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Coupling solid-phase microextraction and high-performance liquid chromatography for direct and sensitive determination of halogenated fungicides in wine

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Abstract

A solid-phase microextraction (SPME) method coupled to high-performance liquid chromatography with diode array detection (HPLC–DAD) for the analysis of six organochlorine fungicides (nuarimol, triadimenol, triadimefon, folpet, vinclozolin and penconazole) in wine was developed. For this purpose, polydimethylsiloxane–divinylbenzene-coated fibers were utilized and all factors affecting throughput, precision, and accuracy of the SPME method were investigated and optimized. These factors include: matrix influence, extraction and desorption time, percentage of ethanol, pH, salt effect and desorption mode. The performed analytical procedure showed detectability ranging from 4 to 27 $\mu g\ l^{-1}$ and precision from 2.4 to 14.2% (as intra-day relative standard deviation, RSD) and 4.7–25.7% (as inter-day RSD) depending on the fungicide. The results demonstrate the suitability of the SPME–HPLC–DAD method to analyze these organochlorine fungicides in red wine.

Keywords: Wine; Food analysis; Pesticides; Organochlorine compounds

1. Introduction

Pesticides are used on an increasingly wider scale throughout the world. Fungicides are widely used in extensive farms such as vineyards. The negative influence of pests on vineyard, especially to those of cryptogamic origin, is obvious in symptoms such as shriveling, blighting, decay and tissue destruction. A large part of the phytotoxic products used in agriculture in La Rioja (Spain) to prevent pest damage is employed for viniculture. All pest and disease agents disrupt vine physiology and, thereby, can influence fruit yield and quality to some degree. However, agents that attack berries directly have the greatest impact on fruit quality. These include three of the major fungal grape vine pathogens, namely *Botrytis cinerea* (grey rot), *Plasmopara viticola* (mildew) and *Uncicula necator* (oidium) [1]. For this reason, the winegrower uses different pesticides, mainly fungicides, to control the pests that affect the vines, but

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the possibility exists that residues of these products can pass from grape to must and, later, to wine [2,3] with the resulting risk to the consumers' health.

This fact led to the drawing up of several European Community Directives stipulating maximum residue limits (MRLs) for viniferous grapes. The European Union has not yet established MLRs for wine, except for EC 94/30, where the MRL for procymidone in wine is reported (0.5 mg kg^{-1}) [4].

Analytical methods for the determination of pesticide residues in must and wine, are usually performed by multi-step procedures [5,6]. The most frequently used are gas chromatography (GC) with nitrogen–phosphorus [7], electron-capture [8] or mass spectrometric [9,10] detection and liquid chromatography with different detectors [11,12]. These methods are mainly based on supercritical fluid extraction (SFE), solid-phase extraction (SPE) and liquid–liquid extraction (LLE) [13–17].

Solid-phase microextraction (SPME) was first reported by Arthur and Pawliszyn [18] in 1990 and is now widely accepted, supported by an ever-increasing number of new publications [19,20]. SPME consists of an absorption and a desorption step. Most SPME methods developed until now are used in combination with GC placing the fiber in the hot injector of the gas chromatograph, where the analytes are thermally desorbed. SPME and high-performance liquid chromatography (HPLC) were first coupled in 1995 [21] but in numerous fields, such as pesticide in wine analysis, it has not been so fully explored.

For SPME–HPLC coupling, the extraction procedure is similar to that used for GC analysis. The main difference between SPME–HPLC and SPME–GC is the second step, the desorption procedure. In HPLC analysis, thermal desorption at high temperature creates practical problems such as degradation of the polymer, and furthermore, many non-volatile compounds cannot be completely desorbed from a fiber. Solvent desorption is thus proposed as an alternative method of SPME–HPLC coupling. An organic solvent (static desorption) or the mobile phase (dynamic desorption) is used to desorb the analytes from the SPME fiber. In order to apply this desorption Chen and Pawliszyn reported an SPME–LC system with a desorption chamber and a six-port valve [21] called a SPME–HPLC interface.

Based on our own survey of the literature, more than 200 articles on SPME–HPLC have been published in different fields. The fungicides determined in this study (nuarimol, folpet, vinclozolin, penconazole, triadimenol and triadimefon) have been extracted by SPME–GC in water [22,23] and different fruit [24] and vegetables [25], and also in grapes, must and wine [26] However, to date, none of the fungicides cited have been extracted by SPME–HPLC.

Therefore, the aim of this study is to develop an SPME–HPLC method using polydimethylsiloxane–divinylbenzene (PDMS–DVB) 60 μm fibers, to determine, nuarimol, folpet, vinclozolin, penconazole, triadimenol and triadimefon in red wine samples, these being the most commonly applied in vineyards in La Rioja (North of Spain).

2. Experimental

2.1. Instrumentation

The isocratic LC system used was a Hewlett-Packard (Palo Alto, CA, USA) series 1050 pump. The diode-array detector was a Hewlett-Packard Model 1040. The column was a Sherisorb ODS2 (150 mm×4.1 mm I.D., 5 μm particle size) with a guard column (10×4 mm) from Tracer (Barcelona, Spain).

SPME was performed with commercially available 60 μm PDMS–DVB coated fibers and housed in the appropriate manual holder (Supelco, Bellefonte, PA, USA). For magnetic stirring, 4-ml US Environmental Protection Agency (EPA) screw-cap vials supplied with a PTFE-lined septum (Kimble Glass, Vineland, NJ, USA) and a 0.2-in. stir bar were used (1 in.= 2.54 cm). The magnetic stirrer was a Metrohm (Herisau, Switzerland) 728 Model.

The SPME–HPLC interface includes a desorption chamber and a six-port Valco valve. The interface was purchased from Supelco.

2.2. Reagents

All the solvents used in this study were HPLC grade and tested for spectral purity: acetonitrile (Scharlau, Barcelona, Spain) and methanol (Merck,

Darmstadt, Germany). LC-grade water was prepared by purifying demineralized water in a Milli-Q water filtration system (Millipore, Milford, MA, USA). All solvents were filtered through a 0.22-μm Millipore membrane filter type GVWP and mobile phases were degassed by a Selecta Ultrasounds System (Selecta, Barcelona, Spain) before utilization.

Nuarimol (NUA), penconazole (PEN), vinclozoline (VIN), folpet (FOL), triadimenol (TRMN) and triadimefol (TRMF) standards were supplied by Dr. Ehrenstorfer (Augsburg, Germany) with a certified purity higher than 99.0%.

The Rioja red wines used in the study were obtained from Covila Bodegas (Lapuebla, Rioja, Spain). These wines were obtained from grapes from experimental vineyards that had never treated with these fungicides and in which only inorganic pesticides were used. Their standard compositions were 13% (v/v) ethanol, pH 3.7, 2.1 g l^{-1} reductive sugars, 4.5 g l^{-1} total acidity and 0.5 g l^{-1} volatile acidity.

2.3. Preparation of the standards and the spiked wine samples

The standards were used to prepare a 10 ml stock solution containing 1000 μg ml^{-1} of each analyte in isopropanol and were preserved at −42 °C in a freezer. A stock mixed standard solution of 100 μg ml^{-1} was prepared weekly in mobile phase.

The stock mixed standard was diluted daily to the required concentration with mobile phase. Stock and working mixed standards were preserved at 4 °C in a refrigerator.

Spiked wine samples for the extraction procedure were prepared by the addition of an appropriate amount of a working standard solution to 3 ml of wine. The spiked samples were stirred and left to stand for 15 min to allow the solution to stabilize. All the experiments were carried out in triplicate.

2.4. Chromatographic separation

With a Spherisorb C_{18} column, acetonitrile–water (50:50) as mobile phase, and isocratic elution at a flow-rate of 0.9 ml min^{-1}, all the analytes were eluted in less than 25 min and detected with good peak resolution.

Under these chromatographic conditions, all these compounds together were simultaneously determined by diode-array detection (DAD). As any drawbacks in UV detection arose from matrix interferences, all fungicides were detected at 200 nm, except FOL, which was detected at 225 nm (its secondary maxima of absorbance).

2.5. Conditioning of fibers for HPLC use

The effects of different solvents on an SPME fiber will vary. It is best to condition the fiber with the mobile phase or solvent to which it will be exposed. Then, the fibers were conditioned and placed in the desorption chamber allowing the mobile phase to pass though the interface (dynamic mode) for 40 min, according to the supplier's instructions.

2.6. SPME procedure

SPME was carried out by introducing 3 ml of aqueous samples into 4-ml screw-cap vials. As wine behaves similarly to a buffered matrix the pH extraction was close to 4 (wine pH). The 60 μm PDMS–DVB fiber was then immersed in the sample for 30 min at ambient temperature (21 °C). The samples were stirred with a magnetic stirrer during extraction. Following this, the analytes were desorbed from the fiber and introduced into the chromatographic system by the SPME–HPLC interface, either by dynamic or static mode.

As a precautionary measure, as the fiber had been desorbed in organic solvents, it was dried for several minutes prior to starting the next extraction.

3. Results and discussion

3.1. Optimization of desorption process

PDMS–DVB fibers were chosen as an extraction coating due to the results obtained in preliminary trials.

Two modes of desorption are possible in SPME–HPLC: dynamic and static desorption. The SPME–HPLC interface was evaluated in both the dynamic and static modes of desorption with water samples containing 0.5 mg l^{-1} of each analyte. For this

comparative study an extraction time of 15 min and ambient temperature were initially selected for the extraction process.

Several experiments were carried out in the static mode. First, the desorption chamber was filled with mobile phase (acetonitrile–water, 50:50) for each desired period of time (1–7 min). All the cases, in a subsequent analysis presented carry-over on the fiber. Static desorption using acetonitrile and methanol was also evaluated. These solvents were introduced into the desorption chamber by adding 500 μl with a luer-tipped glass syringe. All the trials carried out in the static mode with methanol and acetonitrile also presented carry-over and it was necessary to study the dynamic mode.

The fiber, after extraction, was introduced into the desorption chamber and the valve was immediately switched from the load to the inject position and mobile phase at 0.9 ml min^{-1} was passed through the desorption chamber for a time ranging from 1 to 6 min in order for complete desorption to be carried out. The results for the analytes in the dynamic mode for ranged desorption times are shown in Fig. 1. A desorption time of 4 min was selected, as after this period of time peak areas did not increase significantly and in a subsequent analysis no peaks appeared at the retention time of the analytes, neither was there any evidence of carry-over. After this desorption time, the fiber could be removed from the SPME–HPLC interface and prepared to make a further extraction.

3.2. Optimization of the extraction process

The efficiency of analyte extraction by an SPME method can vary widely depending upon the matrix influence [27], the adsorption time [22], the addition of salt to the sample and the pH of the sample [28]. To study the extraction process, the wine samples were spiked containing 0.25 mg l^{-1} and the desorption parameters were fixed at the previously optimized values.

In order to check the matrix influence, it is assumed that ethanol, which is one of the major constituents of wines, can induce some variations of the affinity coefficient [29] of the organic pesticide compounds between the polymeric stationary phase and the aqueous solution in the SPME.

Extraction of 0, 5, 10 and 15% alcoholic solutions (the last one corresponds to the maximum alcoholic concentration in wines) of the six studied fungicides at a level of 0.25 mg l^{-1} were performed and analyzed in triplicate. The results of this study are shown in Fig. 2. In all cases, the extraction efficiencies decreased when ethanol was present in solution. The reduction variation was much more significant between 0 and 10% than between 10% and the other concentration. The extracted amounts were approximately the same when the alcoholic percentage was between 10 and 15%.

To correlate these observations concerning extraction of fungicides from ethanolic aqueous solutions by SPME with those obtained by the same method in natural matrices, analysis of five commercial red wines (with an alcoholic concentration at 10–12%) were performed in triplicate. Taking into account that the time necessary to get a maximal extraction did not depend (in most cases) on the percentage of ethanol, extraction time was fixed at 15 min. Previous to this analysis, no traces of studied

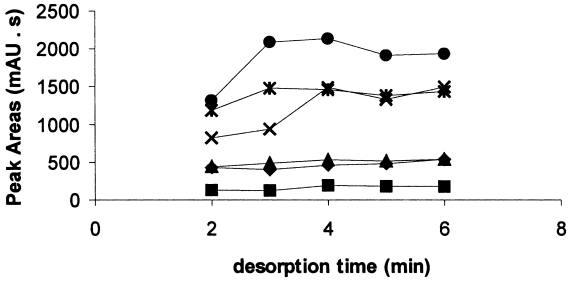

Fig. 1. Optimization of desorption time in the dynamic mode. Mobile phase: acetonitrile–water (1:1). ♦ NUA, ■ TRMN, ▲ TRMF, × FOL, * VIN, ● PEN.

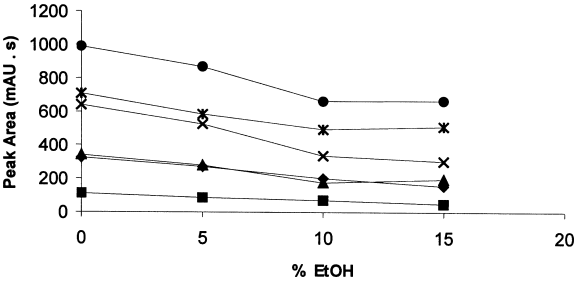

Fig. 2. Influence of ethanol in SPME efficiency. The fiber was exposed to ethanol–water solutions containing 0.25 mg l^{-1} of each fungicide. Each point is the average of the three data points. ♦ NUA, ■ TRMN, ▲ TRMF, × FOL, * VIN, ● PEN.

fungicides were detected in wine samples with this method. Wines samples were spiked at a level of 0.25 mg l^{-1} of each fungicide. Then, 3-ml aliquots were extracted in triplicate by SPME. Results were compared with those obtained from a 10% alcoholic aqueous solution. The results in Table 1 show that the influence of ethanol on the efficiency of the SPME method of extracting these residues in water or in natural wines seems to be of the same order.

In contrast to results obtained by other authors [29] as there were no changes in the extracted amounts, it may be concluded that other wine constituents do not interfere with the extraction in our case (with a 60 μm PDMS–DVB-coated fiber and fungicides studied). As no changes are witnessed in ethanolic water (10–15%) in the extraction amounts, then in commonly commercialized wine with alcoholic concentration between 10 and 15% the extraction must be of the same order.

These results indicate that SPME is a powerful extraction tool for fungicide residues in wines, but other parameters have to be taken into account as wines are natural matrices and are much more complex than water. Therefore, the influence of other parameters has been studied directly in wine samples.

Further experiments were carried out which focused on determining the extraction time of SPME. Triplicate wine samples containing all the analytes were extracted with the PDMS–DVB fiber for periods of time ranging from 5 to 140 min. Fig. 3 shows the adsorption time profiles obtained for the analytes. As can be observed, the PDMS–DVB

Table 1
Reduction indices for peak areas, 100(peak area)$_{matrix}$/(peak area)$_{water}$, in SPME of wines and water solutions containing 10% ethanol compared with pure water

	Reduction indices (%)	
	Wines (n=5) (mean±SD)	10% EtOH (n=5) (mean±SD)
NUA	1.75±0.16	1.62±0.26
TRMN	2.08±0.09	1.70±0.13
TRMF	2.10±0.27	1.96±0.06
FOL	1.76±0.21	1.91±0.10
VIN	1.47±0.12	1.44±0.25
PEN	1.50±0.07	1.49±0.12

Wines samples spiked at a level of 0.25 mg l^{-1} of each fungicide. Ratios of five average values.

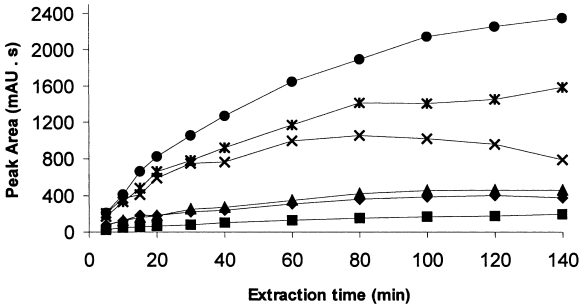

Fig. 3. The effect of equilibrium time on the chromatographic response of selected fungicides. The fiber was exposed to spiked wine containing 0.25 mg l^{-1} of each fungicide. Each point is the average of the three data points. ♦ NUA, ■ TRMN, ▲ TRMF, × FOL, * VIN, ● PEN.

allowed all analytes (except PEN) to attain equilibrium in 70 min. So as not to excessively increase the total analysis time, the extraction time was set at 30 min [30,31]. Although the analytes need more than 30 min to reach the equilibrium, the obtained response for all of them exhibited good reproducibility and it was considered suitable to achieve the detection limit required by legislation on MRLs of these pesticides [32].

Most authors state that effect of pH is not a controlling variable in wine [15]. Wine is a natural buffer matrix, therefore its pH is difficult to modify; other studies carried out the extraction process at a pH close to 4 (this value corresponds to the pH of commercial wines). In this study the effect of pH has been studied in simulated matrices (13% ethanolic water) and the results were in agreement with those mentioned above. Different values of pH present no change in the amount extracted. The extraction process in wine was carried out at wine pH.

Another extraction parameter which has a well-established effect in conventional extraction methods is the salting out effect obtained by adding ionic salts to the wine sample. This effect has also been studied in SPME applications mainly by the addition of NaCl and alternatively divalent salts such as Na_2SO_4 [33] or $MgSO_4$ [15]. Most authors agree on the positive effect of the addition of sodium chloride to the sample on the extraction efficiency of most compounds; however, some discrepancies have been found and no direct relation between extraction efficiency and salt addition has been witnessed in some cases [22]. In other cases, the addition of

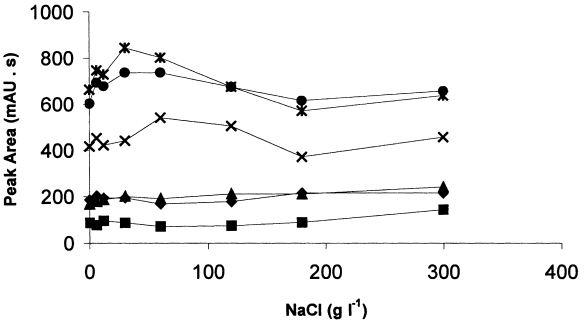

Fig. 4. The effect of salt addition on extraction efficiency of selected fungicides. The fiber was exposed for 30 min to spiked wine containing 0.25 mg l^{-1} of each fungicide. ♦ NUA, ■ TRMN, ▲ TRMF, × FOL, * VIN, ● PEN.

divalent salts was not chosen due to its hygroscopic properties which can cause problems in the determination of the concentration of fungicides [34].

The salting out effect was tested by the addition of NaCl (0 to 300 g l^{-1}). The fibers were cleaned with Milli-Q water after each analysis to avoid damage due to crystallization of NaCl. Fig. 4 shows the influence of NaCl on the detector response area. The responses obtained for NUA, TRMN and TRMF were similar to the ones obtained without the addition of NaCl. In the cases of FOL, VIN and PEN the optimum extraction level was observed at a range of 30–60 g l^{-1} of NaCl, but the signals extraction were not significantly increased, therefore the addition of salt was not considered necessary to carry out the extraction process.

3.3. Calibration curves, detection limits and precision data

With the aim of testing the precision of this SPME method, the intra-day relative standard deviations (RSDs) were determined by performing 10 consecutive extractions at two different levels of concentration under the selected conditions. The same standards were also analyzed at intervals over a 2-week period ($n=10$) in order to determine the inter-day RSDs. The results shown in Table 2 can be deemed excellent beside the values usually obtained with SPME methods.

Similarly, the linearity of the chromatographic responses of all pesticides was studied in spiked wine samples, within the concentration range 20–250 μg l^{-1}. Each solution was run in triplicate. The correlation coefficients were greater than 0.990 in all cases.

The calculation of the detection limits was based on a $2N/m$ ratio, where N is the noise and m is the slope of the respective calibration equation. Table 2 shows the limits of detection (LODs) in wine samples obtained for each pesticide using the PDMS–DVB with HPLC–DAD. All the LODs were lower than MRLs of each fungicide tolerated under current legislation.

3.4. Analysis of red wine samples

The effectiveness of the proposed method for the determination of these fungicides in red wine was tested by performing replicate analyses of five different samples of Rioja red wine. As the target analytes were not found in these wine samples, triplicate aliquots of each sample were artificially spiked with 250 μg l^{-1} and subsequently analyzed using the proposed SPME–HPLC–DAD method with the PDMS–DVB fiber. The average concentrations obtained in the analysis of these spiked

Table 2
Linearity, detection limits and precision data of the analytical method using PDMS–DVB fibers

	Slope (mAU s/mg l^{-1})	Intercept (mAU s)	r	LOD (μg l^{-1})	Intra-day RSD (%, $n=10$)		Inter-day RSD (%, $n=10$)	
					0.05 mg l^{-1}	0.25 mg l^{-1}	0.05 mg l^{-1}	0.25 mg l^{-1}
NUA	891.06	4.31	0.9935	9	8.86	2.39	16.56	7.83
TRMN	479.15	4.31	0.9941	27	12.91	9.25	18.09	15.35
TRMF	990.55	10.77	0.9980	17	14.24	3.31	11.30	7.62
FOL	962.00	20.37	0.9978	10	9.99	7.89	25.69	13.09
VIN	3372.38	40.04	0.9953	4	4.58	8.57	5.7	4.68
PEN	4228.23	26.36	0.9943	5	11.95	4.96	14.73	11.44

samples correspond to mean recoveries ranging from 95±14 to 103±5% for a significance level of 0.05. The corresponding HPLC–DAD chromatograms obtained by the PDMS–DVB fiber for a red wine sample, are shown in Fig. 5. The obtained chromatogram shows the presence of several non-identified compounds in the sample (t_R<5 min). However, such eluted compounds do not interfere with the determination of the analytes of interest. The method also has the advantage of measuring peak purity and confirming the isolated fungicide by UV spectra.

The effect of particulate matter on the PDMS–DVB fiber is unknown, but they appear to reduce the fiber life, possibly by covering the fiber surface irreversibly after several extractions. For this, wine samples were filtered through a 0.45 μm membrane after spiking. In this way, fibers were used more than 50 times for wine samples or about 100 times in water assays.

These results show that the proposed methodology is suitable for the determination of these fungicides in red wine.

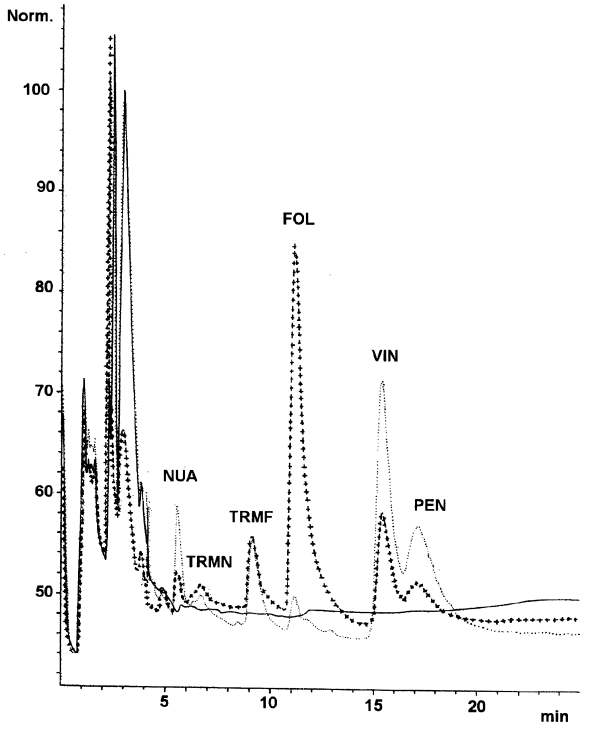

Fig. 5. Typical SPME–HPLC chromatogram of a red wine sample. Normal line: un-spiked wine (λ=200 nm); dotted lines: spiked wine containing 0.25 mg l^{-1} of each fungicide, + line (λ=225 nm), ··· line (λ=200 nm).

4. Conclusions

Six organoclorine fungicides were successfully determined by SPME–HPLC–DAD in real red wine samples. Compared with common extraction methods SPME can be performed very easily. It was possible to analyze one sample every 50 min. The linearity of the method has been researched and the analyzed fungicides presented adequate correlation coefficients. The detection limit values were below 28 μg l^{-1} in all cases. Method precision was researched and RSDs (intra- and inter-day) were good.

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