantageous microliter-per-minute mobile phase flowrate, increasing sensitivity and reducing chances for

thermal decomposition. Fig. 3 reports the ion profiles relative to the analysis of tap water spiked with chloramben, bentazone, 2,4-DB, MCPB, dinoseb, dinoterb and pentachlorophenol at a concentration of 20 ng/l. 1 liter of water was passed through a SPE cartridge packed with 0.5 g of graphitized carbon black (GCB). The cartridge was extracted with 6 ml of methylene chloride–methanol (60:40 by volume) basified with 0.016 M KOH. The eluate was brought to neutrality adding 100 μl of a 7% solution of trifluoroacetic acid in water. The organic solvents were removed by evaporation leaving a 100 μl aqueous solution of the extracted pesticides at the bottom of the vial. As required by large volume injection, a given amount of TFA was added to the vial prior to the injection procedure. A 50 μl volume of the sample was injected in actual chromatographic conditions and via the particle beam LC–MS inter-
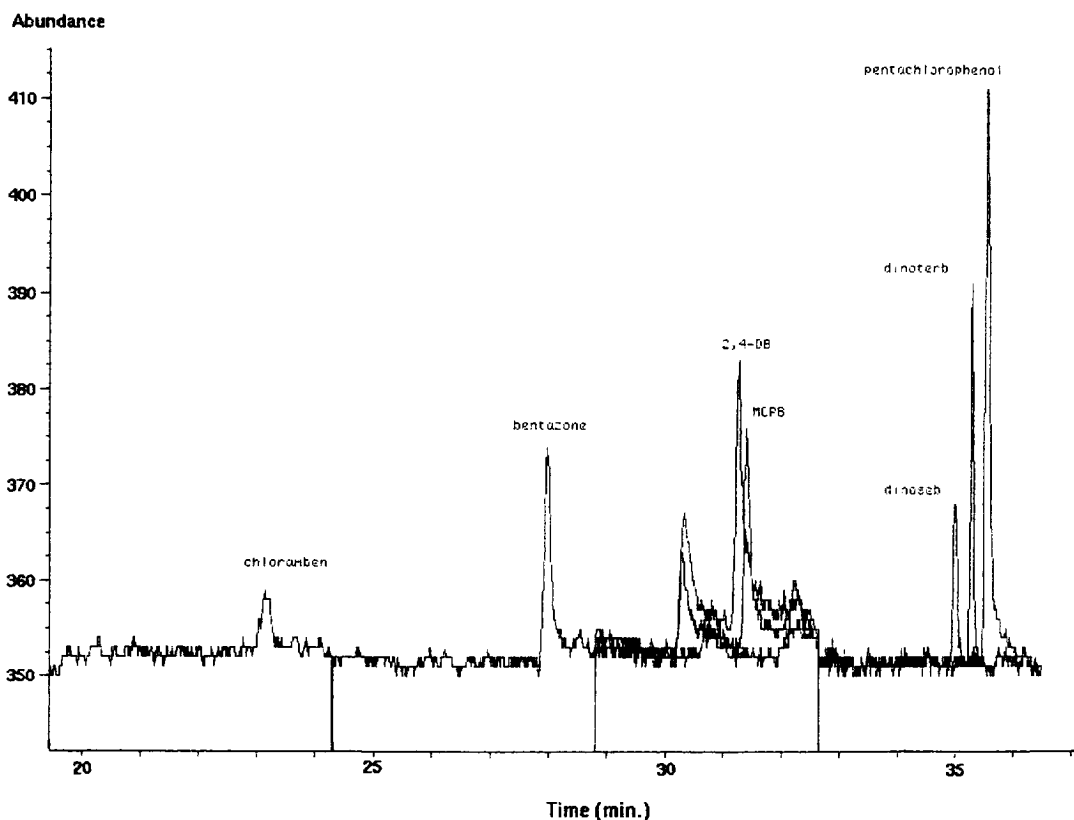

Fig. 3. Ion profiles relative to the analysis of tap water spiked with 20 ng/l of chloramben (m/z 205), bentazone (m/z 198), 2,4-DB (m/z 162), MCPB (m/z 142), dinoseb (m/z 211), dinoterb (m/z 225), pentachlorophenol (m/z 266) performing a large volume injecion of the extract (50 μl).

face. The mass spectrometer was operating in the SIM mode monitoring a characteristic ion for each analyte as reported in the figure legend. The LC–MS run was subdivided into four time programs in order to maximize the dwell time for each monitored ion and increasing sensitivity. As confirmed in the ion chromatogram the method detection limit is well below 20 ng/l for several compounds such as pentachlorophenol, dinoterb, dinoseb and bentazone and for all of them it represents a consistent improvement with respect to any previous similarly based methods.

In conclusion, this procedure permits the analysis of very dilute sample of acidic pesticides by micro-HPLC, taking advantage of the reduced flow-rate when it is coupled to mass spectrometry. The acquisition of high informative spectra like those obtained with a particle beam interface is performed better at lower mobile phase flow-rates like those employed in capillary HPLC. Large volume techniques come into play supplying the correct amount of sample. The proposed method is simple, reliable and effective and does not require special expertise or complex instrument modifications.

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