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Application of hollow fibre liquid phase microextraction for the multiresidue determination of pesticides in alcoholic beverages by ultra high pressure liquid chromatography coupled to tandem mass spectrometry

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Abstract

An alternative method has been developed to determine more than 50 pesticides in alcoholic beverages using hollow fibre liquid phase microextraction (HF-LPME) followed by ultra high pressure liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS), without any further clean up step. Pesticides were extracted from the sample to the organic solvent immobilized in the fibre and they were desorbed in methanol prior to chromatographic analysis. Experimental parameters related to microextraction such as type of organic solvent, extraction time and agitation rate have been optimized. The extraction method has been validated for several types of alcoholic beverages such as wine and beer, and no matrix effect was observed. The technique requires minimal sample handling and solvent consumption. Using optimum conditions, low detection limits (0.01-5.61 µg L^{-1}) and good linearity (R^2 > 0.95) were obtained. Repeatability and interday precision ranged from 3.0 to 16.8 % and from 5.9 to 21.2 %, respectively. Finally the optimized method was applied to real samples and carbaryl, triadimenol, spyroxamine, epoxiconazole, triflumizol and fenazaquin were detected in some of the analyzed samples. The obtained results indicated that the new method can be successfully applied for extraction and determination of pesticides in alcoholic beverages, increasing sample throughput.

Keywords: Pesticides; Wine; Beer; Liquid phase microextraction; Hollow fibre; UHPLC-MS/MS

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1. Introduction

Nowadays, a wide range of herbicides, insecticides and fungicides are used in grape production to protect against insects, fungi, molds, and other agents that may affect crop yield [1]. When pesticides are used in or on plant products, residues can occur on the raw agricultural commodities and they can be transferred to the processed and finished food products [2]. Despite of several European Community Directives have fixed maximum residue limits (MRLs) for viniferous grapes [3], no uniform limits have been established for pesticides in wine, although there is a worldwide trend towards setting specific MRLs in wine, ranging from 0.01 to 2 mg L^{-1} [4]. Therefore, the determination of pesticides residue levels in this type of samples is of special concern to ensure the safety consumption of these products. In this sense, the development of multiresidue analytical methods for the determination of pesticides is essential to monitor and control the use of agrochemical in vineyards.

Basically capillary gas chromatography (GC) [5,6] or liquid chromatography (LC) [7,8] have been the selected techniques for the analysis of pesticide residues in beverages, although capillary electrophoresis (CE) has been also used [9]. However, in the last few years, the use of LC coupled with mass spectrometry detectors (LC-MS), such as single quadrupole, triple quadrupole or time of flight [10] has improved the sensitivity and has been rapidly becoming an accepted technique in pesticide residue analysis for regulatory purposes. Nevertheless, one of the main problems associated with this combination is the ion suppression due to matrix effects, so the selected extraction technique should minimize this effect [11].

Traditionally, routine methods involve several sample preparation steps such as extraction, clean-up and concentration before instrumental analysis, using liquid-liquid extraction (LLE) or solid phase extraction (SPE) as extraction techniques [6,9,12]. However, these sample preparation steps are tedious, time-consuming and they consume large amount of solvents. On the contrary, new microextraction techniques such as solid phase microextraction (SPME) [13,14], stir bar sorptive extraction (SBSE) [15,16] and liquid phase microextraction (LPME) [17,18] have been introduced because they are easy, fast and solvent-free or less organic solvent consumption techniques. Among these techniques, liquid phase microextraction using hollow fibre membranes (HF-LPME) [19] provides mechanical stability and protection to the organic solvent because the use of a membrane or hollow fibre, which is simple, effective, low cost, uses microliters of organic solvents and provides an excellent sample clean up ability, obtaining very clean extracts. One of the main advantages of this technique over SPME is that the acceptor solution in hollow fibre was effectively protected within the fibre which served as filter, while SPME suffers from the presence of sample particles [20]. Besides, HF-LPME can also show some selectivity because of the pores in its wall. In this sense, large molecules, which can be soluble in extracting solvent, may not be extracted.

Basically, the piece of porous polypropylene hollow fibre is impregnated with a water-immiscible solvent and the analytes are extracted by passive diffusion from the sample into the hydrophobic organic solvent supported by the fibre (two phase HF-LPME). On the other hand, the analytes can be extracted through the organic solvent immobilized in the pores of the fibre and further into a new aqueous phase in the lumen of the fibre (three phase HF-LPME). Whereas two phase mode has been mainly used for hydrophobic compounds, which were analyzed by GC, three phase mode has been preferably used for ionisable compounds [21], using LC or CE as analytical techniques [22]. Traditionally, in the two phase mode, the organic solvent comprises the pores of the membrane and the lumen inside the hollow fibre, although there is a recent work, which only use the organic solvent immobilized in the pores of the hollow fibre as acceptor phase [23], and the organic solvent adsorbed in the fibre was desorbed after the extraction step prior GC analysis. This procedure could be an interesting alternative to the three phase system when the fibre can be desorbed in a compatible solvent for LC analysis, minimizing fibre handling due to the acceptor is not injected into the lumen of the fibre, and only a desorption step is necessary prior LC analysis.

The aim of the present study is the development of a new and simple extraction procedure, based on HF-LPME for the extraction of 51 pesticides from alcoholic beverages, prior to determination by ultra high performance liquid chromatography (UHPLC) coupled to tandem mass spectrometry

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Deleted: LPME is a extraction technique which implies the use of a microdrop of organic solvent to extract the selected compounds [19]. Nevertheless, one of the main problems of this technique is drop stability, so novel microextraction techniques that involve the protection of the organic solvent have been introduced [20]. Among them,

Deleted: provides mechanical stability and protection to the organic solvent, and it is usually termed as liquid phase microextraction using hollow fibre membranes (HF-LPME) [21]

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(MS/MS), possibly eliminating the matrix effects normally founded by LC-MS/MS when other extraction techniques are employed.

The combination of a simple and fast extraction technique such as HF-LPME together with the use of UHPLC-MS/MS permits the increase in sample throughput and the suitability of the proposed method for routine analysis.

2. Experimental

2.1. Reagents and materials

Certified pesticide standards were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany), Riedel-de-Haën (Seelze-Hannover, Germany) and Chemservice (Milan, Italy). Purities were always > 95.0 %. Stock standard solutions of individual compounds (with concentrations ranging from 200 to 300 mg L^{-1}) were prepared by exact weighing of the powder or liquid and dissolution in 50 mL of methanol (MeOH), which were then stored under refrigeration (T ≤ 5ºC). A multipesticide working standard solution (2 µg L^{-1} concentration of each compound) was prepared by appropriate dilution of the stock solutions with MeOH and stored under refrigeration (T ≤ 5ºC). MeOH (HPLC grade solvent) was obtained from Sigma (St. Louis, MO, USA). Acetonitrile (ACN, HPLC grade solvent) was supplied by J.T. Baker (Deventer, The Netherlands). Ethyl acetate (EtAc, residue analysis grade) and dichloromethane (DCM, residue analysis grade) were supplied by Riedel-de Haën and 1-octanol from Sigma. Other reagents were trioctylphosphine oxide (TOPO; ≥ 99 %; Aldrich, Steinheim, Germany), dihexyl ether (DHE; ≥ 97 %; Fluka, Buchs, Switzerland) and NaCl (≥ 99.5 %; Panreac, Barcelona, Spain). Q3/2 Accurel PP hydrophobic polypropylene hollow fibre tubing (200 µm wall thickness, 600 µm i.d., and 0.2 µm pore size) was obtained from Membrana GmbH (Wuppertal, Germany). 10-µL syringe plungers were provided by Hamilton (Bonaduz, Switzerland). Highly purified water (milli-Q Millipore, Bedford, USA) was used throughout for the preparation of mobile phase and samples.

2.2. Instruments and apparatus

Chromatographic analyses were performed with an Acquity UPLC™ system (Waters, Milford, MS, USA) and separations were achieved using an Acquity UPLC™ BEH C18 column (100 × 2.1 mm, 1.7-µm particle size) from Waters.

MS/MS detection was performed using an Acquity TQD tandem quadrupole mass spectrometer (Waters, Manchester, UK). The instrument was operated using an electrospray (ESI) source in positive mode. The ESI source was set as follows: capillary voltage 3.5 kV, extractor voltage 5 V, source temperature 110 °C, desolvation temperature 350 °C, cone gas flow 80 L h^{-1} and desolvation gas flow 600 L h^{-1} (both gases were nitrogen). Collision-induced dissociation was performed using argon (99.999 %) as collision gas at a pressure of 4×10^{-3} mbar in the collision cell. The multiple reaction monitoring (MRM) transitions and the cone and collision energy voltages applied are summarized in Table 1. Data acquisition was performed using MassLynx 4.0 software with QuanLynx software (Waters). A Reax-2 rotary agitator from Heidolph (Schwabach, Germany), a Unitronic 320 OP longitudinal agitator and an ultrasound bath from JP Selecta (Barcelona, Spain) were available for sample extraction. An analytical balance was used in standard preparation.

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2.3. Sample preparation by HF-LPME

The HF-LPME procedure shown in Figure 1 was prepared as follows: hollow fibres were cut into 2-cm pieces which were completely inserted into a 10-µL syringe plunger. The fibre was impregnated with 1-octanol (1 min) and then placed into a 15-mL screw top vial containing 15 ml of homogenised sample. In the optimized procedure, the vial was carefully closed and put into a rack in the rotary agitator for 45 min at 90 rpm. After the extraction stage, wine traces and plunger were removed by using tweezers and only the fibre was put into a 2-mL vial containing 1.5 mL of MeOH. This second vial was placed into a rack and then in the rotary agitator for 5 min at approximately 30 rpm in order to perform the desorption of the analytes from the fibre to the

solvent. Finally, the fibre was removed from the vial with tweezers and 5 µL of sample were injected in the UHPLC-MS/MS system.

2.4. UHPLC-MS/MS analysis

Chromatographic analyses were carried out using gradient elution with a mobile phase prepared from MeOH (eluent A) and 0.01 % formic acid in water (eluent B). The analysis started with 25 % of eluent B, which was linearly increased up to 90 % in 5 min (hold 1 min). This composition was held for further 2 min. Afterwards, a re-equilibration time of 1.5 min was included and so, a total running time of 11 min was obtained. The flow rate was 0.35 mL min^{-1} and the column temperature was set at 35ºC. Aliquots of 5 µL of sample extract were injected.

2.5. Samples

A variety of commercial wine and beer brands were purchased from local supermarkets in Almeria (Spain); other samples were obtained from home-made productions. All samples were analysed following the procedure described below and those samples showing the absence of the target compounds were used as blank samples in the preparation of standards and recovery studies.

3. Results and Discussions

Several set-ups can be used in HF-LPME, which implies three-phase or two phase systems. Recently, Zorita et al., [23] described a new one, which only uses as acceptor phase the organic solvent immobilised in the pores of the hollow fibre, minimizing sample handling and reducing extraction time. After the extraction, the fibre is transferred to a vial containing another solvent and the analytes are desorbed from the fibre to this last solvent. This procedure was used as an alternative to conventional two phase system. Bearing in mind the different polarity of the pesticides selected in this study (see Table 1), this set up could be an interesting alternative to two or three phase system, because depending on the desorption solvent, different pesticides could be desorped from the fibre, to a stripping solvent, so this approach was selected for the extraction of the selected pesticides.

On the other hand, the chromatographic analysis was based on a previously reported UHPLC-MS/MS methodology [24].

3.1. Optimization of the HF-LPME parameters

Different parameters that influence the extraction of the pesticides by HF-LPME were optimized, selecting peak areas as response. The optimization was carried out using blank white wine spiked with 25 µg L^{-1} of each pesticide, using 2 cm of fibre and 1.5 mL of desorption solvent; this volume enables the fibre to be completely immersed in the solvent (see Fig. 1)

First, the organic phase immobilized in the pores of the membrane was studied, as well as the solvent used to reextract the analytes from the membrane after the extraction (stripping solvent). The selection of the organic phase immobilized in the pores of the membrane was selected taking into account that solvent should be immiscible with the sample and stable (low volatility), so DHE, and 1-octanol were studied. Furthermore, DCM and EtAc were also tested because they are mainly used for the extraction of pesticides in conventional liquid-liquid extraction. The obtained results for metazachlor, linuron, terbuthylazine, cyprodinil and triflumizole are shown in Figure 2. When 1-octanol was used as organic solvent, better results were obtained for most of the pesticides. In consequence, it was used as organic solvent immobilized in the fibre, although for some pesticides such as linuron, promecarb, propyzamide, pencycuron, trifluoxystrobin, fluazifop buthyl and fenazaquin, better results were obtained when DHE was used. It can be observed that lower results were obtained when DCM and EtAc were used, maybe due to the volatility of these solvents.

In relation to the stripping solvent, MeOH and ACN were used considering that they are LC-MS compatible solvents. Both solvents provided similar results, but better peak shape was obtained when methanol was used, so further experiments were carried out using 1-octanol as solvent immobilized in the fibre and MeOH as stripping solvent.

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Bearing in mind that some of the compounds are relatively polar, such as cicloxidim, tepraloxidim, bensulfuron methyl and iodosulfuron methyl (log K_{ow} < 2), trioctylphosphine oxide (TOPO) was added in order to enhance the polarity of the membrane. Several concentrations of TOPO, from 1 to 10 % (w/v) were checked but no improvement of the extraction was observed for these pesticides, whereas the peak area of the less polar compounds decreased, so TOPO was not added in further experiments.

It is well known that the addition of salt can increase or decrease the extraction of analytes using the HF-LPME device [25]. The effect of addition of NaCl to blank white wine was studied in the range from 0 to 20 % (w/v); however, this modification did not improve significantly the extraction of the pesticides, except for spiroxamine, fenpropimorf and bensulfuron methyl. Besides, it was observed that the extraction efficiency considerably decreased at high values of NaCl, so further extractions were carried out without the addition of NaCl.

One important parameter that affects the extraction is the agitation of the sample. Traditionally, agitation was done by horizontal shaking, although stirring was also checked [25]. In this work, rotary agitation was also checked using an overhead shaker. Figure 3a shows the obtained results when horizontal shaker and rotary agitation was compared for carbaryl, epoxiconazol, paclobutrazol and sethoxydim (similar results were obtained for the rest of assayed pesticides), observing that better results were obtained when the overhead shaker (rotary agitation) was used.

Then, shaking speed was studied, and Figure 3b shows the obtained results for promecarb and trifloxystrobin (the same profile was observed for the rest of pesticides). It can be observed that extraction increased with the agitation rate of the sample up to 90 rpm. Higher speed was not selected taking into account the formation of air bubbles in the hollow fibre and the loss of the organic solvent.

Bearing in mind that analytes need time for their mass transfer through the sample to the liquid membrane, extraction time is an important parameter that should be optimized. Figure 4 shows the obtained results for desethyl terbuthylazine and azaconazol (time profile for simazine, and ofurace are similar), diethofencarb and bitertanol (time profile for the rest of pesticides is similar) when extraction time was studied between 15 and 240 min. It can be observed that for desethyl terbuthylazine peak area kept constant at short extraction time (15-30 min) and then decreased; whereas for diethofencarb and related compounds, peak area increased until 60 min, and when extraction time was increased, the signal slightly decreased. This behaviour was described previously [27,28], and the decreased of peak area can be due to pesticides were extracted back to the sample together with the loss of organic solvent. Considering sensitivity and analysis time, an extraction time of 45 min was selected as optimal condition.

Desorption conditions were also evaluated, using 1.5 mL of methanol as stripping solvent. First, sonication, horizontal shaking and rotary agitation were studied. The best results were obtained when rotary agitation was applied at 30 rpm. Second, desorption time was evaluated from 5 to 60 min, showing in Figure 5 the obtained results for monolinuron, paclobutrazol and fenazaquin (the same resuls were obtained for the rest of pesticides). The same signal was obtained when short desorption times (5-10 minutes) were applied, and the signal does not decrease with longer desorption time, so in order to increase sample throughput, 5 min was selected as desorption time. The efficiency of back extraction of the selected pesticides was confirmed by performing two consecutive methanol desorptions. The obtained results indicate that in the second desorption, the signal was always lower than 5 % of the signal obtained for the first desorption, so a second desorption was not necessary.

The extraction procedure with the optimized conditions was applied to spiked blank white wine (25 µg L^{-1}), and the mean value of extraction efficiency and relative standard deviation were obtained (Table 2), where it can be observed that good repeatability was achieved. It must be indicated that although for some pesticides such as simazine, desethyl terbuthylazine, chlorotoluron, diuron and bensulfuron methyl, extraction efficiency was lower than 10 %, HF-LPME is an efficient clean-up procedure for the extraction of pesticides from alcoholic beverages as it can be observed in Figure 6, where a spiked blank white wine (25 µg L^{-1}) was injected directly in the UPLC-MS/MS

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chromatogram (Figure 6a) and extracted by the optimized HF-LPME procedure prior chromatographic determination (Figure 6b). It can be noted that pesticides were not observed when wine was directly injected due to the matrix components, whereas the extracted wine provide a clean chromatogram, where only the pesticides were detected, and no interfering peaks appear on the chromatogram, indicating the efficiency as clean up technique of the HF-LPME.

3.2. Study of matrix effect on the extraction process

Ethanol is one of the major constituents of wine and beer and it is well known that it can influence the extraction of pesticides in alcoholic beverages [29], and sometimes it is necessary to dilute the sample prior to extraction in order to reduce alcoholic content. In this work, the effect of dilution was studied, fixing a total extraction volume of 15 mL, adding several volumes of wine (from 2.5 to 15 mL) and filled with water up to 15 mL, using a final concentration of pesticides of 25 µg L^{-1} in the final solution. The extracted amounts were approximately the same when the sample volume was between 5 and 15 mL (data not shown), obtaining lower responses when only 2.5 mL, as it was observed previously [13], so in order to minimize sample handling and to increase sensitivity, no dilution of sample was carried out.

The use of HF-LPME could be affected by matrix affect, so this influence over the extraction process and UHPLC-MS/MS determination of the pesticides in different alcoholic beverages, was studied. In this work, five beverages (white, red and sparkling wine, alcoholic and non-alcoholic beer) were selected for evaluation of matrix effect, analyzing the extraction efficiency when the indicated matrices were spiked with 25 µg L^{-1} of pesticides. Table 2 shows the relative recoveries determined when a white wine was used as representative matrix. No significant difference between the different matrices is observed, indicating that the selected matrices do not have a significant influence on the extraction procedure. Furthermore, recovery ranged from 72.7 to 112.0 %, indicating the reliability of the extraction procedure in the checked matrices. This means that the analytical procedure does not depend on the matrices evaluated and the HF-LPME devices provides clear extracts without matrix effect during UHPLC-MS/MS determination.

3.3. Validation

The optimized HF-LPME method was validated in terms of linearity, accuracy, precision, selectivity and lower detection limits.

Identification of the pesticides was based on the retention time windows (RTWs), defined as the retention time averages ± three standard deviations of the retention time when ten blank white wine samples spiked at 25 µg L^{-1} were analysed (Table 1) in five consecutive days. The identity was then confirmed by acquisition of two MS/MS transitions, bearing in mind European guidelines [30]. Selectivity of the method was evaluated by running control blank samples. The absence of any signal at the same retention time as target pesticides indicated there were no matrix interferences that may give a false positive signal.

Linearity of the method was tested by spiking blank white wine samples within the range from 5 to 100 µg L^{-1}, before the HF-LPME procedure was applied. Peak area was selected as response and good linearity was found for all pesticides at concentrations within the tested interval, with linear determination coefficients higher than 0.95 (Table 3).

Precision of the overall method was studied by performing repeatability and interday precision. Repeatability was evaluated spiking blank white wine samples at two concentration levels (5 and 25 µg L^{-1}), performing 5 replicates for each level (Table 3). For interday precision, two spiked samples at 5 and 25 µg L^{-1} were analysed daily for a period of 5 days (Table 3). It can be observed that repeatability and interday precision, expressed as RSD, were lower than 20 %, except for some pesticides such as simazine, iprovalicarb and triadimenol, which values were slightly higher than 20 % at low concentrations, indicating the good precision of the extraction procedure.

Limits of detection (LODs) and quantification (LOQs) were calculated analyzing spiked blank white wine samples at low concentrations. LODs and LOQs were determined as the lowest concentration of the analytes that produce chromatographic peak at a signal to noise ratio of 3 and

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10, respectively (Table 3). In general, low LODs and LOQs were obtained. For instance, LOQ ranged from 0.03 (fenazaquin and fenbuconazol) to 5.62 µg L^{-1} (isoproturon), which were similar or lower than other reported results [9,12]. It should be indicated that the MRLs fixed by the European legislation are referred to wine grapes or cereals, not to derivate products such as wine or beer; however it is worth noting that LOQ levels are below the values established MRLs for this type of commodities.

3.4. Analysis of real samples

The applicability of the method was evaluated by the analysis of 5 samples of beer (alcoholic and non-alcoholic) and 15 samples of wine, including a sparkling wine and two home-made wine (red and white) prepared by local farmers. In order to assure the quality of the results when the proposed method was applied, an internal quality control was carried out in every batch of samples. This quality control implies a spiked blank sample at 25 µg L^{-1} in order to check the reliability of the proposed method for each pesticide, a calibration curve and a blank reagent.

The obtained results are shown in Table 4 and no pesticides were detected in the analyzed beers. However, some pesticides, mainly insecticides and fungicides, such as spiroxamine, carbaryl and triadimenol, were detected in a few wine samples. Only one sample (S13), which corresponds to a home-made wine, presents high amounts of pesticides (carbary and triadimenol), suggesting that grapes have been treated with these compounds. Whereas triadimenol concentration was ten times lower than the MRL established for wine grapes [31], carbaryl concentration was higher than the MRL established for wine grapes. These values indicate that more work should be carried out in order to establish definitive MRLs in wines in order to assure the correct use of pesticides in this type of crops. On the other hand, epoxiconazole, triflumizole and fenazaquin were detected at trace levels.

Finally, figure 7 shows the UHPLC-MS/MS chromatograms for three positive results of carbaryl, triflumizole and fenazaquin at 138.7, 0.4 and 0.3 µg L^{-1} respectively in three wine samples, observing the high selectivity of the procedure due to the high clean-up efficiency of HF-LPME coupled with UHPLC-MS/MS.

4. Conclusion

A new method is proposed for the simultaneous extraction of 51 pesticides in wine and beer using HF-LPME. Microextraction method is very selective and negligible matrix effects were found when extracts were analysed by UHPLC-MS/MS. The method combines the advantages of UPLC-MS/MS, to separate and identify the pesticides, with HF-LPME in terms of reduction of organic solvents, simplicity and elimination of carry over effect through the use of disposable membranes. so HF-LPME can be used as an easy clean-up procedure of complex matrices. The sensitivity of the method allows the determination of the pesticides at trace levels in alcoholic beverages such as wine and beer. However, the extraction procedure is not automated and one of the main drawbacks is the operator skill should be high in order to get reproducible results. Despite of extraction time could be considered too long, a large number of samples can be extracted simultaneously, increasing the capacity of the method. Thus, 12 samples can be analyzed in less than 3 hours, including sample preparation and determination, so the method could be used in routine analysis.

Acknowledgements

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Figure Captions

Figure 1. Schematic of HF-LPME extraction: (a) immobilization of the organic phase in the fibre; (b) sample extraction; (c) removing the fibre from the sample; (d) desorption in methanol; (e) removing the fibre before chromatographic separation.

Figure 2. Effect of the organic solvent on the peak area of metazachlor, linuron, terbuthylazine, cyprodinil and triflumizole. Extraction conditions: 15 mL of white wine spiked with 25 µg L^{-1} of each pesticide; stripping solvent: methanol; extraction time: 30 min.; type of agitation: shaking (80 oscillations min^{-1}); desorption time: 10 min (shaking). Abreviations: DHE: dihexylether; OCT: 1-octanol; DCM: dichloromethane; EtAc: Ethyl acetate.

Figure 3. Effect of the shaking on the extraction. (a) Influence of the type of agitation on the peak area of carbaryl, paclobutrazol, epoxiconazol and sethoxydim. Extraction conditions: 15 mL of white wine spiked with 25 µg L^{-1} of each pesticide; organic solvent: 1-octanol; stripping solvent: methanol; extraction time: 30 min; desorption time: 10 min (shaking). (b) Influence of the agitation speed on the extraction of promecarb (•) and trifloxystrobin (▲). Extraction conditions: 15 mL of white wine spiked with 25 µg L^{-1} of each pesticide; organic solvent: 1-octanol; stripping solvent: methanol; extraction time: 30 min; agitation: rotary; desorption time: 10 min (shaking).

Figure 4. Extraction time profile of desethyl terbuthylamine (●), azaconazol (♦), diethofencarb (▲) and bitertanol (✕). Extraction conditions: 15 mL of white wine spiked with 25 µg L^{-1} of each pesticide; organic solvent: 1-octanol; stripping solvent: methanol; agitation speed: 90 rpm (rotary); desorption time: 10 min (rotary).

Figure 5. Study of the desorption time for monolinuron, paclobutrazol and fenazaquin. Extraction conditions: 15 mL of white wine spiked with 25 µg L^{-1} of each pesticide; organic solvent: 1-octanol; stripping solvent: methanol; extraction time: 45 min; agitation speed: 90 rpm (rotary).

Figure 6. Comparison of a: (a) UHPLC-MS/MS chromatogram of a spiked white wine (25 µg·L^{-1}) injected directly into the chromatographic system, and (b) the same spiked wine extracted by the optimized HF-LPME procedure prior chromatographic separation.

Figure 7. UHPLC-MS/MS extracted chromatograms of three positive real samples showing quantification (upper) and confirmation (lower) transitions: (a) white wine containing carbaryl at 138.7 µg L^{-1} (b) white wine containing triflumizole at 0.4 µg L^{-1} and (c) red wine containing fenazaquin (0.3 µg L^{-1}).

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Table 1
Logarithm of octanol-water partition coefficient (log K_{ow}), retention time windows and MS/MS conditions

Pesticide	Log K_{ow}	RTW (min)	Cone voltage (V)	Quantification transition[a]	Confirmation transition[a]
Simazine	2.1	3.32-3.43	35	202.1 > 132.1 (18)	202.1 > 96.1 (25)
Desethyl terbuthylazine	1.9	3.53-3.67	20	202.3 > 146.1 (16)	202.3 > 78.9 (25)
Carbaryl	1.8	3.54-3.68	20	202.1 > 145.1 (10)	202.3 > 127.0 (30)
Monolinuron	2.2	3.63-3.77	28	215.1 > 126.1 (18)	215.1 > 148.1 (15)
Chlorotoluron	2.5	3.79-3.94	25	213.2 > 72.1 (15)	213.2 > 46.2 (15)
Metobromuron	2.4	3.83-3.96	28	259.1 > 170.0 (20)	259.1 > 148.1 (20)
Atrazine	2.5	3.88-4.03	30	216.1 > 174.1 (18)	216.1 > 96.1 (25)
Metazachlor	2.1	3.89-4.06	15	278.3 > 134.0 (20)	278.3 > 210.2 (10)
Lenacil	2.3	3.92-4.10	20	235.2 > 153.0 (15)	235.2 > 136.0 (30)
Fensulfothion	2.2	3.94-4.11	30	309.1 > 281.2 (15)	309.1 > 157.0 (26)
Isoproturon	2.5	3.97-4.13	30	207.2 > 72.1 (30)	207.2 > 165.2 (15)
Diuron	2.8	4.05-4.20	30	233.1 > 72.0 (25)	233.1 > 160.0 (18)
Azaconazole	2.2	4.08-4.23	20	300.1 > 159.0 (23)	300.1 > 231.0 (16)
Iodosulfuron methyl	1.1	4.08-4.28	25	530.4 > 163.2 (16)	530.4 > 390.2 (16)
Bensulfuron methyl	0.6	4.18-4.30	40	411.3 > 149.2 (18)	411.3 > 182.2 (18)
Diethofencarb	3.0	4.30-4.45	20	268.3 > 226.3 (10)	268.3 > 152.1 (20)
Sebuthylazine	3.2	4.33-4.51	25	230.4 > 174.1 (18)	230.4 > 95.9 (25)
Propazine	3.0	4.36-4.53	25	230.4 > 188.2 (16)	230.4 > 146.6 (20)
Linuron	3.0	4.37-4.54	30	249.1 > 160.0 (18)	249.1 > 182.1 (18)
Spiroxamine	2.8	4.38-4.62	25	298.3 > 144.2 (20)	298.3 > 100.1 (33)
Methiocarb	3.1	4.44-4.59	22	226.1 > 169.2 (10)	226.1 > 121.1 (18)
Terbuthylazine	3.2	4.46-4.60	28	230.2 > 174.1 (15)	230.2 > 96.1 (25)
Paclobutrazol	3.2	4.51-4.67	25	294.2 > 70.0 (25)	294.2 > 125.0 (25)
Flutalonil	3.7	4.52-4.66	27	324.4 > 242.3 (25)	324.4 > 262.3 (20)
Promecarb	3.2	4.53-4.68	23	208.2 > 151.2 (9)	208.2 > 109.1 (15)
Prometryn	3.1	4.54-4.69	20	242.0 > 157.9 (17)	242.0 > 200.3 (25)
Propyzamide	3.2	4.61-4.72	25	256.2 > 190.0 (16)	256.2 > 173.0 (23)
Triazophos	3.3	4.66-4.80	35	314.2 > 162.1 (18)	314.2 > 119.1 (34)
Tepraloxidim	1.5	4.69-4.85	20	342.4 > 250.3 (12)	342.4 > 166.1 (20)
Iprovalicarb	3.2	4.70-4.82	20	321.4 > 119.1 (15)	321.4 > 203.3 (8)
Triadimenol	3.2	4.73-4.86	25	296.2 > 70.0 (12)	296.2 > 99.1 (12)
Fenhexamide	3.5	4.78-4.90	25	302.2 > 97.1 (25)	302.2 > 55.1 (30)
Epoxiconazole	3.4	4.82-4.95	25	330.2 > 121.1 (20)	330.2 > 141.1 (20)
Tebutam	3.0	4.88-4.98	27	234.3 > 90.9 (18)	234.3 > 192.2 (18)
Metolachlor	2.9	4.91-5.02	20	284.3 > 252.4 (15)	284.3 > 176.3 (23)
Fenbuconazol	3.2	4.91-5.02	30	337.3 > 70.1 (25)	337.3 > 125.0 (20)
Diflubenzuron	3.9	4.97-5.07	25	311.1 > 158.1 (12)	311.1 > 141.1 (22)
Cyprodinil	4.0	4.97-5.09	20	226.0 > 108.1 (20)	226.0 > 93.1 (20)
Thiazopyr	3.9	5.05-5.16	35	397.2 > 377.2 (28)	397.2 > 335.1 (22)
Furmecyclox	3.6	5.13-5.26	20	252.5 > 170.2 (13)	252.5 > 110.0 (20)
Spinosad	4.5	5.14-5.47	30	732.7 > 142.2 (30)	544.5 > 142.2 (25)
Bitertanol	4.0	5.29-5.39	25	338.3 > 269.3 (10)	338.3 > 99.2 (15)
Pencycuron	4.7	5.40-5.45	40	329.3 > 125.1 (19)	329.3 > 218.3 (14)
Trifloxystrobin	4.5	5.43-5.51	30	409.3 > 186.2 (18)	409.3 > 206.2 (14)

Triflumizole	5.1	5.51-5.59	23	346.2 > 73.1 (17)	346.2 > 278.2 (11)
Clethodim	5.1	5.53-5.62	20	360.3 > 164.1 (20)	360.3 > 268.3 (12)
Cycloxydim	1.4	5.53-5.62	22	326.3 > 280.4 (13)	326.3 > 180.2 (20)
Fluazifop buthyl	4.5	5.60-5.67	35	384.3 > 282.3 (20)	384.3 > 91.0 (30)
Sethoxydim	4.5	5.68-5.76	23	328.5 > 178.1 (18)	328.5 > 282.3 (11)
Hexythiazox	2.5	5.87-5.95	25	353.3 > 228.1 (15)	353.3 > 168.1 (20)
Fenazaquin	5.5	6.26-6.37	30	307.3 > 161.2 (16)	307.3 > 147.1 (16)

[a] Collision voltage (eV) is given in brackets

Table 2
Extraction efficiency and mean relative recoveries of pesticides in different matrices spiked at 25 μg L^{-1}

Pesticide	% E[a]	Relative recovery (%)[b]				
		White wine	Red Wine	Sparkle wine	Non-alcoholic beer	Beer
Simazine	3.6 (6.4)	83.3 (7.2)	86.1 (14.7)	109.4 (6.1)	102.2 (4.0)	93.9 (6.5)
Desethyl terbuthylazine	2.5 (3.2)	99.2 (5.7)	96.8 (14.1)	112.0 (7.7)	104.0 (5.6)	111.6 (12.7)
Carbaryl	4.9 (3.6)	73.5 (10.8)	76.3 (5.9)	80.4 (11.5)	71.0 (8.4)	75.9 (4.6)
Monolinuron	4.3 (3.6)	97.7 (12.2)	75.8 (13.3)	85.6 (9.6)	71.2 (13.9)	73.0 (11.5)
Chlorotoluron	3.6 (8.2)	97.2 (8.4)	103.9 (4.3)	108.3 (5.6)	90.0 (8.9)	91.7 (13.4)
Metobromuron	4.9 (5.3)	94.3 (6.4)	87.3 (15.6)	90.2 (6.4)	106.1 (13.5)	109.0 (8.3)
Atrazine	7.0 (10.5)	94.0 (7.8)	97.4 (9.8)	109.7 (4.8)	108.6 (15.9)	95.1 (8.8)
Metazachlor	2.2 (10.7)	80.0 (3.8)	87.3 (7.8)	79.1 (10.9)	90.0 (10.9)	80.9 (9.1)
Lenacil	3.4 (6.3)	73.5 (5.7)	108.2 (5.6)	82.9 (7.9)	82.4 (14.7)	75.3 (11.5)
Fensulfothion	6.9 (12.6)	97.4 (8.6)	97.1 (6.3)	73.0 (8.1)	75.1 (12.0)	76.2 (10.2)
Isoproturon	4.2 (13.8)	87.5 (10.5)	102.5 (5.3)	103.8 (12.2)	98.8 (5.5)	103.8 (8.6)
Diuron	4.5 (17.0)	102.8 (9.9)	106.0 (9.5)	108.8 (11.1)	111.2 (11.6)	98.0 (15.2)
Azaconazole	2.6 (16.0)	85.0 (6.4)	84.0 (8.6)	108.0 (10.8)	104.0 (4.0)	90.0 (11.0)
Iodosulfuron methyl	7.8 (8.5)	90.3 (7.3)	82.8 (13.2)	93.1 (12.6)	80.0 (11.4)	88.2 (12.3)
Bensulfuron methyl	4.6 (15.7)	104.1 (8.3)	73.9 (15.7)	86.5 (12.6)	103.5 (8.3)	76.1 (7.6)
Diethofencarb	7.8 (11.0)	97.4 (9.0)	96.2 (14.8)	104.4 (8.9)	111.5 (5.7)	103.8 (11.7)
Sebuthylazine	8.7 (5.6)	89.4 (8.5)	98.6 (5.7)	109.2 (13.7)	90.3 (7.1)	98.2 (4.9)
Propazine	9.7 (6.2)	91.7 (6.5)	73.7 (10.3)	102.3 (5.0)	92.9 (4.4)	85.7 (12.3)
Linuron	11.1 (14.9)	94.1 (15.1)	74.2 (13.9)	87.4 (12.3)	78.6 (12.9)	74.4 (11.9)
Spiroxamine	3.0 (16.9)	80.0 (10.3)	76.7 (6.9)	92.7 (6.8)	101.3 (12.8)	106.7 (11.8)
Methiocarb	13.1 (14.0)	86.9 (8.1)	77.7 (12.7)	87.9 (15.9)	85.6 (12.4)	83.2 (12.6)
Terbuthylazine	10.4 (11.1)	92.3 (6.1)	79.2 (12.4)	109.0 (8.2)	100.6 (9.4)	109.4 (6.8)
Paclobutrazol	8.2 (5.1)	105.8 (5.7)	93.7 (11.8)	88.2 (9.9)	103.7 (4.4)	108.5 (6.1)
Flutalonil	17.9 (10.2)	100.0 (9.0)	96.9 (15.0)	89.7 (11.4)	101.6 (16.0)	96.9 (8.9)
Promecarb	11.1 (6.4)	93.2 (4.8)	72.4 (15.6)	72.2 (7.5)	80.7 (11.8)	79.0 (6.9)
Prometryn	6.2 (8.4)	95.8 (9.3)	96.1 (15.7)	103.9 (10.1)	97.1 (6.6)	104.2 (13.7)
Propyzamide	12.3 (8.0)	104.9 (9.4)	87.5 (9.1)	75.4 (6.0)	87.2 (6.9)	97.6 (11.2)
Triazophos	15.5 (10.0)	91.5 (8.5)	80.4 (9.3)	81.7 (12.5)	95.6 (9.0)	82.6 (4.7)
Tepraloxidim	7.7 (3.7)	96.9 (11.8)	93.1 (8.1)	104.9 (15.4)	94.3 (5.8)	109.7 (5.9)
Iprovalicarb	13.1 (17.2)	91.7 (12.4)	90.3 (15.8)	110.0 (10.5)	104.3 (8.8)	106.3 (8.5)
Triadimenol	9.4 (13.9)	88.3 (7.0)	79.8 (4.7)	109.7 (5.1)	104.7 (6.0)	105.5 (6.0)
Fenhexamide	14.4 (12.2)	92.8 (6.5)	81.9 (4.5)	91.4 (8.3)	87.2 (6.9)	87.9 (5.1)
Epoxiconazole	15.0 (9.3)	103.6 (9.8)	98.5 (6.5)	109.5 (6.9)	110.1 (8.5)	102.4 (6.9)
Tebutam	14.5 (4.9)	102.5 (7.5)	101.2 (7.7)	105.0 (6.1)	105.4 (4.0)	107.7 (8.0)
Metolachlor	20.8 (12.7)	85.2 (4.1)	91.5 (14.8)	87.1 (8.6)	93.3 (15.1)	82.9 (12.5)
Fenbuconazol	25.5 (11.7)	98.1 (6.5)	102.0 (6.0)	97.0 (7.4)	109.3 (15.0)	108.2 (12.9)
Diflubenzuron	28.0 (8.5)	106.7 (8.3)	94.6 (7.0)	98.5 (15.4)	93.8 (14.9)	101.2 (7.3)
Cyprodinil	9.3 (12.3)	93.8 (6.9)	84.6 (11.1)	103.6 (14.5)	101.6 (11.8)	109.5 (9.9)
Thiazopyr	17.9 (9.3)	99.4 (5.4)	93.8 (6.4)	93.7 (8.0)	97.6 (15.9)	102.3 (13.6)
Furmecyclox	17.5 (7.0)	106.3 (8.4)	97.7 (7.3)	99.5 (7.2)	108.0 (10.6)	106.2 (10.0)
Spinosad	3.9 (7.4)	93.3 (9.4)	87.2 (4.7)	83.1 (13.0)	109.7 (7.8)	95.9 (7.2)
Bitertanol	21.2 (11.5)	109.9 (8.9)	97.7 (11.9)	91.0 (11.6)	87.6 (8.3)	102.7 (6.2)
Pencycuron	21.9 (10.1)	100.0 (6.1)	98.5 (10.5)	107.6 (13.7)	106.7 (5.5)	103.5 (12.7)
Trifloxystrobin	24.8 (10.0)	105.6 (9.7)	93.0 (13.8)	99.3 (10.2)	98.9 (12.9)	110.6 (10.8)
Triflumizole	20.2 (11.4)	82.7 (5.7)	76.4 (12.0)	71.2 (4.8)	70.3 (4.1)	71.4 (10.3)
Clethodim	17.7 (16.7)	86.1 (9.1)	93.2 (6.2)	96.2 (7.1)	92.2 (13.7)	92.3 (4.0)
Cycloxydim	21.6 (9.8)	83.5 (10.8)	78.2 (12.5)	87.9 (6.0)	80.6 (7.3)	80.3 (4.5)
Fluazifop buthyl	27.7 (4.3)	99.5 (7.9)	106.4 (5.1)	99.8 (15.3)	102.8 (10.9)	85.6 (11.5)

Sethoxydim	18.0 (14.0)	89.7 (4.7)	76.6 (7.1)	80.2 (13.8)	72.7 (7.7)	84.4 (5.5)
Hexythiazox	27.9 (9.9)	102.0 (5.4)	93.5 (8.8)	84.6 (6.0)	98.6 (10.9)	103.8 (10.2)
Fenazaquin	27.2 (8.3	88.3 (5.9)	83.2 (8.4)	81.7 (10.8)	78.2 (9.0)	82.0 (12.6)

[a] Mean extraction efficiency. Relative standard deviation is given in brackets (n = 5)
[b] Relative standard deviation (RSD) is given in brackets (n = 3)

Table 3
Validation parameters of the optimized HF-LPME procedure

Pesticide	R^2	LOD (μg L^{-1})	LOQ (μg L^{-1})	Repeatability	
				5 μg L^{-1}	25 μg L^{-1}
Simazine	0.9520	1.50	5.00	15.2 (21.2)[b]	3.9 (12.0)
Desethyl terbuthylazine	0.9820	0.10	0.30	6.7 (8.9)	4.0 (6.6)
Carbaryl	0.9886	1.20	3.80	12.9 (19.1)	8.1 (11.3)
Monolinuron	0.9567	2.00	5.00	11.5 (15.0)	8.1 (13.3)
Chlorotoluron	0.9729	1.00	3.40	10.1 (14.4)	5.8 (13.2)
Metobromuron	0.9970	1.90	5.30	14.2 (15.9)	10.3 (13.0)
Atrazine	0.9661	1.10	3.80	9.6 (12.6)	4.9 (9.3)
Metazachlor	0.9734	1.78	4.28	8.7 (10.4)	1.8 (4.5)
Lenacil	0.9883	1.60	5.10	9.5 (14.2)	4.7 (9.9)
Fensulfothion	0.9806	1.40	4.90	6.3 (14.4)	3.7 (8.9)
Isoproturon	0.9973	1.69	5.62	11.4 (16.2)	7.2 (12.8)
Diuron	0.9718	1.57	5.22	7.9 (10.8)	7.4 (8.6)
Azaconazole	0.9742	0.71	2.35	5.8 (12.3)	3.3 (8.4)
Iodosulfuron methyl	0.9923	0.70	2.33	12.7 (14.3)	6.1 (8.5)
Bensulfuron methyl	0.9889	1.50	5.00	14.2 (18.9)	9.8 (14.6)
Diethofencarb	0.9624	0.50	1.66	8.3 (13.5)	6.6 (11.4)
Sebuthylazine	0.9666	1.00	3.00	7.5 (16.8)	5.9 (18.5)
Propazine	0.9842	0.71	2.37	6.3 (13.4)	4.0 (10.2)
Linuron	0.9736	0.38	1.28	4.0 (12.7)	3.6 (10.9)
Spiroxamine	0.9932	1.10	3.67	7.2 (13.4)	5.2 (10.9)
Methiocarb	0.9954	0.78	2.60	8.4 (10.2)	5.9 (9.0)
Terbuthylazine	0.9535	0.51	1.70	10.7 (18.9)	3.7 (8.4)
Paclobutrazol	0.9827	1.00	3.33	9.2 (16.2)	6.5 (12.8)
Flutalonil	0.9952	1.42	4.26	9.6 (15.0)	6.7 (8.0)
Promecarb	0.9962	1.70	5.67	12.3 (13.2)	6.4 (12.9)
Prometryn	0.9650	1.60	5.33	9.7 (11.3)	5.3 (10.1)
Propyzamide	0.9702	1.50	5.00	12.4 (17.4)	5.1 (12.9)
Triazophos	0.9588	0.41	1.37	12.3 (14.5)	7.4 (10.9)
Tepraloxidim	0.9948	1.50	5.00	15.0 (18.4)	7.7 (18.2)
Iprovalicarb	0.9967	1.54	5.13	12.3 (20.5)	11.6 (13.2)
Triadimenol	0.9523	1.00	3.33	11.5 (20.7)	4.5 (9.9)
Fenhexamide	0.9503	0.09	0.30	6.1 (9.3)	3.0 (8.2)
Epoxiconazole	0.9910	0.15	0.50	6.5 (12.3)	4.8 (6.1)
Tebutam	0.9544	0.20	0.67	6.2 (14.3)	4.4 (7.3)
Metolachlor	0.9873	0.42	1.40	5.9 (10.4)	4.1 (5.9)
Fenbuconazol	0.9891	0.01	0.03	5.4 (5.6)	4.0 (6.3)
Diflubenzuron	0.9549	0.70	2.33	11.7 (14.8)	10.7 (11.2)
Cyprodinil	0.9634	0.09	0.30	4.3 (9.1)	3.3 (5.2)
Thiazopyr	0.9639	0.03	0.10	5.6 (10.0)	4.1 (9.1)
Furmecyclox	0.9702	0.07	0.23	6.5 (8.7)	4.6 (7.9)
Spinosad	0.9951	0.11	0.37	9.2 (10.9)	5.6 (9.3)
Bitertanol	0.9918	0.01	0.04	8.9 (12.5)	4.8 (8.3)
Pencycuron	0.9799	0.03	0.10	8.6 (12.3)	4.5 (7.4)
Trifloxystrobin	0.9762	0.50	1.67	9.4 (18.1)	7.5 (14.1)
Triflumizole	0.9764	0.05	0.18	8.7 (17.6)	4.2 (8.7)
Clethodim	0.9978	1.00	3.33	14.1 (14.5)	7.2 (9.4)
Cycloxydim	0.9994	1.40	4.20	14.3 (18.7)	10.4 (10.6)
Fluazifop buthyl	0.9787	0.09	0.30	9.3 (12.2)	7.1 (10.9)
Sethoxydim	0.9839	1.00	3.33	14.8 (19.6)	7.3 (11.2)

Hexythiazox	0.9889	0.80	2.67	16.8 (18.5)	6.6 (14.2)
Fenazaquin	0.9840	0.01	0.03	4.6 (7.6)	3.8 (6.1)

[a] Expressed as relative standard deviation (RSD) of 5 replicates
[b] Interday precision is given in brackets as RSD (n = 5)

Table 4
Concentration of pesticides detected in analyzed samples

Pesticide	Pesticides concentration (μg L^{-1})						
	S1	*S9*	*S10*	*S11*	*S13*	*S17*	*S19*
Carbaryl					138.7		
Spiroxamine	16.4						6.0
Triadimenol		8.5		49.2	163.6		
Epoxiconazole						1.9	
Triflumizole			0.4				
Fenazaquin		0.3					

Figure 1

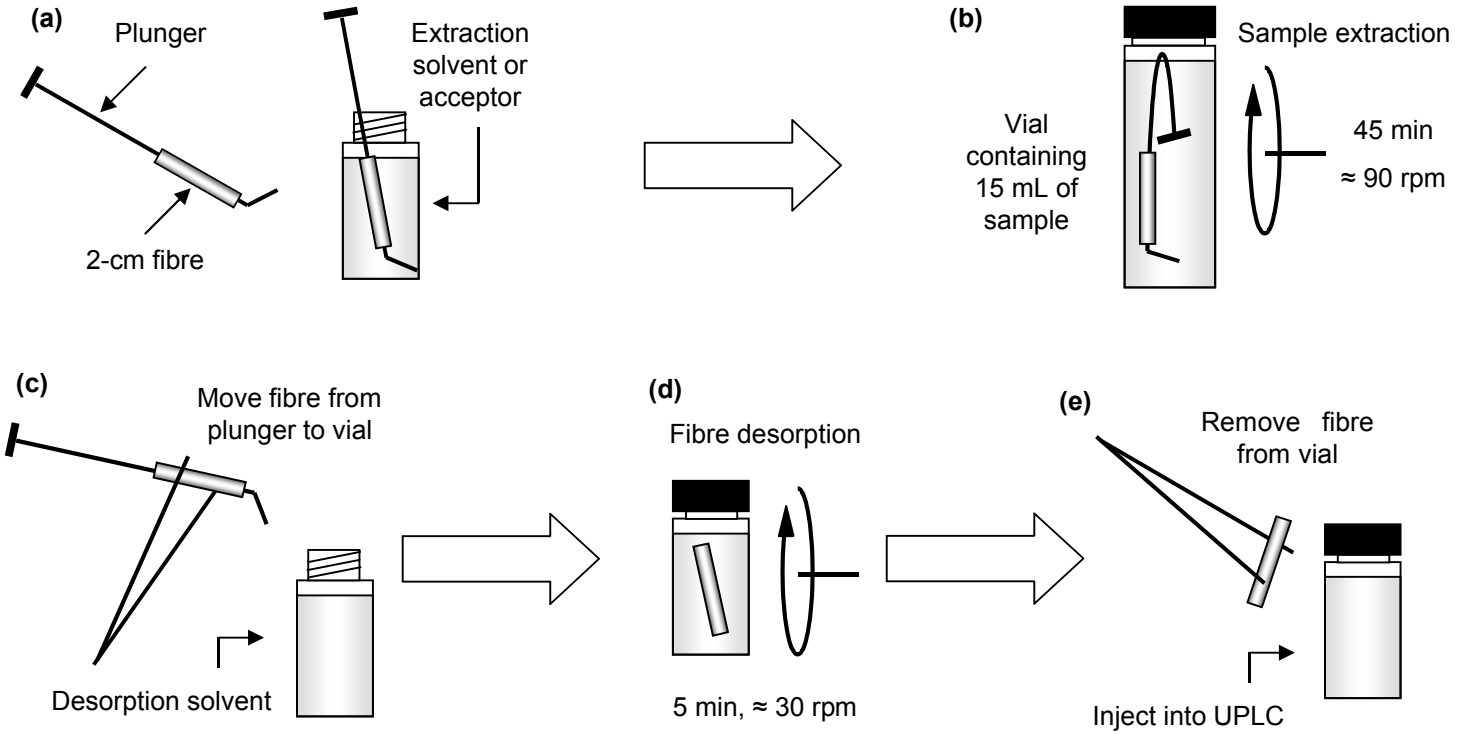


Figure 1

Figure 2

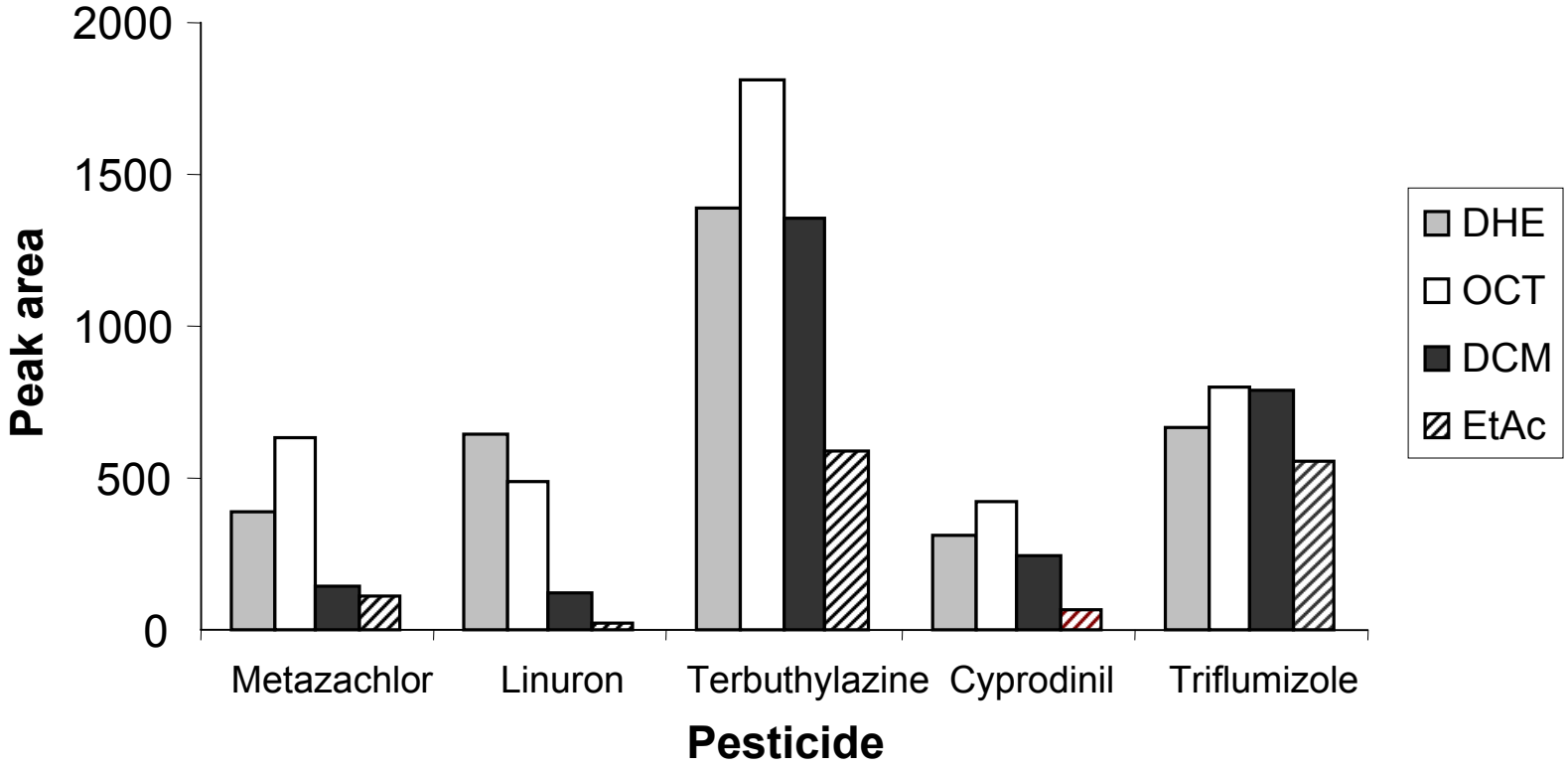
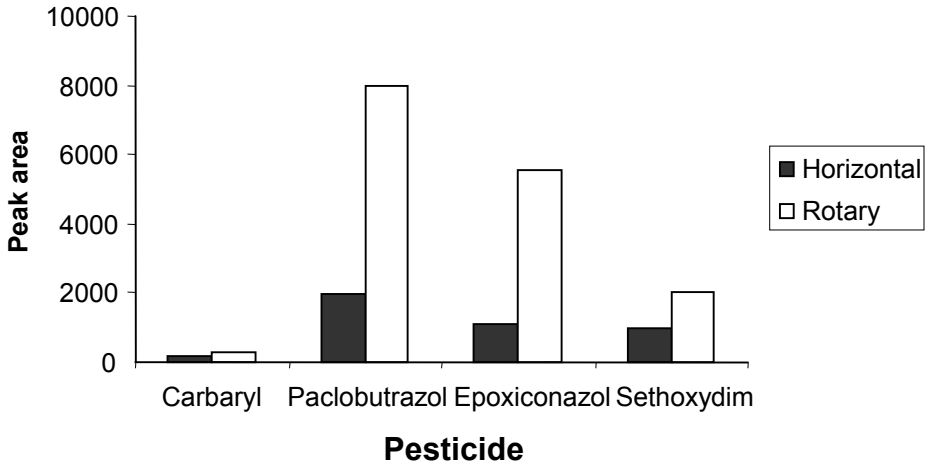

2000
1500
1000
500
0
Peak area
Metazachlor
Linuron
Terbuthylazine
Cyprodinil
Triflumizole
Pesticide
DHE
OCT
DCM
EtAc

Figure 3

a)


10000
8000
6000
4000
2000
0
Peak area
Horizontal
Rotary
Carbaryl
Paclobutrazol
Epoxiconazol
Sethoxydim
Pesticide

b)

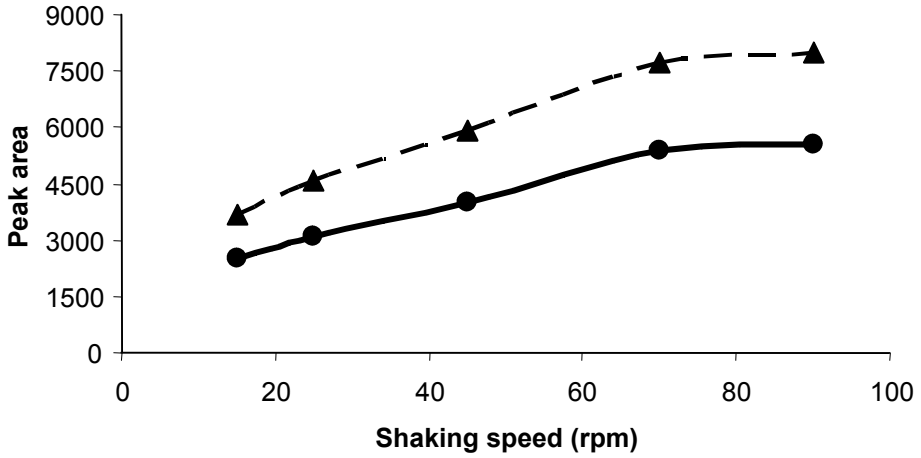

9000
7500
6000
4500
3000
1500
0
Peak area
0
20
40
60
80
100
Shaking speed (rpm)

Figure 4

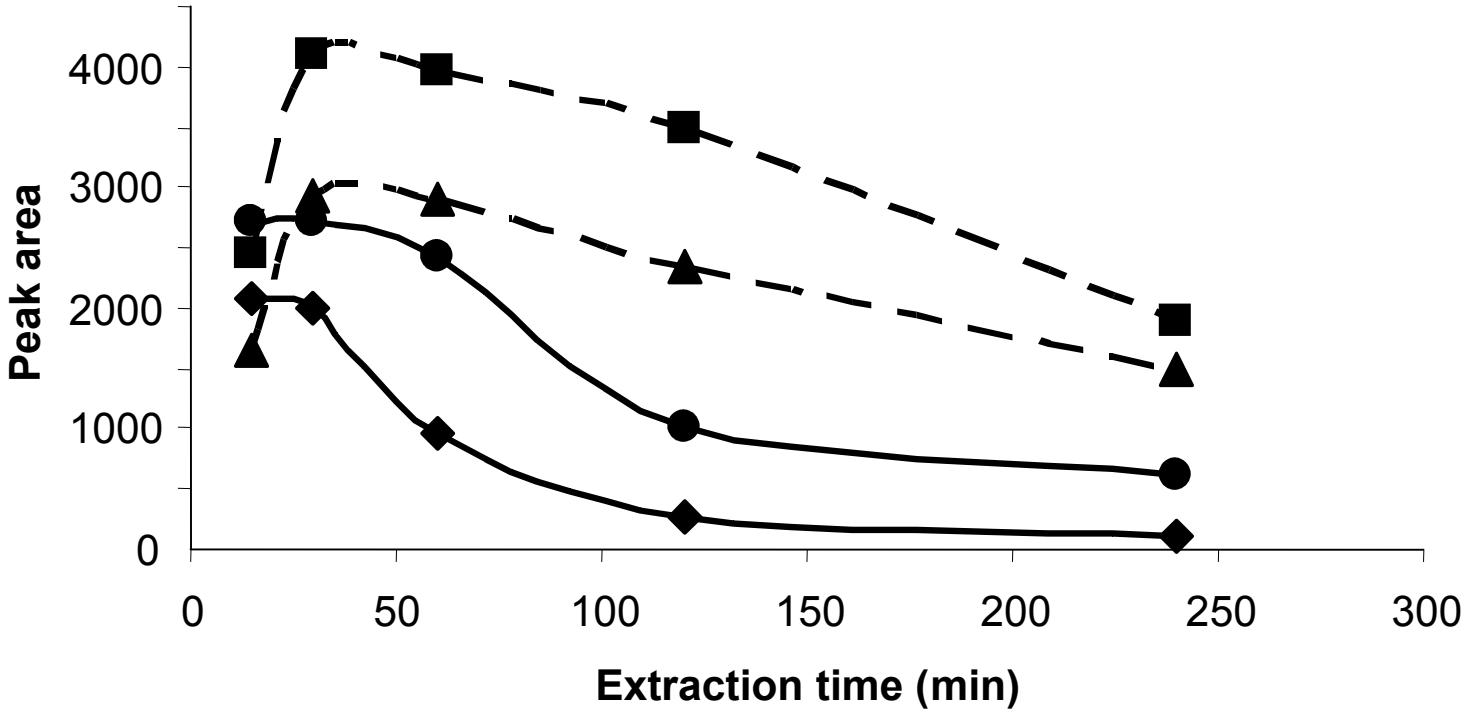

Peak area
4000
3000
2000
1000
0
0
50
100
150
200
250
300
Extraction time (min)

Figure 5

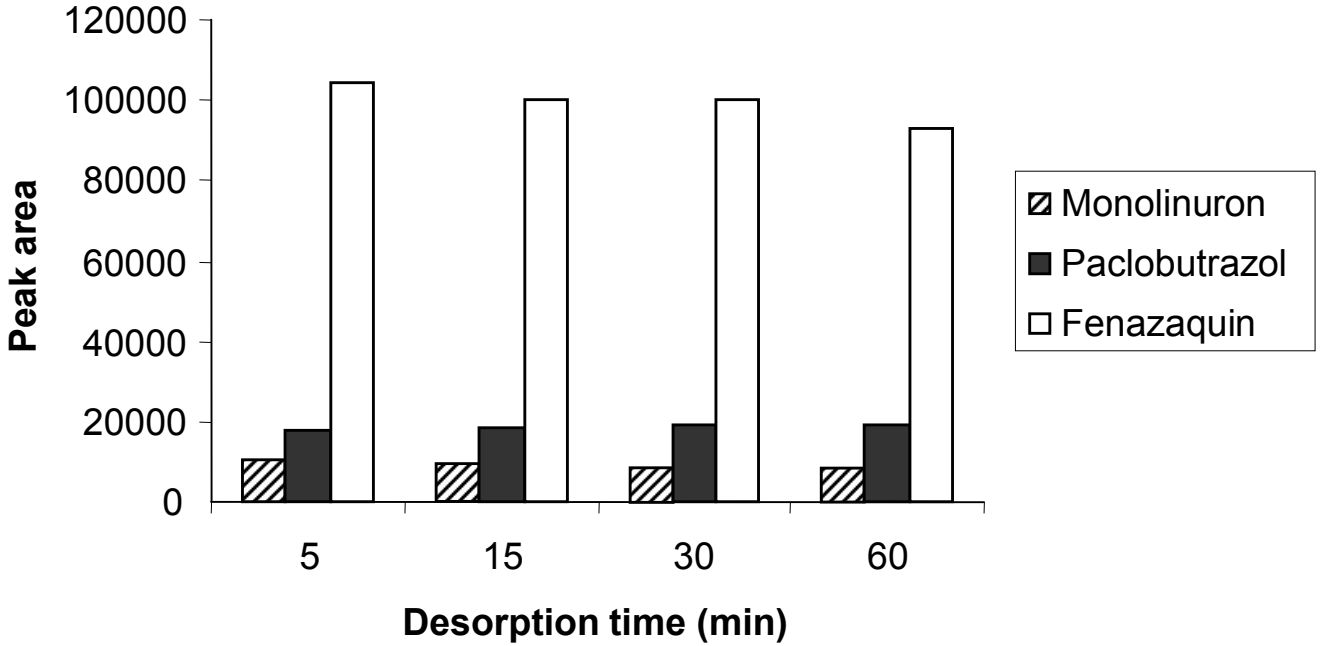

120000
100000
80000
60000
40000
20000
0
Peak area
5
15
30
60
Desorption time (min)
Monolinuron
Paclobutrazol
Fenazaquin

Figure 6

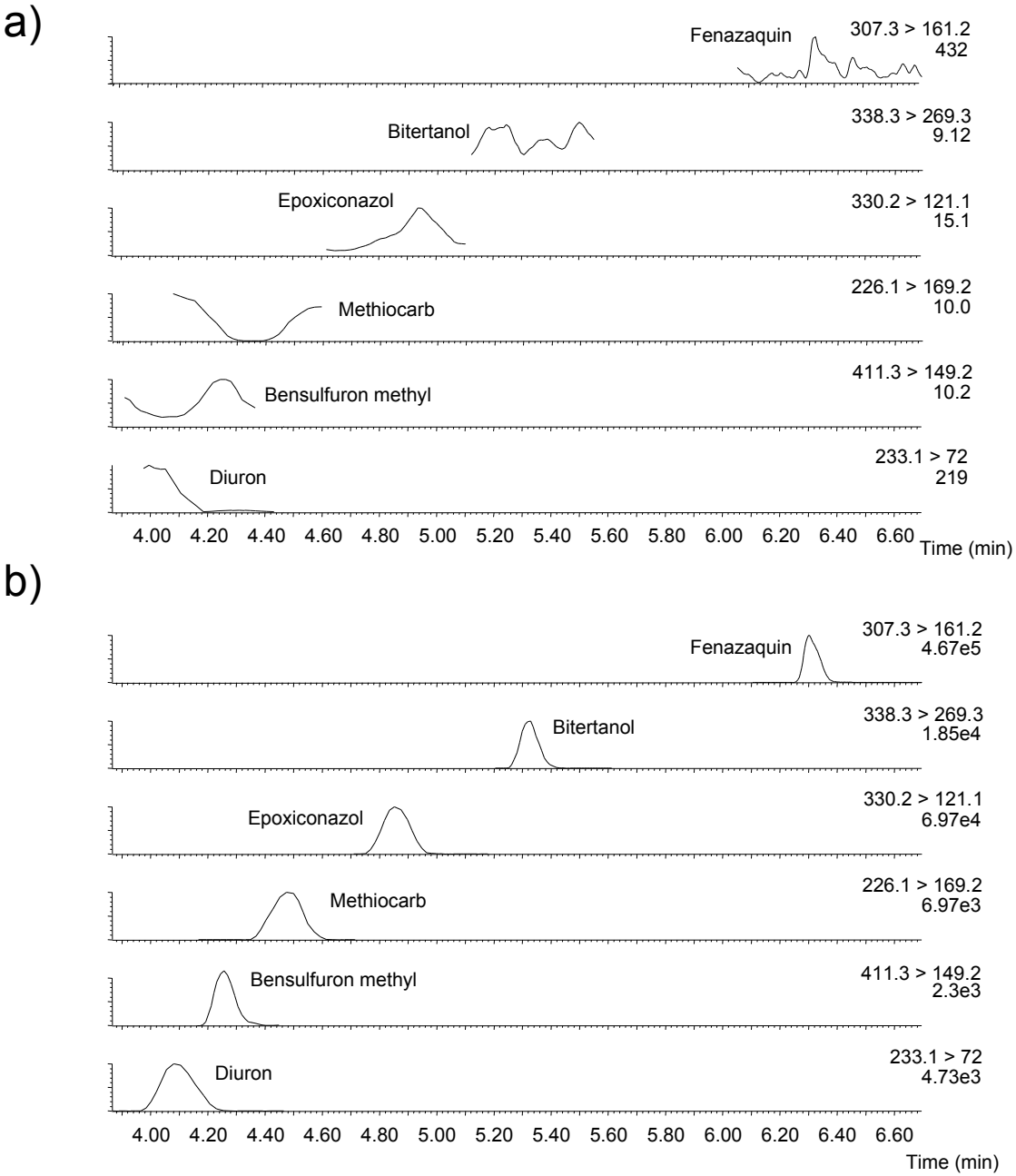
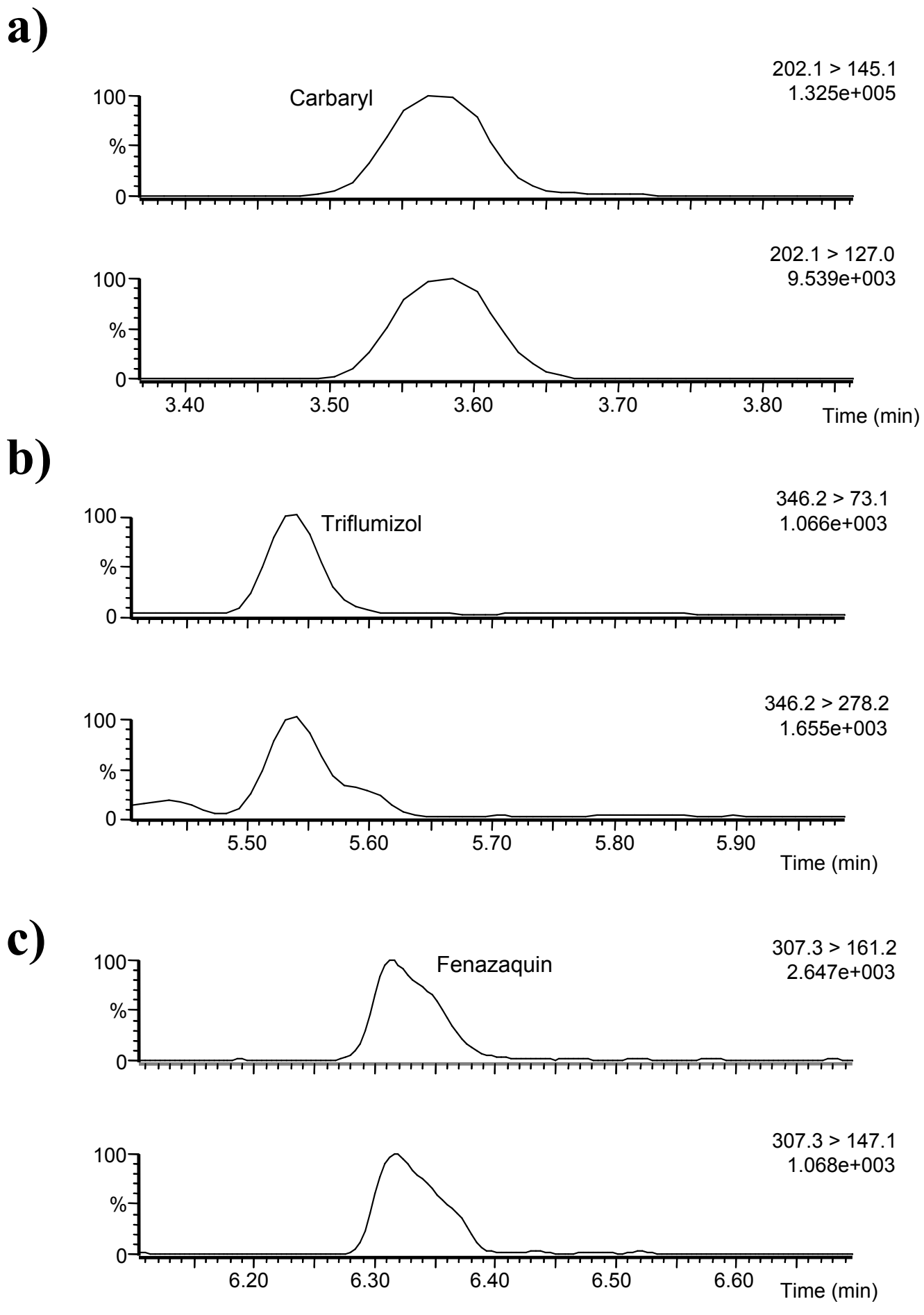

a)
Fenazaquin
307.3 > 161.2
432
Bitertanol
338.3 > 269.3
9.12
Epoxiconazol
330.2 > 121.1
15.1
Methiocarb
226.1 > 169.2
10.0
Bensulfuron methyl
411.3 > 149.2
10.2
Diuron
233.1 > 72
219
4.00 4.20 4.40 4.60 4.80 5.00 5.20 5.40 5.60 5.80 6.00 6.20 6.40 6.60
Time (min)
b)
Fenazaquin
307.3 > 161.2
4.67e5
Bitertanol
338.3 > 269.3
1.85e4
Epoxiconazol
330.2 > 121.1
6.97e4
Methiocarb
226.1 > 169.2
6.97e3
Bensulfuron methyl
411.3 > 149.2
2.3e3
Diuron
233.1 > 72
4.73e3
4.00 4.20 4.40 4.60 4.80 5.00 5.20 5.40 5.60 5.80 6.00 6.20 6.40 6.60
Time (min)

Figure 7


a)
Carbaryl
202.1 > 145.1
1.325e+005
202.1 > 127.0
9.539e+003
100
%
0
3.40
3.50
3.60
3.70
3.80
Time (min)
b)
Triflumizol
346.2 > 73.1
1.066e+003
346.2 > 278.2
1.655e+003
5.50
5.60
5.70
5.80
5.90
Time (min)
c)
Fenazaquin
307.3 > 161.2
2.647e+003
307.3 > 147.1
1.068e+003
6.20
6.30
6.40
6.50
6.60
Time (min)

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