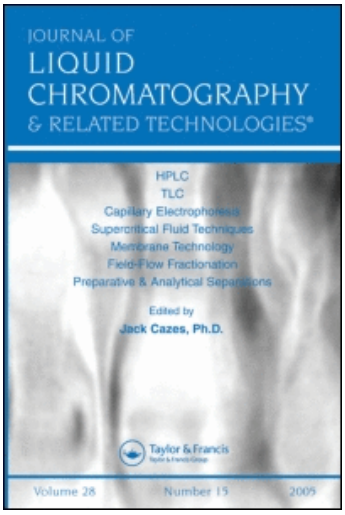

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DETERMINATION OF AZOLE PESTICIDES IN HUMAN SERUM BY COUPLED COLUMN REVERSED-PHASE LIQUID CHROMATOGRAPHY USING ULTRAVIOLET ABSORBANCE AND MASS SPECTROMETRIC DETECTION

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ABSTRACT

Analysis of some azole pesticides (triadimenol, triadimefon, and tetraconazole) in serum samples was carried out using a coupled column reversed-phase liquid chromatography with ultraviolet detection and liquid chromatography with electrospray mass spectrometry detection. A 5 μm GFF-II, internal surface reversed phase (ISRP, Pinkerton) column coupled to a 3 μm reversed phase, Microsphere C_{18} column were used for LC–LC-UV analysis. Linear dynamic ranges were established over more than two orders of magnitude. The applicability of the method to the determination of triadimenol, triadimefon, and tetraconazole in spiked serum samples was evaluated. Recoveries of spiked compounds range between 70.1% and 82.1% and 83.9% and

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100.7% at fortification levels of 0.2 and 0.05 μg/mL of each compound, respectively. The relative standard deviation (RSD) is lower than 7.0% in all cases, and the limits of quantitation (LOQ) calculated range from 5 and 10 μg/L.

For LC–ES–MS analysis the lineal range, detection limits, precision, and recovery of the analytical method after the clean up step with solid phase extraction using a C_{18} cartridge, were determined. The results obtained with both detectors were compared. The methods have been fully validated and utilized for analysis of triadimenol, triamidefon, and tetraconazole in serum samples of five agricultural workers from Almería (Spain).

Key Words: Coupled column LC (LC–LC); ISRP column; Column liquid chromatography–mass spectrometry; Serum analysis; Azole pesticides

INTRODUCTION

The widespread use of pesticides in agriculture and cattle-breeding has necessitated analytical methods for residue analysis of these compounds.

Measurement of human exposure can be done either directly or indirectly. Direct measurement involves determination of the pesticide residues in the media through which the exposure occurs, such as measurement of pesticides adsorbed on clothing worn by workers.[1–3] These techniques provide a calculable measure of human exposure under actual conditions. However, this type of measurement is not always possible and, in addition, does not consider other multiple sources of entry of pesticides into the human body, such as ingestion, or through respiration and dermal absorption.

Indirect methods of exposure assessment can also be used. These measurements more accurately reflect total exposure from all routes and sources of exposure.

The characterization of exposure and risk to human health from azole pesticides demands the availability of reliable data on the levels of those compounds in human tissues and fluids. We have chosen the analysis of serum samples.

Triadimefon, triadimenol, and tetraconazole are systemic fungicides that act by inhibition of steroid demethylation. They are used for the control of powdery mildews in cereals, fruits, cucurbits, tomatoes, ornamentals, and other crops.

In the technical grade, triadimenol contains two diastereoisomers (A and B) in a ratio of A : B of 7 : 3. Triadimenol is the principal metabolite of triadimefon

in animals. Triadimenol-A is excreted in urine (31–52%) and faeces (37–55%) and triadimenol-B is excreted in urine (14–47%) and faeces (44–78%).[4]

Residues of these compounds are analyzed commonly by GLC based techniques with ECD,[5] NPD,[6,7] and MS[7,8] detection for the determination of these compounds in different matrices such as vegetables,[5,6,8] fruits,[7] and waters.[9] Liquid chromatography with MS-MS detection has been used too.[10]

Different authors[11,12] have reported papers comparing single and coupled column systems in HPLC, concluding that the LC–LC system provide advantages over a single column system both in selectivity and sensitivity. Whereas interest has grown for using LC–LC in analysis of biological samples,[13,14] analysis of pesticides based on LC–LC in biological samples is scarce.[15]

Conventional methods for analysis of the target molecule in serum samples require extensive sample preparation and often introduce substantial experimental errors. In the case of complicated matrices, a C_{18} step before the LC–LC analysis is used to recover analytes or to clean up impurities from the sample,[16–19] getting a synergic effect of clean up, so when analyzing a serum sample using SPE and a single column system, a masked chromatogram is obtained. The SPE step also allows increasing the sensitivity of detection.[20,21]

A LC–LC configuration employing specially developed precolumn phases enables the clean up of complicated matrices, as serum, into an HPLC set-up, followed by subsequent analysis of the triadimefon, triadimenol, and tetraconazole analytes. The serum matrix compounds are quantitatively eluted in the void volume of the precolumn due to the restricted access given by the pore size of the packing.[22–25]

Internal surface reversed-phase (ISRP) supports are the most popular RAMs.[12,26] The development of ISRP silica materials by Pinkerton for assay of drugs in serum, milk, and urine by direct injection on to the LC column, has been successfully used for field analysis of drugs in biological samples[27] and pesticides in water[28] and soil samples.[29]

The use of a LC–LC-UV system achieves fast separation and selective and sensitive analysis of triadimefon, triadimenol, and tetraconazole. The sensitivity of the system is increased by the generation of narrow peaks on the second column, and its specificity is increased by the simultaneous generation of two sets of retention data for each substance.

The aim of the present work was to investigate the potential of LC–LC, using UV and ES–MS detection for determination of triadimefon, triadimenol, and tetraconazole in human serum samples. Essential in this work, was the use of a column packed with ISRP material as a preliminary separation column, which provided efficient separation of large interfering molecules and the analytes. The advantages of this approach are the possibility of obtaining very clean extracts, simplifying and automating extraction, and clean-up procedures.

EXPERIMENTAL

Materials, Chemicals, and Solvents

Triadimefon, triadimenol, and tetraconazole (content >99%) were obtained from Dr. S. Ehrenstorfer (Promochem, Wesel, Germany). Acetonitrile (HPLC-grade) from Merck (Darmstadt, Germany). HPLC-grade water was obtained by purifying demineralized water in a Milli-Q system (Millipore, Bedford, MA). Mobile phases were filtered by vacuum suction over a 0.45 μm filter (Millipore), mixed by volume, and subsequently degassed with helium before use.

Stock standard solutions of analytes were prepared by dissolving accurately weighed amounts of each pesticide in acetonitrile (500 μg/mL). Working standard solutions were made by diluting aliquots of the stock standard solutions to the appropriate concentrations in water. Fresh solutions were prepared daily. Aliquots of these working standard solutions were used to prepare calibration standards or to spike serum samples. The diluted solutions were kept in a refrigerator at 4°C.

Disposable 3 mL SPE cartridges containing 500 mg C_{18} were from Waters (Milford, MA). Cartridges were preconditioned sequentially with 15 mL CH_3CN and 10 mL Milli-Q water.

LC–LC-UV Analysis

The system consists of an isocratic Model 510 LC pump (P-1) and a gradient Model 600 LC pump (P-2) from Waters, a Rheodyne six-port injection valve (Model 7725), a Type 7000 high pressure column switching valve (V) from Rheodyne (Berkeley, CA), a Model 486 variable wavelength UV-Vis detector (D) from Waters set at 210 nm.

A 50 × 4.6 mm I.D. first separation column (C-1) packed with 5 μm GFF-II (ISRP, Pinkerton) from Regis and a 100 × 3 mm I.D. second separation column (C-2) packed with 3 μm Microsphere C_{18} (Chrompack, Middelburg, Netherlands) were used.

Recording of chromatograms and quantitative measurements of peak heights were performed with a Baseline 810 (Waters).

The mobile phases were set at a flow rate of 1 mL/min. A mixture of CH_3CN–H_2O (40 : 60, v/v) was used as the first mobile phase (M-1), while the second one (M-2) consisted of a CH_3CN–H_2O gradient (see Table 1).

A schematic diagram of a dual-column system used in the present study is shown in Fig. 1. The analytical process was as follows: a 1 mL aliquot of serum extract was injected into the sample loop, and was transferred to the pre-separation column at a flow rate of 1 mL/min (status B). In 2 min from the injection, the

Table 1. Mobile Phase Gradient (M-2) Used for the Determination of Triadimenol (A, B), Triadimefon and Tetraconazole

Time (min)	Acetonitrile (%)	HPLC Water (%)
0.0	40	60
2.0	40	60
4.0	50	50
12.0	50	50
14.0	40	60

switching valve was turned to the alternate position, so that the first separation column and main separation column were connected together (status A).

The mobile phase used in the first separation column (pump 2) carrying analytes from the first separation column to the main column was M-2 (described before). After duration of 3.6 min, the switching valve was turned back to the initial position to disconnect the first separation column and the main separation column, so as to re-equilibrate the former with the mobile phase in the pre-separation (status B). Analytes were eluted from the main column with the mobile phase used for the main separation (pump 2) (M-2), (see Table 1). The time required for one analytical run was approximately 14 min.

LC–ES–MS Analysis

The system consists of a Hewlett Packard (Waldbronn, Germany) series 1100 with a HP Chem Station for recordings of chromatograms and quantitative

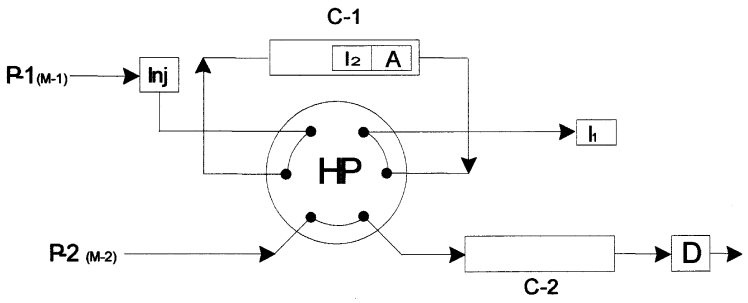

Figure 1. Schematic presentation of separation procedure involved in coupled-column RPLC. Inj = Injector; C-1, C-2 = first and second separation column, respectively; M-1, M-2 = mobile phases; I_1, I_2 = interferences; A = target analytes; HP = high-pressure valve; D = detector.

measurements. A gradient Model G 1311 A pump from Hewlett Packard, an autosampler, Hewlett Packard 1100, injecting aliquots into a 20 μL sample loop was used.

A 100×3 mm I.D. column packed with 3 μm Microsphere C_{18} (Chrompack, Middelburg, Netherlands) was used. The mobile phase was set at a flow rate of 0.8 mL/min. The solvent program was acetonitrile–0.4% formic acid in water (45 : 55, v/v) in isocratic mode. A Hewlett Packard G 1948 A Platform benchtop mass spectrometer, consisting of a ES interface and a single quadrupole, was used for detecting and quantifying target compounds in the LC column effluent. This was introduced into the ES interface through a 30 cm length of 0.25 mm diameter PEEK capillary tube. The MS was operated in the positive ion mode by applying to the capillary a voltage of 3000 V. The source temperature was maintained at 350°C. Ions were generated using highly pure nitrogen as a drying gas at flow rate of 10 L/min and nebulizing gas at a pressure of 50 psi *g*.

Previous to the LC–MS system, a sample clean up step with the ISRP column was made, injecting 1 mL of sample into the HPLC system and the fraction eluting between 2 and 3.6 min from the HPLC system was collected. Twenty microliters of this extract was injected into the LC–MS instrument.

LC–MS chromatograms were obtained by operating in the time-scheduled, one selected ion monitoring (SIM) acquisition mode. Quantitation ion (m/z) values were 296, 294, and 372 for triadimenol, triadimefon, and tetraconazole, respectively, applying a fragmentor voltage of 60 V in all cases. Fragment ions were obtained by controlling the potential difference between sample and skimmer cones. This provides structurally significant fragment ions in the intermediate ion transport region by cone-induced fragmentation (CIF).

Sample Preparation Procedure

A volume of 8 mL CH_3CN was added to 10 mL of human serum to precipitate proteins, mixed on a vortex mixer during 60 s, centrifuged (20 min at 1600 *g*), passed through to a preconditioned 500 mg C_{18} cartridge, and dried with air for 10 min. (the cartridge was not allowed to dry during conditioning). The sample was eluted with 8 mL of acetonitrile, dried under a gentle stream of nitrogen and redissolved in 2 mL of LC-water. An aliquot of 1 mL was finally injected into the liquid chromatography system.

Handling and Storage of Serum Samples

The serum samples used as controls were collected from the local hospital blood bank. Real human serum samples were collected from occupationally

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exposed, volunteer agricultural workers of Almería. All samples were frozen at −20°C until analysis.

RESULTS AND DISCUSSION

Analysis by LC–LC–UV

The applicable UV absorption [wavelength (λ) and molar absorptivity (ε°)] and the retention time of the analyte on the stationary phase largely determine the sensitivity and selectivity that can be obtained. A wavelength of compromise of 210 nm was chosen for measuring analytical signals of the azole pesticides. Molar absorptivity was 33,000, 34,000, and 59,000 L/(mol cm) for triadimenol, triadimefon, and tetraconazole, respectively. Lower wavelengths increase the sensitivity of the method, but increase the number of interfering compounds belonging to matrix sample.

A short column packed with 5 μm GFF II internal surface reversed material (Pinkerton) was selected as C-1 column due to its capability to separate the early-eluting polar interferences. It is advisable to select a C-2 column with a higher separation power than C-1. This will provide some flexibility when optimizing the eluotropic strength of M-2, in order to reach a proper compromise between the required separation efficiency on the second column and the desired peak compression (sensitivity of detection). A 3 μm C_{18} column was selected as the second column (C-2), because of the high separating capability of the RP18 material.

Different mobile phases of acetonitrile : water, were assayed; the acetonitrile content ranged between 20 to 100% for M-1. Percentages higher than 50% of acetonitrile gave broader peaks and overlap took place. A mixture of CH_3CN–H_2O (40 : 60, v/v) was chosen because it provided narrower peaks, but the resolution between analyte peaks were lower than unity. The linear gradient optimum conditions for M-2 can be seen in Table 1.

Injection volume between 200 and 5000 μL were assayed. Good peak shapes were obtained when a 1 mL of sample was injected. This volume was selected as a compromise between the sensitivity required and speed of analysis. With these mobile phases, a clean-up volume of 2 mL (sample injection included) and a transfer volume of 1.6 mL were determined by connecting C-1 with the UV detector. Triadimenol shows two peaks because of a diastereoisomerization process.

To the analysis of samples of complex matrix, it is necessary to combine SPE clean up with column switching to remove large interfering solutes from the extract. Figure 2 shows chromatograms corresponding to a human serum control sample spiked to a 0.05 μg/mL level of each compound and the same non-spiked

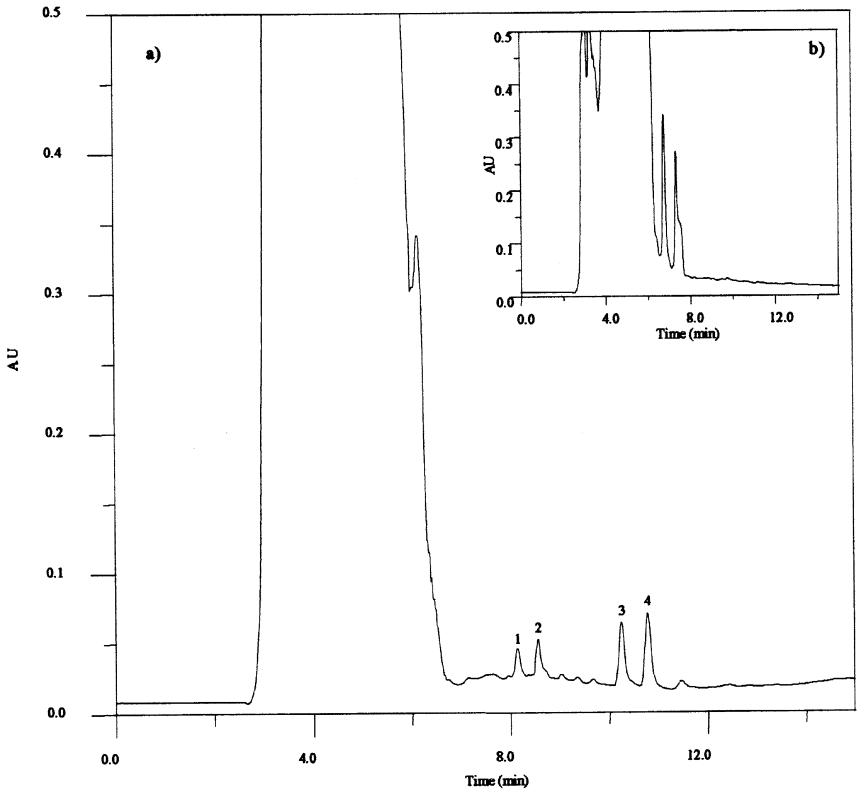

Figure 2. LC–LC–UV chromatogram of a serum sample at 0.05 μg/mL of each compound and analyzed with the proposed method (a), peak numbers: 1 = triadimenol B, 2 = triadimenol A, 3 = triadimefon, 4 = tetraconazole, and the same sample non-spiked (b). Wavelength 210 nm.

sample. Retention time windows (RTWs), defined as retention time (t_R) averages ± 3 standard deviation of retention time are shown in Table 3.

Quantification of peaks was performed by an external standard method, using peak height measurements. To determine calibration graphs (six points, $n = 3$), control serum was spiked with known amounts of each compound (0.005–1 μg/mL) after extraction. The regression coefficients were >0.996 in all cases. Table 2 shows these results.

In order to reduce the matrix effect, calibration was done using uncontamined matrix solutions spiked with the analytes, instead of solvent solutions.

Table 2. Calibration Curves for the Analytes in Human Serum Samples (Five Points, $n = 3$)

Compound	Calibration Curve	r	RTW (min)
	LC–LC–UV		
Triadimenol B	$Y = 85097X - 160$	0.999	7.9–8.5
Triadimenol A	$Y = 99457X + 196$	0.996	8.4–8.9
Triadimefon	$Y = 197768X + 1122$	0.999	10.2–10.4
Tetraconazole	$Y = 223672X + 3041$	0.997	10.6–11.1
	LC–ES–MS		
Triadimenol	$Y = 16807X - 18$	0.999	3.9–4.2
Triadimefon	$Y = 186080X + 560$	0.993	4.8–5.1
Tetraconazole	$Y = 161645X - 68$	0.997	5.0–6.3

Recovery studies were performed on serum samples spiked with 0.05 and 0.2 μg/mL of each compound ($n = 3$). The extracts were analyzed as previously described. Average recoveries between 70.1 and 100.7% were obtained. Relative standard deviation (RSD) ranged between 2.9–7.0%. Table 3 summarizes detection (LODs) and quantitation (LOQs) limits calculated, respectively, as 3 and 10 times the respective standard deviation (five injections) of the baseline signal corresponding to a blank matrix extract chromatogram at the analyte retention times, divided by the respective slopes of the calibration curves of the analytes. Limits of quantitations ranged between 5 and 10 μg/L and the LODs ranged between 1.5 and 3 μg/L.

The two columns maintained their performance during the time of experiments and readjustments of column-switching conditions (clean up and transfer time) were not necessary.

Table 3. Mean Recoveries (% R), RSD, LOD, and LOQ (μg/L) for Triadimenol, Triadimefon, and Tetraconazole in Human Serum Samples Spiked at Two Levels ($n = 3$). Analysis by LC–LC–UV

Compound	R (RSD)[a] (%)	R (RSD)[b] (%)	LOD (μg/L)	LOQ (μg/L)
Triadimenol B	79.9 (7.0)	89.8 (4.4)	3	10
Triadimenol A	82.1 (6.5)	100.7 (5.0)	2.4	8
Triadimefon	70.1 (6.9)	87.8 (2.9)	2.7	9
Tetraconazole	74.1 (6.3)	83.9 (4.8)	1.5	5

[a]Spiking levels: 0.2 μg/mL.
[b]Spiking levels: 0.05 μg/mL.

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Analysis by LC–ES–MS

Figure 3 shows the LC–ES–MS spectrum of the analytes obtained with an optimal mobile phase, CH_3CN–0.4% formic acid in water (45 : 55, v/v), and applying a fragmentor voltage of 60 V. The MS detector was operated in the two-ion SIM acquisition mode. The quantitation ions chosen for SIM were 296, 294, and 372 for triadimenol, triadimefon, and tetraconazole, respectively. A LC–ES–MS analysis of working standard solutions of analytes was performed and a library spectral was created.

Calibration graphs were obtained for different concentrations of analytes in spiked serum samples (0.005 and 0.1 μg/mL). The regression coefficients were higher than 0.993 in all cases. Table 2 summarizes this result.

The extraction recovery of target pesticides was determined by comparing the peak high in samples, which had been spiked with the analyte prior to extraction, with those for samples to which the analyte had been added post-extraction. Each experiment was repeated three times. Average recoveries were between 78.1 and 80.9%. The RSD was in the range 8.7–12.3. The analytes were searched by its retention time windows and confirmed by comparison with the ES–MS spectra in the library. A positive confirmation required a minimum spectral fit of >700 and the signal-to-noise level $S/N > 3$ (for identification ions). For quantification, S/N was set at >10. Chromatograms of target analytes in serum obtained using LC–ES–MS, is represented in Fig. 4. Limits of quantitations ranged between 13 and 19 μg/L.

Due to the sample pre-concentration process, the amount of interfering ions in the MS detector is very high, in comparison with the amount of target ions from the analytes. This causes the masking of the analytical signals and low reproducibility. Important variations of the signals of the analytes is a consequence of this problem. The repeatability of the process is poor.

The chromatographic system also needs to be conditioned with 2 or 3 solvent injections after each analysis to be ready for the next one.

This demonstrates that the pre-concentration of the sample (in order to achieve high sensitivity, and low LOD and LOQ) is better when a UV-Vis detector is employed; in this case, the purpose is achieved without any difficulty related with interferences.

CONCLUSIONS

The determination of triadimenol, triadimefon, and tetraconazole in human serum is feasible using both UV or MS detection modes. Only the LC–LC–UV method offers good linearity and sensitivity for the determination of target pesticides at trace levels. The LC–LC–UV method has better regression

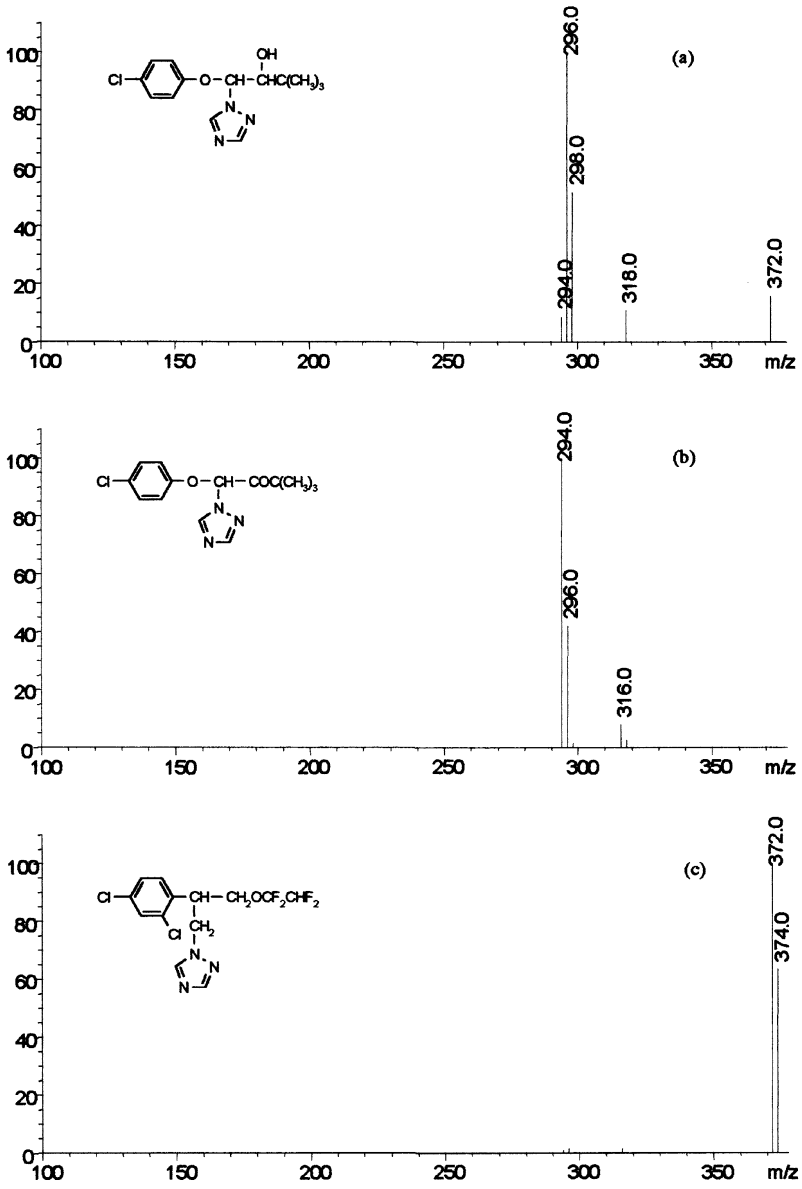

Figure 3. LC–ES–MS spectra and structural formulae: (a) triadimenol, (b) triadimefon, (c) tetraconazole.

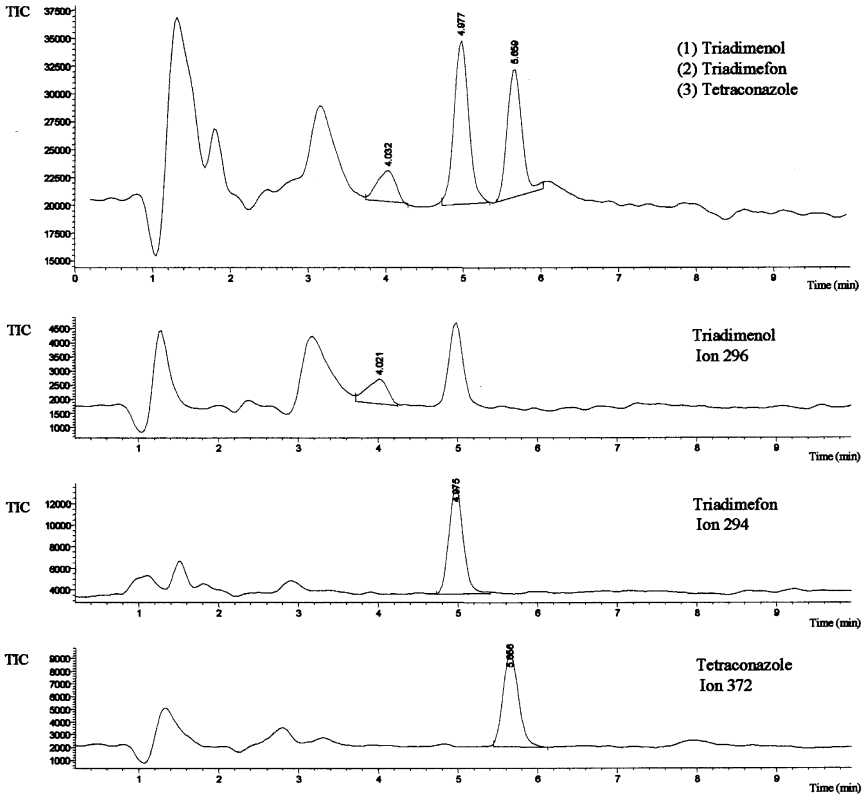

Figure 4. Chromatograms of target analytes in human serum spiked with 0.05 μg/mL of each analyte using LC–ES–MS. Peak numbers: 1 = triadimenol, 2 = triadimefon, 3 = tetraconazole.

coefficient values and better recoveries (lower RSD) than LC–ES–MS. Besides, the LC–LC–UV method is more economical and sensitive than the LC–ES–MS method, which provides sufficient structural information for identification.

Using solid phase extraction, the sensitivity was increased. Thus, a more sensitive procedure has been developed in this paper, by the addition of a preconcentration step on SPE cartridges previous to the RPLC–LC-UV and LC–ES–MS analysis. The detection limits achieved, makes it possible to perform determinations of serum samples from azole pesticides exposed workers with both methods. The pre-concentration of analytes in the extract is accompanied by the same concentration of matrix components (interferences). The mass spectrometer receives a high amount of analytes, but in a low proportion vs. the high amount of

interferents. If this technique is used, it is advisable to reduce, to the minimum, the amount of undesirable components, even if this implies a reduction of the concentration of the analytes.

The analysis of serum samples of five occupational workers in agriculture from Almería (Spain) did not detect triadimenol, triadimefon, and tetraconazole.

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