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Solid-Phase Microextraction Liquid Chromatography/Tandem Mass Spectrometry To Determine Postharvest Fungicides in Fruits

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A method to determine five postharvest fungicides (dichloran, flutriafol, *o*-phenylphenol, prochloraz, tolclofos methyl) in fruits (cherries, lemons, oranges, peaches) has been developed using solid-phase microextraction (SPME) coupled to liquid chromatography (LC) with photodiode array (DAD), mass spectrometry (MS), or tandem mass spectrometry (MS/MS) with ion trap detection. Extraction involved sample homogenization with an acetone/water solution (5:1), filtration, and acetone evaporation prior to fiber extraction. The pesticides were isolated with a fused-silica fiber coated with 50-μm Carbowax/template resin. The effects of pH, ion strength, sample volume, and extraction time were investigated, and their impact on the SPME-LC/MS was studied. Dynamic and static modes of desorption were compared and the variables affecting desorption processes in SPME-LC optimized. Static desorption provided the best recoveries and peak shapes. Recoveries at the limit of quantification (LOQ) levels were between 10% for prochloraz and 60% for *o*-phenylphenol, with relative standard deviations from 13.6% for prochloraz to 3.1% for *o*-phenylphenol. The versatility of the method was also exhibited by its excellent linearity in the concentration intervals between 0.0005 and 5 mg kg^{-1} for dichloran and 0.01−10 mg kg^{-1} for tolclofos methyl and prochloraz. LOQs ranged from 0.25 to 1 μg g^{-1} using DAD, from 0.002 to 0.01 μg g^{-1} using LC/MS, and from 0.0005 to 0.01 to μg g^{-1} using LC/MS/MS. LOQs obtained in the present study using LC/MS and LC/MS/MS are lower than maximum residue limits established for all the fungicides in any matrix studied. The method enables to determine polar pesticides at low-microgram per gram levels in fruits.

Fungicides are widely used in industry, in agriculture, and at home for a number of purposes, which comprise: protection of crops and seedlings in the field, in the storage process, and during shipment; suppression of mildews that attack painted surfaces and control of slime in paper pulps.[1] Fungicides vary enormously in their potential for causing adverse effects in humans.[2] Historically, some of the most tragic epidemics of pesticide poisoning occurred because of erroneous consumption of seed grain treated with organic mercury or hexachlorobenzene.[3] In general, most fungicides currently in use are unlikely to cause frequent or severe systemic poisoning since a large number of them have low inherent toxicity in mammals and are inefficiently absorbed.[4] By reason of current concern about the possibilities of chronic health problems and environmental effects, including groundwater contamination and the presence of their residues in food for human consumption, many of those that were commonly used in the past are now under review, since several studies determined that ∼90% of fungicides could be carcinogenic in experimental animals.[5,6]

Dichloran (nitro derivative), flutriafol (triazole), *o*-phenylphenol (biphenyl), prochloraz (imidazole), and tolclofos methyl (thiophosphate) are some of the chemical structures commercially available as fungicides for different crops. Animal bioassays have shown that *o*-phenylphenol is highly effective in causing bladder cancer in male F344 rats[7] and is also suspected of interfering with the hormone systems of humans and wildlife.[8] *o*-Phenylphenol has been found to cause cancer in rats treated at doses up to 15 mg kg^{-1} by a mechanism based on protein binding[7] that does not occur in humans; correlation of the rat-based ingestion data to humans is no longer considered to be reliable. The available data are inadequate to evaluate whether exposure to *o*-phenylphenol causes cancer in humans. On the contrary, little is known about the environmental significance and toxicology of the other fungicides. They deserve particular attention because of their

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widespread use, their persistence, and the lack of scientific evidence on their long-term effects.[5,6]

Fungicide analysis has been carried out by chromatographic separations as gas chromatography (GC),[9−12] liquid chromatography (LC),[13−15] and capillary electrophoresis (CE),[16,17] coupled to a variety of detectors. GC/MS is still a common separation technique in fungicide residue analysis because of its sensitivity and selectivity and the easy identification of compounds from the mass spectra.[10,11] However, the required derivatization, poor GC properties, and the instability of some analytes limit the use of GC/MS in favor of LC or EC methods, which do not require derivatization.[9,11] The development of routine and reliable LC/MS instruments has been a relatively long process that was successful 10 years ago when simple quadrupole devices with atmospheric pressure ionization sources began to be used for pesticide residues determination.[18−21] LC/MS has been a revolutionary technique, and technical progress is still rapid on the mass spectrometry front, especially with the development of tandem mass spectrometry.[22,23] The improvement of tandem mass spectrometry over traditional MS methods is that it uses two stages of mass analysis, one to preselect an ion and the second to analyze fragments induced. The high MS selectivity largely compensates for the lack of chromatographic resolving power. Although single quadrupoles are the less expensive and probably easier to handle, the fragmentation of molecules is insufficient for an unambiguous identification and quantification of fungicides in complex matrixes, as for example in extracts of fruits, even using collision-induced dissociation (CID). When coelution occurs, the resulting multiple-component spectrum is definitively useless. This limitation may render them uncompetitive in some applications with respect to the most recent tandem mass spectrometry (MS/MS), which has rapidly become popular since the appearance of triple quadrupole and ion trap (IT) instruments, becoming one of the most selective and sensitive analytical tools.[24,25] Various examples of the application of IT for determining polar herbicides in water have been described,[26−32] but its use in the analysis of pesticide residues in fruits only has been reported for chlormequat[33−35] and four pesticides (aldicarb, carbendazim, imidacloprid, propiconazole)[36] in conventional extracts.

Sample preparation is essential to increase sensitivity and to achieve a more efficient, practical, and reliable method for the analysis of fungicides in fruit samples. Ideally, a sample preparation method should be fast, simple, and capable to isolate a wide range of compounds with very different chemical structures and properties. Until now, organic solvent extraction,[9−12] solid-phase extraction (SPE) with disposable columns[13,14,16] or tandem extraction procedures coupling both[15] has been used for the extraction of the samples. However, these methods are laborious, time-consuming, and require large volumes of samples and toxic solvents.

On-line sample preparation and miniaturization have been two significant trends in the development of extraction procedures for the last two decades. Solid-phase microextraction (SPME) is an extraction technique that uses a fused-silica fiber coated externally with an appropriated stationary phase, which integrates sampling, extraction, concentration, and analyte desorption into a single procedure.[37] SPME is usually combined with GC or GC/MS for a wide variety of compounds including pesticides, agrochemicals, and other contaminants in food samples.[37,38] Some advantages of SPME, as compared with other sample preparation techniques, are the savings in solvent purchase and disposal cost and the potential to improve detection limits because all the analytes adsorbed in the fiber are transferred to the column for analysis. On the other hand, one disadvantage is that the method is not suitable for weakly volatile or thermally labile compounds as most pesticides are. In 1994, a simple interface coupling on-line SPME desorption and LC/MS was designed permitting their combination and solving this problem.[39] Since then, SPME has been combined with LC/MS for the determination of different contaminants such as polyciclic aromatic hydrocarbons,[40] phenolic compounds[41,42]

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alkylbenzenesulfonates[43] and polar pesticides[44] in water samples. To date, no report describing the on-line coupling of SPME desorption and LC/MS for analysis of fungicides in fruit samples has been published.

The present study establishes a procedure for the determination of relatively polar fungicides (dichloran, flutriafol, *o*-phenylphenol, prochloraz, tolclofos methyl) in fruits by SPME-LC/IT-MS (MS/MS) with atmospheric pressure chemical ionization (APCI). The selectivity of LC/MS/MS is compared with LC/DAD and LC/MS. To our knowledge, this is the first work describing a food application of SPME combined on-line with LC/IT-MS/MS. It incorporates the high capacity of concentration offered by SPME for enrichment of analytes and the selectivity obtained using LC/IT-MS/MS.

EXPERIMENTAL SECTION

Materials and Standards. Fungicides (dichloran, flutriafol, *o*-phenylphenol, prochloraz, tolclofos methyl) were supplied by Riedel-de Haën (Seelze, Germany). Individual stock solutions were prepared by dissolving 100 mg of each compound in 100 mL of methanol and were stored in glass-stopper bottles at 4 °C. Standard working solutions, at various concentrations, were prepared daily by appropriate dilution of aliquots of the stock solutions in methanol.

HPLC-grade methanol was purchased from Merck (Darmstadt, Germany), acetone from Promochem (Wesel, Germany), and sodium chloride from Panreac (Barcelona, Spain). Deionized water (<8 MΩ cm resistivity) was obtained from the Milli-Q SP Reagent Water System (Millipore, Bedford, MA). All the solvents and solutions were filtered through a 0.45-μm cellulose filter from Scharlau (Barcelona, Spain) before use.

A manual fiber holder for SPME and the SPME−HPLC interface were purchased from Supelco (Bellefonte, PA). The six types of fibers tested, 7-, 30-, and 100-μm poly(dimethylsiloxane) (PDMS), 85-μm polyacrylate, 60-μm poly(dimethylsiloxane)/divinylbenzene (PDMS/DVB), and 50-μm Carbowax/templated resin (CW/TPR) were obtained from the same manufacturer. The new fibers were conditioned in methanol with stirring for 30 min, and the used ones were cleaned in methanol with stirring for 15 min before extraction.

Liquid Chromatography and Liquid Chromatography/Mass Spectrometry. Chromatographic conditions were the same in all the equipment. The separation was achieved on an analytical column Luna C_{18} (250 × 4.6 mm i.d, 5 μm) preceded by a Securityguard cartridge C_{18} (4 × 2 mm i.d.), both from Phenomenex. The isocratic mobile phase was methanol/water (85:15 v/v) with a flow rate of 0.6 mL min^{-1}.

Liquid chromatography-UV detection was carried out using a Merck-Hitachi LC system equipped with an L-7100 pump, a Rheodyne Model 7125 injector (20 μL loop), an L-4250 UV−Vis detector and a Millennium software. The UV−Vis detector was operated at 210 nm.

Liquid chromatography, with single quadrupole mass spectrometry, was accomplished on a Hewlet-Packard (Palo Alto, CA) HP-1100 series LC/MSD with the HP Chemstation software version A.06.01 consisting of HP-1100 autosampler with a 100-μL loop, a G1312A binary pump, and a G1345A diode array UV−vis detector coupled in series with a mass-selective detector equipped with APCI interface.

The parameters of the MS detector were optimized by evaluating the sensitivity (signal-to-noise ratio) and fragmentation of each analyte, at a concentration of 10 μg mL^{-1}, which was injected into the flow of the mobile phase using flow injection series (FIA) and detected in the full-scan mode. The injection volume was set at 5 μL. Optimal operating conditions of the APCI interface in negative mode were as follows: vaporizer temperature, 450 °C; nebulizer gas, nitrogen at a pressure of 60 psi; drying gas, also nitrogen, at a flow rate of 4 L min^{-1}; temperature of 350 °C; capillary voltage, 4000 V; and corona current 25 μA. Full-scan LC/MS chromatograms were obtained by scanning from m/z 100 to 400. Time-scheduled selected-ion monitoring (SIM) of the most abundant ion of each compound was used for quantification.

LC/IT-MS was performed using the Esquire3000 ion trap LC/MS(n) system (Brucker Daltonik GmbH) and Agilent 1100 series LC system that includes a quaternary pump, an autosampler, and a variable-wavelength detector. The mass spectrometer was equipped with an APCI source and operated in negative polarity at a temperature of 450 °C. The conditions were as follows: nebulizer pressure 60 psi and drying gas flow 60 L min^{-1} at a temperature of 350 °C. The Esquire3000 was tuned for fungicides, optimizing the voltages on the lenses in the ExpertTune mode of the Daltonic Esquire Control software while infusing a standard solution (10 μg mL^{-1}) by a syringe pump at a flow rate of 250 μL h^{-1}, which was mixed with the mobile phase at 0.6 mL min^{-1} by means of a T piece. The optimized tune parameters were set for groups of compounds via time segments definition.

The mass spectrometer was run in full scan and multiple reaction monitoring (MRM) modes. Ions were detected in ion charge control mode. Full-scan mode was performed with a target of 70 000 and maximum accumulation time of 100 ms at m/z range from 100 to 400 u. MRM was carried out setting the target at 50 000 and maximum accumulation time at 50 ms. Negative ions were detected at unit resolution (scan speed 10 300 u s^{-1}). Four scans were summarized for one spectrum, resulting in a spectral rate of 0.4 Hz. MS/MS was performed by sequential selected product ion monitoring, isolation of the parent ion, and CID with helium. Conditions, optimized for product ions, were also regulated via time segment definition, the details of which are given in Table 1.

Sample Collection and Preparation. Ten samples of each fruit collected from different markets were tested. As far as possible, the samples were taken at various places distributed throughout the lot (size ∼50 kg). Cherry, lemon, orange, and peach samples weighed ∼1 kg and consisted of at least 10 individual fruits. They were analyzed unwashed, with the peel intact and, in the case of cherries and peaches, stoned. The samples were chopped into small pieces, and a 200-g portion was homogenized in a Bapitaurus food chopper (Taurus, Berlin, Germany)

SPME Procedure. One gram of homogenized sample was placed in an Erlenmeyer flask and homogenized with 5 mL of acetone by sonication over 15 min. The resulting suspension was centrifuged at 3000 rpm during 20 min, filtered through a filter,

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Chart 1. Diagram of the SPME-LC/MS System

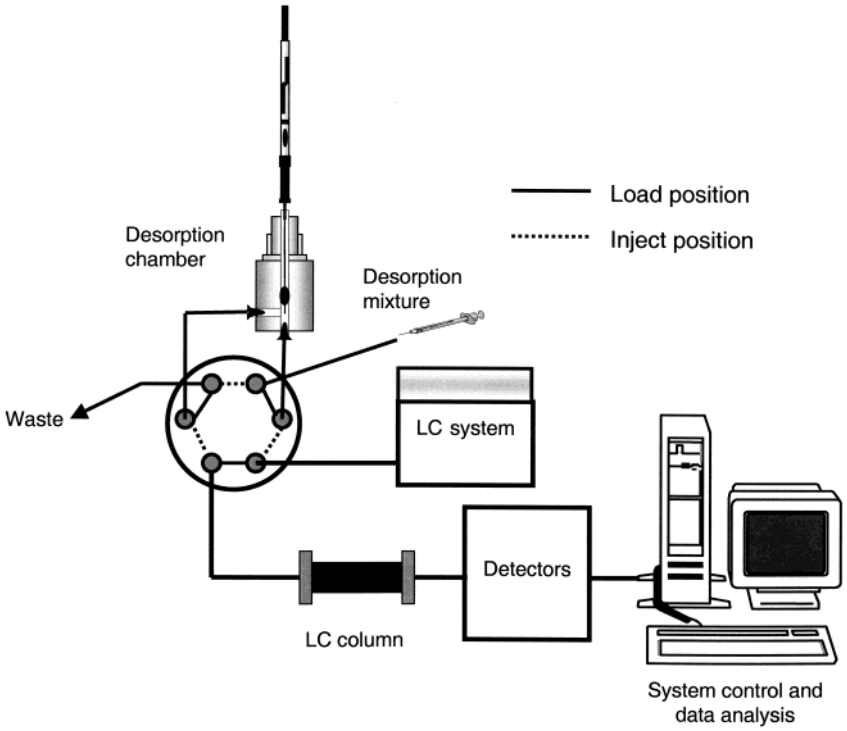


Table 1. APCI-MS Conditions Time Schedule

	0−9 min	9−12 min	12−15 min
capillary (V)	1500	1500	1500
skimmer (V)	-62	-41.8	-51.64
capillary exit (V)	-153.5	-183	-112
Octopolo 1. dc (V)	-11	-3	-3.00
Octopolo 2. dc (V)	-0.5	-0.7	-0.5
tap driver (relative value)	32.5	28	29
Octopolo reference (V)	99	82	128
lens 1 (V)	-0.5	3.45	3.46
lens 2 (V)	45.54	55	58
MRM	204	169	356
parent ion isolated (*m*/*z*)		205	286
MRM fragmentation			
cutoff (%)	100	120 (169)	140 (356)
		100 (205)	140 (287)
amplitude (%)	1.4	2.0 (169)	2.0 (356)
		2.0 (205)	0.8 (287)
ions monitored (*m*/*z*)	161.6	140.7	174.6
		174.6	176.6

and collected in a graduated conical cylinder (15 mL). The acetone was evaporated at 50 °C under a nitrogen stream. The remainder aqueous phase was placed into a 2-mL screw cap vial containing a small magnetic stirring bar, adjusted to a volume of 1 mL, and mixed with 300 mg of NaCl. The SPME fiber was immersed directly in the sample solution with continuous stirring for different times (20−160 min) at ∼1000 rpm. Desorption was performed in a static mode: the fiber was placed into the desorption chamber, which has been filled with methanol/water (70:30 v/v), for various times (2−15 min). After desorption, the handle of the injection valve was turned from the load to the inject position; the content of the desorption chamber was flushed directly onto the LC column by the mobile-phase flow. The SPME fiber was continuously exposed to the mobile-phase flow during the analysis, then the injection valve was returned to the load position, and the fiber was withdrawn into the needle and pulled out of the interface. Chart 1 shows a diagram of the SPME coupled to LC/MS/MS. The SPME−LC interface was placed between the autosampler and the analytical column and was manually turned whereas the autosampler software was programmed to perform a blank run.

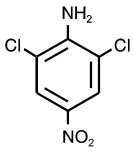
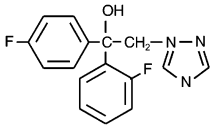
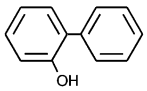
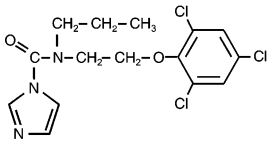
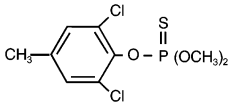
Extraction recoveries were determined by spiking fresh samples (1 g) with volumes between 50 and 100 μL of the working mixtures at appropriate concentrations. They were then allowed to stand at room temperature for 3 h. Samples were spiked with acidic pesticides at six concentration levels ranging from the limits of quantification (LOQs) to 100 times the LOQs. The concentrations used on the fruit were selected to cover from the LOQ to at least 2 orders of magnitude to calculate linearity. Using MS detection, this working range was considered appropriate since real samples with contents higher than MRLs (∼1 mg kg^{-1} for most of the compounds) occur only rarely. This concentration range is in line with that used in industry since the postharvest-treated fruit should contain the residues always at levels lower than MRLs.

RESULTS AND DISCUSSION

Mass Spectra. Dichloran, flutriafol, *o*-phenylphenol, prochloraz, and tolclofos methyl have a very distinct course in the IT because their skeletal structures are quite different. The chemical structures and molecular weights of the studied compounds together with the main ions obtained by IT LC/MS and LC/MS/MS with negative ion (NI) APCI mode, with their relative abundances and tentative assignation, are shown in Table 2.

The MS spectra attained for the studied fungicides were quite similar to those reported in a previous work[14] for LC-APCI-MS

Table 2. Structures of the Five Fungicides Studied and Their Mass Spectra Obtained by LC/MS and by LC/MS/MS

Compound	Structure	Mr	*m/z* (relative intentisity (%)) [tentative ions]	
			MS	Product ions
Dichloran		206	<u>205</u>/207 (100/66) $[M-H]^-$	<u>175/177</u> (100/66) $[M-H-NO]^-$
Flutriafol		301	300 (20) $[M-H]^-$ <u>204</u> (100) $[M-H-C_6H_5F]^-$	189 (50) $[M-H-C_6H_5F-NH]^-$ 176 (15) $[M-H-C_6H_5F-N_2]^-$ <u>162</u> (100) $[M-H-C_6H_5F-CH_2N_2]^-$ 108 (34) $[M-H-(C_6H_5F)_2]^-$
o-phenylphenol		170	<u>169</u> (100) $[M-H]^-$	<u>141</u> (100) $[M-H-CO]^-$ 115 (10) $[M-H-CO-(CH{\equiv}CH)]^-$ 93 (25) $[M-H-C_6H_6]^-$
Prochloraz		376	<u>356/348</u> (50/33) $[M-Cl+O]^-$ 194/196/198 (100/99/33) $[Cl_3C_6H_2O]^-$	<u>177/179</u> (100/66) $[OHCl_2C_6H_2O]^-$
Tolclofos methyl		301	<u>285/287</u> (100/66) $[M-CH_4]^-$ 175/177 (60/40) $[CH_3C_6H_2Cl_2O]^-$	<u>175/177</u> (100/66) $[CH_3C_6H_2Cl_2O]^-$

Underlined ions are those selected for isolation

using single quadrupole. The only ion obtained for dichloran and *o*-phenylphenol was the deprotonated molecular ion $[M - H]^-$. The flutriafol mass spectrum showed the deprotonated molecular ion and a fragment ion originating from the loss of fluorobenzene (−96 amu), which was the base peak of the spectrum. The prochloraz spectrum presented two fragments, at *m*/*z* 356 and 195, corresponding to the substitution in the molecule of a chlorine atom by oxygen and to the 2,4,6-trichlorophenolate, respectively. Tolclofos methyl also displayed two predominant fragments corresponding to the loss of a methyl group of the molecule and to the 2,6-dichloro-*p*-methylphenolate. These results showed that APCI in NI mode cannot give the deprotonated molecular ion, but fragments with losses that resemble electron impact (EI) fragmentation such as the loss of a methyl group (−15 amu), loss of a fluorobenzene (−96 amu), or replacement of a chlorine atom by oxygen, which is in agreement with the observations published in recent articles that some kind of EI ionization takes place in NI APCI mode.[20,27]

The base peak of the mass spectrum (either the deprotonated molecular ion or the fragment ion) was subjected to the first stage of MS/MS for all studied compounds, except prochloraz, for which the second most abundant ion, *m*/*z* 356, was chosen to obtain product ions of high *m*/*z* value.

The MS^2 analysis of the five fungicides with the APCI interface in NI mode provided product ions for all of them. Dichloran, tolclofos methyl, and prochloraz showed only one product ion of *m*/*z* 175 for the two first compounds and *m*/*z* 177 for the third one corresponding to 3,5-dichloro-*p*-aminophenolate, 2,6 dichloro-*p*-methylphenolate, and 2,6-dichloro-*p*-diphenolate, respectively. Meticulous examination of the isotopic profile of the mass spectra confirmed the identity of these fragments. The doublet signal in the mass spectrum indicated the contribution of chlorine isotopes (^{35}Cl: ^{37}Cl = 100:66), which confirmed that the three ions retained two chlorine atoms.

The MS^2 spectrum of the deprotonated molecular ion of *o*-phenylphenol and the fragment ion of flutriafol originating from the loss of fluorobenzene showed various product ions, the tentative structure assignments of which are given in Table 2.

Calibration curves were established on the five substances analyzed at six different concentrations injecting 20 μL (25−250 μg L^{-1} for the lowest concentrations to 1000−10 000 μg L^{-1} for the highest concentrations) by LC/MS/MS in MRM mode, followed by extraction of the signal of the most abundant daughter ions. The linearity of the analytical responses recorded was adequate, with correlation coefficients greater than 0.995. Analytical performance of the LC/MS/MS method was evaluated by intraday and interday precisions and limits of detection (LODs). The repeatability was calculated by five daily replicate analyses of a methanolic solution of all the analytes, at concentrations from 0.1 to 10 μg mL^{-1}, depending of the compound. The reproducibility was carried out for five successive days using the same solution. The RSDs of the peak area varied from 3.4 to 7.6% for intraday and from 5.6 to 12.9% for interday precisions and those of retention times oscillated from 0.2 to 0.8% and from 0.3 to 1.2% for run-to-run and day-to-day precisions, respectively.

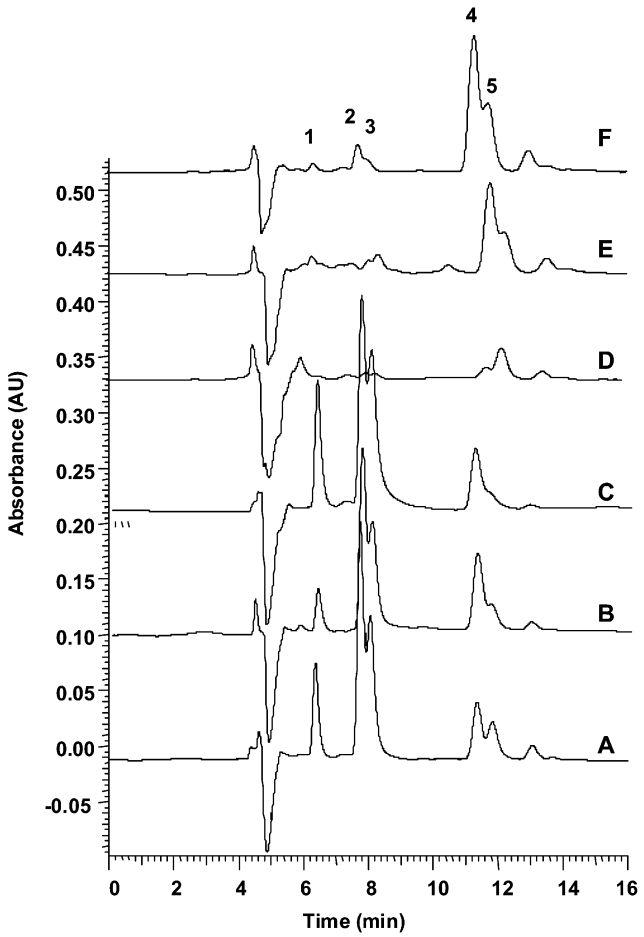


Figure 1. Comparison of different fiber coatings for the SPME of the five fungicides from water: (A) CW/TPR; (B) polyacrylate; (C) PDMS/DVB; (D) PDMS, 7 μm; (E) PDMS, 30 μm; (F) PDMS, 100 μm. The amounts of each fungicide spiked into 1 mL of water were 0.25 μg for *o*-phenylphenol and 0.5 μg for the other compounds. Peak identification: 1, flutriafol; 2, *o*-phenylphenol; 3, dichloran; 4, prochloraz; 5, tolclofos methyl.

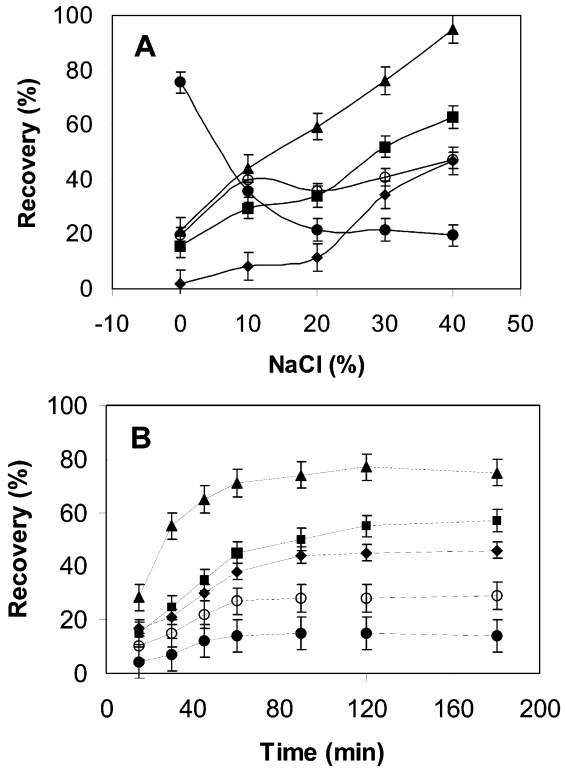


Figure 2. Effect of (A) salt concentration (fiber immersed into each sample solution 90 min) and (B) extraction time (each solution added of 30% NaCl) on the recovery of (▲) dichloran, (♦) flutriafol, (■) *o*-phenylphenol, (●) prochloraz, and (○) tolclofos methyl. The error bars for each point were calculated from triplicate measures.

LODs based on a signal-to-noise ratio of 3:1 were 8 μg L^{-1} for prochloraz, 80 μg L^{-1} for flutriafol, *o*-phenylphenol, and tolclofos methyl, and 120 μg L^{-1} for prochloraz, which corresponded to injected amounts of 0.16, 1.6, and 3.2 ng, respectively. LC/MS/MS LODs were of the same magnitude order as those obtained using LC/MS because the high selectivity of MS/MS achieves reduction of background, improving the signal-to-noise ratio, which is a very important feature when complex samples are analyzed.

Optimization of Conditions for SPME. The first step in the development of a SPME method is the selection of the fiber coating. In the present study, six different fibers, CW/TPR, three PDMS of different thin coating, polyacrylate, and PDMS/DVB, were evaluated for the extraction efficiencies of the five fungicides from water.

Figure 1 shows the comparison of the performance of these fibers for five selected fungicides in water under identical conditions. CW/TPR and PDMS/DVB fibers showed the highest efficiencies for all compounds (82−20%) as compared to the less polar polyacrilate and PDMS (28−1%). In particular, the more polar compounds, flutriafol, *o*-phenylphenol, and dichloran, were recovered only to an insignificant degree by the less polar fibers of PDMS (always less than 8%) (Figure 1D−F). The low polarity compounds used in this comparison, tolclofos methyl and prochloraz, were recovered best on the 100-μm PDMS fiber (85 and 45%, respectively). The PDMS/DVB fiber exhibited slightly better extraction efficiencies (from 82 to 29%) for the analytes than CW/TPR (from 79 to 20%), but the coating of PDMS/DVB, after one or two analyses, was stripped off in the interface during the desorption in water/methanol. Therefore, the CW/TPR fiber was used throughout the following experiments.

The effect of the pH on the sorption of the studied fungicides showed unappreciable changes in the amount absorbed when the pH was varied from 4 to 9. Tests were also performed to determine how the sample volume affected the recovery of fungicides. The best recoveries for all the fungicides were obtained with 1 mL. Tolclofos methyl and prochloraz were not extracted using large volumes (2 and 5 mL).

Experiments were conducted to determine the effect of ionic strength of the sample solution in the recoveries for the five compounds. Extraction efficiencies of flutriafol, dichloran, and *o*-phenylphenol were greatly improved just as increasing the ionic strength of the sample solutions (see Figure 2A) because an increase in ionic strength reduces the compound's solubility in water. The response of tolclofos methyl gradually augmented when the salt concentration was increased to 10%, but the differences found between 10 and 40% NaCl were unappreciable. On the contrary, peak area of prochloraz decreased with the amount of salt. A total of 30% NaCl was added as a compromise to achieve adequate extraction efficiency for all the compounds.

The SPME time profiles of the five fungicides from a stirred aqueous solution (30% NaCl) using the CW/TPR fiber are shown

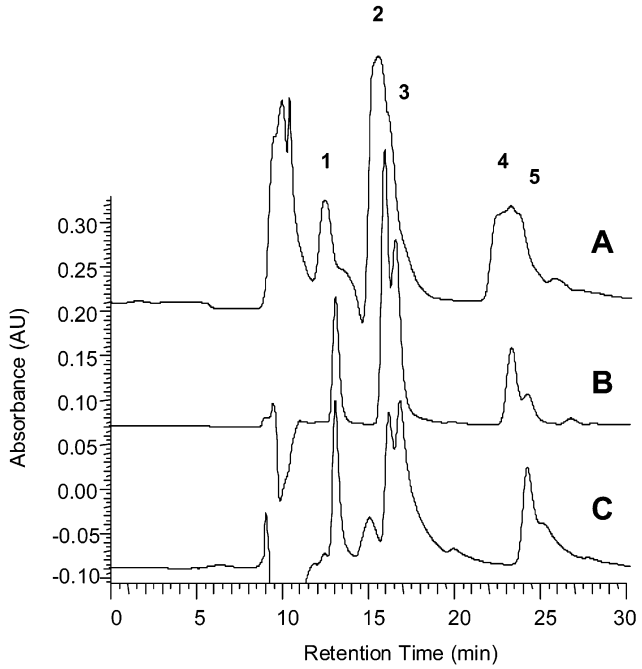


Figure 3. Comparison of chromatograms obtained from different desorption mixtures: (A) only methanol, (B) methanol/water (70:30 v/v), and (C) methanol/water (40:60 v/v). Peak identification as in Figure 1.

in Figure 2B. SPME is governed by the analyte partition between a sample matrix and a coating stationary phase; the time to reach equilibrium depends on the distribution constant between the matrix and the coating. The equilibrium times for the studied compounds ranged from ~60−120 min. However, after 90 min, ≥85% of the equilibrium concentrations were reached.

The desorption conditions by the SPME−LC interface were evaluated in dynamic and static modes with water samples. In the dynamic mode, analytes are continuously desorbed from the fiber by the mobile phase whereas in the static mode, desorption takes place in a small volume of solvent inside the desorption chamber for a certain time. Dynamic desorption of the fiber led to very broad fungicide peaks. In this way, static desorption was demonstrated to be more versatile since parameters such as desorption solvent and time can be modified. A desorption time of 10 min resulted in the total elution of the compounds from the fiber, independent of the solvent used. Desorption was performed with different methanol/water mixtures. Figure 3 displays the effect of the methanol percentage in the desorption mixture, showing that peak shape is very dependent on the methanol/water proportion. The best resolution was achieved using a mixture of methanol/water (70:30 v/v). Other proportions provided wide and badly resolved peaks.

SPME can be applied to aqueous solutions, for this 1 g of fruit sample was extracted with acetone for 15 min by sonication. After centrifuging, filtering, and evaporating, the remainder of the water residue was transferred to an extraction vial, adjusted to 1-mL volume, and NaCl was added. The results showed that there was no difference in the extraction yield of samples spiked before and after the acetone extraction.

Comparison between UV, SIM, and MRM. UV, SIM using the base peak of the mass spectrum of each compound, and MRM of the base fragment product ions were compared for the detection

Table 3. Comparison of LOQs (mg kg^{-1})

compounds	LC-DAD	LC-APCI-MS[a]	LC-APCI-MS[b]	LC-APCI-MS/MS
dichloran	0.5	0.005	0.0005	0.0005
flutriafol	0.5	0.002	0.005	0.005
o-phenylphenol	0.25	0.005	0.005	0.005
prochloraz	1	0.01	0.01	0.01
tolclofos methyl	1	0.01	0.01	0.01

[a] Using single quadrupole. [b] Using ion trap.

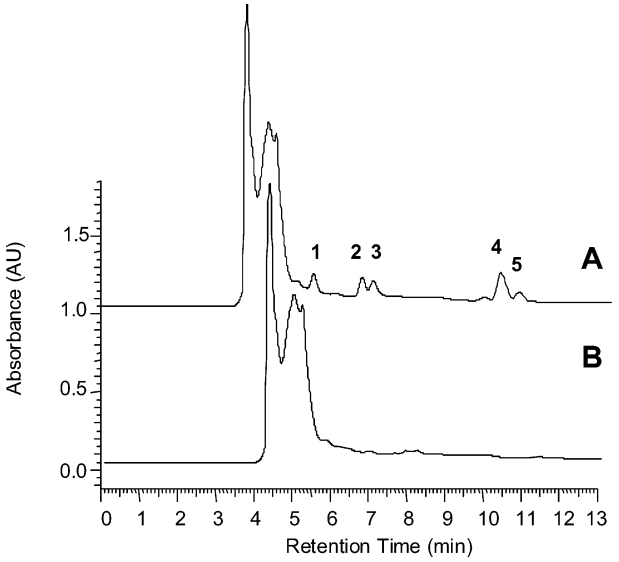


Figure 4. SPME-LC-DAD of (A) untreated lemon spiked at LOQ level and (B) untreated lemon. Peak identification as in Figure 1.

of the five fungicides after SPME for lemon samples. Table 3 outlines the LOQs obtained by different detection systems, which were calculated according to the European Union (EU) guidelines[45] as the lower level that provides acceptable recoveries and repeatabilities (<20%). Using UV detection, LOQs ranged from 0.25 to 1 μg for the overall analytical procedure, which corresponds to a concentration range from 0.25 to 1 mg kg^{-1} for fruit samples. Figure 4 illustrates the chromatograms with UV obtained from the fungicides spiked into lemon. The peaks were generally small and sometimes embedded into impurity peaks.

The LOQs obtained by LC/MS ranged from 0.5 to 10 ng for the overall procedure, which coincides with a concentration range from 0.0005 to 0.01 mg kg^{-1}, depending of the fungicide. Figure 5 presents the SIM chromatograms obtained using the single quadrupole (which was almost identical to those obtained using IT) for lemons. The peaks showed up nicely, but the background noise was high. Figure 6 shows MRM chromatograms by IT for the fungicides spiked into lemon. Distinct peaks appeared for all the compounds with no impurity peaks and very low background noise, which demonstrated that MRM with SPME-LC/MS/MS offered much higher selectivity and the same sensitivity than SPME-LC/MS for determining the five fungicides in the samples. Therefore, MRM was used for further experiments. IT was more sensitive for dichloran whereas single quadrupole gave the highest signal for flutriafol. Prochloraz and tolclofos methyl provided the

(45) European Community. http://europa.eu.int.

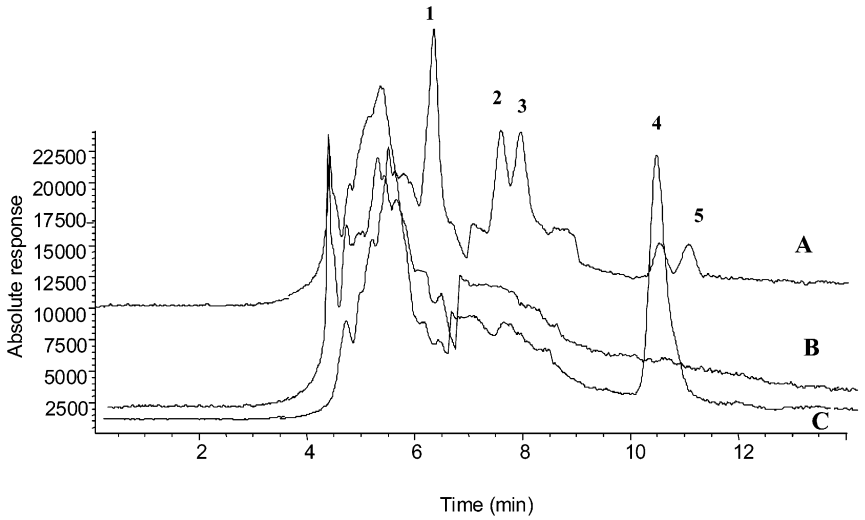


Figure 5. SIM of SPME-LC/MS of (A) untreated lemon spiked at LOQ level, (B) untreated lemon, and (C) lemon sample containing prochloraz. SIM used 0−7 min, *m*/*z* 204; 7−9 min, *m*/*z* 169 and 205; and 9−12 min, *m*/*z* 194 and 285. Peak identification as in Figure 1.

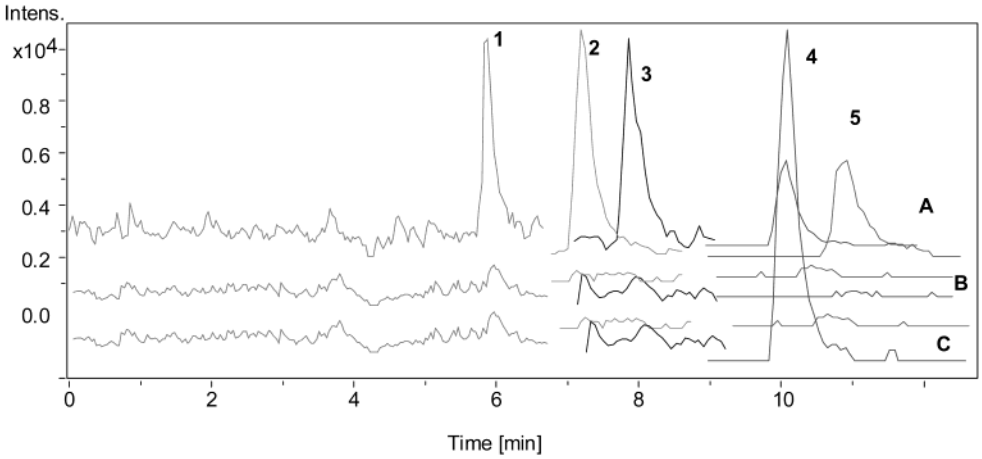


Figure 6. MRM with SPME-LC/APCI-MS/MS of (A) untreated lemon spiked at LOQ level, (B) untreated lemon, and (C) lemon sample containing prochloraz. MRM used 0−7 min, *m*/*z* 204 → 189; 7−9 min, *m*/*z* 169 → 141 and 205 → 175; and 9−12 min, *m*/*z* 356 → 177 and 285 → 175. Peak identification as in Figure 1.

lowest response in both detectors because of the weak ionization of these molecules in APCI.

LOQs were of the same order of magnitude or lower than MRLs established by the EU,[45] Codex Alimentarius Commission of FAO/WHO,[46] and Food and Drug Administration (FDA)[47] of the United States, which were in the interval of 0.01−5 mg kg^{-1} for dichloran, flutriafol, prochloraz, and tolclofos methyl and between 0.1 and 12 mg kg^{-1} for *o*-phenylphenol.

Method Performance. To test the linearities of the calibration curves, various concentrations of pesticides ranging from 0.0005 to 10 μg mL^{-1} were analyzed. The calibration curves were constructed from peak area counts using the MRM mode. As shown in Table 4, a linear relationship was obtained for each fungicide in this range (six-point calibration) from water and cherries by SPME. Greater difference in the regression equations between water and cherry was found for prochloraz, which is reasonable since the differences in the recovery between water (∼20%) and cherry (∼10%) for this pesticide is more marked than for the others. The correlation coefficients were 0.995−0.999, and relative standard deviations were between 0.5 and 12% ($n = 3$).

Recovery data for all the samples analyzed are reported in Table 5 at LOQ and 10 times the LOQ fortification levels. They were calculated by comparing the peak areas obtained from the extract of the spiked fruit sample with those obtained by direct injection of standard solutions. Recoveries of the five fungicides from water and fruit samples were in the range of 20−79% and 5−69%, respectively, using CW/TPR fiber (Table 5). The low recoveries obtained for tolclofos methyl and prochloraz for all the fruits tested were expected because of the partition of the compounds between the stationary phase of the SPME fiber and the sample solutions. Despite the low recoveries, small variations and excellent quantitation can be achieved by SPME as is evidenced in Table 5.

The intra- and interday precision and accuracy for oranges are presented in Table 6. The intraday precision was less than 12%, and the accuracy was in the range 92−103% for the concentrations examined. The interday precision at LOQ concentration was less than 14.6%, and the accuracy was in the range 94−100%.

The chemical stability of the CW/TPR fibers over repeated adsorption and desorption steps was checked. The stability test was performed by determining the fungicides' recovery from orange samples using the same fiber, time after time. Four

(46) Codex Alimentarius Commission. http://www.fao.org.
(47) Food and Drug Administration of the United States. http://wm.cfsan.fda.gov.

Table 4. Data on Regression Equations for the Five Fungicides Extracted from Water and Cherry by SPME-LC/MS/MS[a]

		water		cherry	
compound	concn added (μg mL^{-1})	equation	correlation coefficient (r)	equation	correlation coefficient (r)
dichloran	0.0005−0.5	$y = 163540581x + 2594162$	0.997	$y = 135899920x + 2155712$	0.997
flutriafol	0.005−5	$y = 16760686x + 0.64018$	0.999	$y = 14131560x + 53005$	0.999
o-phenylphenol	0.005−5	$y = 5077772x + 723407$	0.997	$y = 4873125x + 716373$	0.997
prochloraz	0.01−10	$y = 506970x + 463624$	0.995	$y = 202878x + 185005$	0.995
tolclofos methyl	0.01−10	$y = 3510351x + 648002$	0.999	$y = 435600x + 804784$	0.999

[a] The data were subjected to linear regression analysis of peak area (y) of the compound against the spiked concentration (x). For the equations, six plots (each point represents the mean of duplicate determinations) with different concentrations for each compound were used.

Table 5. Recovery for the Five Fungicides from Fruits by SPME

		recovery (%)				
compound	amt added (μg g^{-1})	water	cherries	lemons	oranges	peaches
dichloran	0.0005	71	59	61	60	63
	0.005	79	65	68	66	69
flutriafol	0.005	51	43	42	39	41
	0.05	55	48	45	41	45
o-phenylphenol	0.005	55	53	50	47	50
	0.05	59	56	57	52	51
prochloraz	0.01	20	8	10	5	7
	0.1	22	14	11	8	10
tolclofos methyl	0.01	29	36	22	21	25
	0.1	30	38	24	20	24

Table 6. Intra- and Interday Precision and Accuracy for the Five Fungicides from Oranges by SPME-LC/MS/MS

		intraday			interday		
compound	amt added (μg g^{-1})	amt detected[a] (μg g^{-1})	accuracy (%)	RSD (%)	amt detected[a] (μg g^{-1})	accuracy (%)	RSD (%)
dichloran	0.0005	0.00051 ± 0.00001	102	6.0	0.00049 ± 0.00007	99	9.3
	0.005	0.0049 ± 0.0004	99	5.2			
flutriafol	0.005	0.0052 ± 0.0005	103	8.3	0.0047 ± 0.0003	94.5	7.1
	0.05	0.049 ± 0.0045	99	7.0			
o-phenylphenol	0.005	0.0051 ± 0.0004	102	3.1	0.0050 ± 0.0003	100	6.0
	0.05	0.050 ± 0.005	92	2.8			
prochloraz	0.01	0.012 ± 0.005	103	13.6	0.009 ± 0.004	99	14.6
	0.1	0.13 ± 0.04	99	11			
tolclofos methyl	0.01	0.009 ± 0.002	101	9.1	0.011 ± 0.003	98	14.0
	0.1	0.11 ± 0.02	102	9.1			

[a] The values are means of ±SD of five experiments.

different fibers were submitted to the same process. Until ∼25 consecutive adsorption/desorption cycles, the recoveries remain constant.

Comparing the SPME-LC/MS/MS method with LC/MS methods utilizing matrix solid-phase dispersion[14,15] as the sample preparation technique, the former offered similar performance in terms of linearity but it was clearly more sensitive (LOQs were ∼10 times lower) and precise (relative standard deviations (RSDs) were approximately half). The sensitivity is one of the most important parameters in fungicide residue determination. The main advantages of the SPME were avoidance of long concentration procedures, reduction of organic solvents consumed, and less interference of the matrix compounds. A disadvantage was the limited enrichment capability of some fungicides.

Application of the Method. Of the 40 samples analyzed, pesticide residues were detected in 24 (60%), of which 1 (2.5%) exceeded MRLs involving tolclofos methyl. The results obtained showed that lemons had the greater percentage of contaminated

Table 7. Pesticide Concentrations in Fruits Samples by SPME-LC/MS/MS

matrix	no. sample	fungicide	concn range (mg kg^{-1})
cherries	5	flutriafol	0.32−0.46
lemons	4	dichloran	0.01−0.32
	3	*o*-phenylphenol	0.5−8
	1	tolclofos methyl	0.2
oranges	2	flutriafol	0.005−0.008
	3	dichloran	0.0005−0.25
	4	*o*-phenylphenol	0.5−5
	3	prochloraz	0.01−0.32
peaches	6	flutriafol	0.005−0.25

samples (80%), followed by oranges (70%). The most commonly detected fungicide was flutriafol, followed by dichloran and *o*-phenylphenol. The co-occurrence of two fungicide residues was detected in eight samples (20%). The most common pair of residues was *o*-phenylphenol and prochloraz, but the pairs of dichloran and prochloraz and of dichloran and *o*-phenylphenol were also detected. Table 7 recapitulates the frequency of residue findings and the concentration range of contaminated samples. Typical LC-APCI/MS using single quadrupole and LC-APCI/MS/MS profiles of prochloraz for a lemon sample are shown in Figures 5C and 6C, respectively. The concentration of prochloraz in lemon was 0.2 mg kg^{-1}. Residues of these pesticides were found widely distributed in the samples since they are commonly used in postharvest treatments of the fruits.

CONCLUSIONS

The described SPME-LC/MS/MS method for determining fungicides is fast, selective, linear, precise, and sensitive. Moreover, it can be applied to the analysis of fruits samples after minimal extraction procedure (sonication with acetone, filtration, and evaporation of the organic solvent). The quantitative analysis of fungicides in fruit samples using the LC/MS/MS technique with an ion trap mass spectrometer and the APCI interface correlates well with the quantitative results obtained for the same extracts using LC/MS with CID. MS is between 100 and 1000 times more sensitive than DAD, but results agreed with those obtained with LC-DAD for samples showing no spectral interference. Comparing the SPME-LC/MS/MS method with LC/MS methods utilizing traditional sample preparation techniques, such as SPE or organic solvent extraction, the proposed method presents analogous performance in terms of precision and linearity but it is clearly more sensitive and easier to use. It is also economical since SPME fibers could be used up to 20 times in the case of fruit samples. The manual experimental SPME setup used in this report can be automated to a certain extent, which should further improve the ruggedness of the method replacing the manual six-port valve of the SPME−LC interface with an automated version. This method provided a useful tool for monitoring, food toxicology, and nutrition.

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