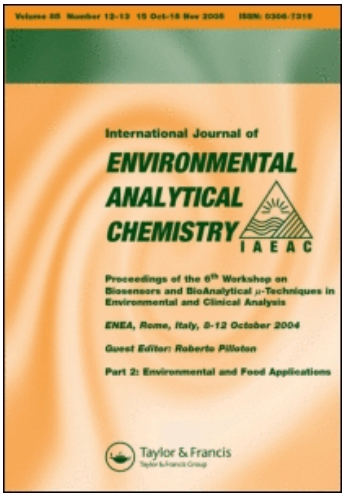

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Residue screening in apple, grape and wine food samples for seven new pesticides using HPLC with UV detection. An application to trifloxystrobin dissipation in grape and wine

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A new analytical procedure was developed and validated, using liquid chromatography, for simultaneous determination of seven new pesticides belonging to different chemical classes (azoles, strobilurines, benzoylureas, and a new oxazolidinedione) in apple, grape and wine food samples. Solvent extraction of pesticides was performed using a cyclohexane-dichloromethane (9 + 1 v/v) mixture and the extract was cleaned-up by solid-phase extraction (SPE) using silica cartridges and elution with tetrahydrofurane. Separation of pesticides flusilazole, fenbuconazole, diniconazole, tebufenozide, famoxadone, trifloxystrobin and flufenoxuron was performed by gradient elution High-Performance Liquid Chromatography (HPLC) with a microbore Hypersil HS C_{18} column in 35 min total time, using mixtures of acetonitrile-water as the mobile phase. Pesticides were detected and quantified at 210 nm with a variable-wavelength UV detector. Recoveries of pesticides from spiked samples, at 0.05–2.0 mg kg^{-1} for apple and grape and at 0.025–0.250 mg L^{-1} for wine, ranged from 82 to 107% for apple and grape and from 87% to 106% for wine with RSD < 12%. The limits of quantification (LOQs) of the method, as a signal to noise ratio equalled 10, ranged from 0.02 to 0.10 mg kg^{-1} for apple and grape and from 0.005 to 0.02 mg L^{-1} for wine samples. The uncertainty associated with the analytical methodology, estimated using the 'bottom-up' approach, was lower than 12.8% for all pesticides and matrices tested. The proposed methodology was applied for the evaluation of trifloxystrobin residue levels in grapes exposed to field treatments and in the must and wine produced from them.

Keywords: food analysis; liquid chromatography; fungicides; insecticides; uncertainty

1. Introduction

Management of a wide range of pests and diseases is important to maintain grape productivity and wine quality. Downy mildew (*Plasmopara viticola*), powdery mildew (*Uncinula necator*) and grey mould (*Botrytis cinerea*) are major diseases and grape moth (*Lobesia botrana*) is a major pest in grape cultivation requiring repeated applications of fungicides and insecticides. This practice may result in undesirable residues on grape and wine, especially when the established pre-harvest interval (PHI) for each product is not

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respected, affecting not only the wine-making process, but also wine quality and consumer safety [1–3]. Although pesticide residues are known to decrease during vinification [4,5], they are generally regulated as maximum residue limits (MRLs) for pesticides in table or vinified grapes [6]. Thus specific lower MRLs for pesticides in grapes and wine are setting in recent years.

Pesticide residues in food gained major significance during recent decades and analytical methods are used and developing worldwide gas chromatography with nitrogen-phosphorus, electron capture or mass spectrometric detection has been widely used to develop methods for multiresidue analysis of various commonly used pesticides [7–10]. However, taking into account that the target compounds usually have a low volatility and are thermally labile, liquid chromatography is the more proper alternative to gas chromatography for pesticide residue analysis. Numerous analytical methods for determining some compounds of these pesticide residues in fruit and vegetables, including grapes and wine, have been reported [11–20]. However, only few studies have been found in the recent literature using HPLC with UV detection, to detect and quantify new pesticide residues in grape and wine samples. Multiresidue methods for simultaneous determination of pesticides of new chemical classes use one or more extraction solvents, such as acetone, ethyl acetate, dichloromethane, petroleum ether, acetonitrile, and hexane. However, some matrix coextractives cause matrix interferences which enhance the responses in the quantitative analysis, resulting in false positive results or inaccurate quantification [21]. Several cleanup modes, such as SPE [22], have been assayed for purification and reduction of these effects.

The objective of this study was to develop a HPLC method for simultaneous residue determination of seven widely used new generation pesticides belonging to different chemical classes (Table 1) in apple, grape and wine samples. The developed method was used to investigate the behaviour of fungicide trifloxystrobin on grape in the field and during the vinification process. Only a few articles have been published on the analysis of trifloxystrobin [17–19], on its effect on the aroma composition of wine [23] and on its removal from red wines [24]. None of these has investigated trifloxystrobin dissipation on grape and its fate from vine to wine.

2. Experimental

2.1 *Materials and standard solutions*

Diniconazole (purity 98.2%, were purchased from Rhone-Poulenc), famoxadone (99.4%, from Dupont), fenbuconazole (99.8%, from Dow AgroSciences), flusilazole (99%, from Dupont) and trifloxystrobin (99.6%) flufenoxuron (99.3%), tebufenozide (99.9%) were purchased from Dr Ehrenstorfer GmbH. The common names, chemical classes, chemical names and main activities of studied pesticides used in vineyard and apple orchard protection [25], are presented in Table 1.

Individual analytical standard stock solutions 1000 mg L^{-1} for all pesticides were prepared in acetone and stored at −18°C in glass vials. An intermediate mixture standard solution containing all compounds, at 100 mg L^{-1} each, was prepared in acetone from the individual stock solutions and stored at −18°C. Spiking mixture solutions were prepared by dilution from the intermediate mixture solution and stored at 4°C. Calibration standard solutions at concentrations 0.05 to 10 mg L^{-1} (eight solutions) in acetonitrile-water (1/1, v/v) were prepared from the intermediate mixture solution.

Table 1. Common and chemical names, classes, main activities and maximum residues limits (MRLs in grape, apple and wine) of the investigated pesticides.

Common names	IUPAC names	Chemical classes	Main activity	Grape MRLs ($mg\,kg^{-1}$)	Apple MRLs ($mg\,kg^{-1}$)	Wine MRLs ($mg\,L^{-1}$)
Diniconazole	(*E*)-(RS)-1-(2,4-dichlorophenyl)-4,4-dimethyl-2-(1*H*-1,2,4-triazol-1-yl)pent-1-en-3-ol	Azole	Fungicide	0.2	0.1	
Famoxadone	(RS)-3-anilino-5-methyl-5-(4-phenoxyphenyl)-1,3-oxazolidine-2,4-dione	Oxazolidi-nedione	Fungicide	2	0.02	
Fenbuconazole	(RS)-4-(4-chlorophenyl)-2-phenyl-2-(1*H*-1,2,4-triazol-1-ylmethyl)butyronitrile	Azole	Fungicide	1	0.4	
Flufenoxuron	1-[4-(2-chloro-α,α,α-trifluoro-p-tolylox)-2-fluorophenyl]-3-(2,6-difluorobenzoyl)urea	Benzoylurea	Insecticide	2	0.5	0.01
Flusilazole	bis(4-fluorophenyl)(methyl)(1*H*-1,2,4-triazol-1-ylmethyl) silane	Triazole	Fungicide	0.2	0.02	0.1
Tebufenozide	*N*-*tert*-butyl-*N*-(4-ethylbenzoyl)-3,5-dimethylbenzohydrazide	Diacylhydrazine	Insecticide	3	1	0.1
Trifloxystrobin	methyl(*E*)-methoxyimino-{(*E*)- α-[1-(α,α,α-trifluoro-m-tolyl)ethylideneaminooxy]-o-tolyl}acetate	Strobilurin	Fungicide	5	0.5	0.3

Note: MRLs in grape, apple ($mg\,kg^{-1}$) European Community 396/2005 & 149/2008 and wine ($mg\,L^{-1}$), other countries (Switzerland, LCBO).

Cyclohexane and dichloromethane were pesticide residue grade, acetonitrile were HPLC far UV grade, and water and tetrahydrofurane were HPLC grade. All solvents were purchased from Labscan (Dublin, Ireland). Commercial formulation (Flint WG 50% w/w trifloxystrobin) was purchased from Bayer Hellas (Greece).

2.2 *Instrumentation and chromatographic conditions*

Chromatographic analyses were performed with a HP 1100 liquid chromatograph (Hewlett-Packard GmbH, Waldbronn, Germany) equipped with a ternary-delivery system, a variable-wavelength UV detector and a HP ChemStation LC 3D chromatography manager data acquisition and processing system with the ability to obtain UV spectra at selected retention times of chromatograms. The analytical column was a Thermo Hypersil HS C_{18} column (250×2.1 mm I.D with 5 μm particle size) with a guard column. The mobile phase was acetonitrile-water delivered at a flow of 0.26 mL min^{-1} with a gradient composition; from acetonitrile-water (55/45, v/v), held for 5 min, to acetonitrile-water (80/20, v/v) in 10 min, held for 10 min, and finally a degrease at acetonitrile-water (55/45, v/v) over 10 min to stabilise the HPLC system before starting the next run, giving a total run-time of 35 min. The injection volume was 20 μL. Before injection, samples were filtered through Titan 2 HPLC nylon membrane filters (17 mm, 0.2 μm pore size). The optimum detection was obtained at 210 nm and the column temperature was maintained at 30°C.

2.3 *Sample preparation*

A simple and one step extraction was used for extracting the investigated pesticides from the matrices using a mixture of cyclohexane-dichloromethane (9/1, v/v) as extraction solvent.

Grape and apple extraction. An aliquot (10 g) of previously homogenised sample was weighed into a centrifuge tube and 10 mL of extraction solvent was added. The mixture was homogenised for 1.5 min using an Ultra Turrax T25 homogeniser at 5000 rpm. After centrifugation 5 mL of organic layer was transferred to a pear shape flask and carefully evaporated to dryness with a rotary evaporator at 40°C and the residue was quantitatively transferred with 1 mL cyclohexane to preconditioned SPE cartridges.

Wine extraction. Ten mL of wine was mixed in a centrifuge tube with 5 mL of extraction solvent and the tube was agitated for 30 min in an orbital shaker. After centrifugation 3 mL of the upper organic layer was transferred to a pear shape flask and carefully evaporated to dryness with a rotary and the residue was quantitatively transferred with 1 mL cyclohexane to preconditioned SPE cartridges.

Clean-up procedure. Isolute SPE cartridges containing 500 mg silica sorbent in 3 mL reservoir (IST Ltd. International Sorbent Technology, Mid Glamorgan, UK) were used for the clean-up of grape, apple and wine sample extracts. The cartridges were preconditioned with 10 mL cyclohexane and after loading of the sample extract (1 mL) were rinsed with 10 mL cyclohexane and 3 mL of a cyclohexane-tetrahydrofurane (90/10, v/v) solution. The pesticides were eluted with 2 mL tetrahydrofurane and the eluent was conducted to dryness under a gentle stream of nitrogen. Residues were redissolved in acetonitrile-water (50/50, v/v) solution (in 1 mL for grape or apple and in 0.5 mL for wine) and the resulting solution was filtered prior to injection into the HPLC system.

On the basis of this extraction procedure, the concentration factor of the sample in the final solution corresponds to 5 g of grape, apple and must matrix mL^{-1} and 12 g of wine matrix mL^{-1}. The concentration factors were different in wine samples in order to obtain lower quantification limit of compounds in wine samples. Unprocessed must and centrifugal must samples were extracted as grape or wine samples, respectively.

2.4 *Samples*

Commercial organic produced apples as well as grapes from untreated vines and wine produced from them were used as uncontaminated samples for the method development. All apple and grape samples were homogenised and 50 g sub-samples were kept frozen until spiking or analysis. Grape samples were also collected from an experimental vineyard (cv Roditis) located in central Greece.

Field experiment. The experiment was divided into four randomised plots (60 plants each). Three of them, used as replicates, were treated with Flint WG on 28 August, 2004; at the recommended application rate of 12.5 g ai $100\,L^{-1}$, using an automated high pressure machine (Euro spray Ecology 2000) and the other one was left untreated to be used as control. Grape samples were randomly collected [26] at 0 (3 h after application), 7, 14 and 28 days after application (DAA). During the sampling period average daily air temperature was 20.6°C, average relative humidity was 64.2% and the total rainfall was 18.8 mm.
Vinification process. The vinification was performed at laboratory scale. Two vinification experiments were performed from the grapes collected at 14 and at 28 DAA. The grape samples were divided into two equal parts, one part was allowed to ferment with the skins (vinification with maceration); the other was pressed, and the resulting must was allowed to ferment (vinification without maceration) at room temperature. A 250 g aliquot of cloudy must was taken and centrifuged at 4000 rpm for 5 min in order to quantify residues in the clear must. After completing the alcoholic fermentation the obtained wine was racked, filtered and analysed for trifloxystrobin residues.

2.5 *Validation studies*

Calibration data, accuracy, precision and LOQs as well as the associated global uncertainty were calculated for the analytical methodology developed. Recovery assays ($n = 5$) were performed by spiking uncontaminated grape and apple (10 g homogenised sample) and wine (10 mL of wine) samples at different levels with spiking solutions. The spiked samples were allowed to equilibrate for one hour before extraction to allow the spiked solution to penetrate the material.

3. Results and discussion

3.1 *Analysis*

The screening method is simple and suitable for routine analysis. The cyclohexane-dichloromethane solution at 9/1 ratio effectively extracted all investigated pesticides. The clean-up procedure was optimised after examining parameters such as rinsing and elution solvent. Finally used elution pattern necessary to recover the analytes from the normal phase SPE cartridges resulted in chromatographic analysis free of extraneous peaks. Figures 1–3 show chromatograms of a mixture standard solution, control and fortified

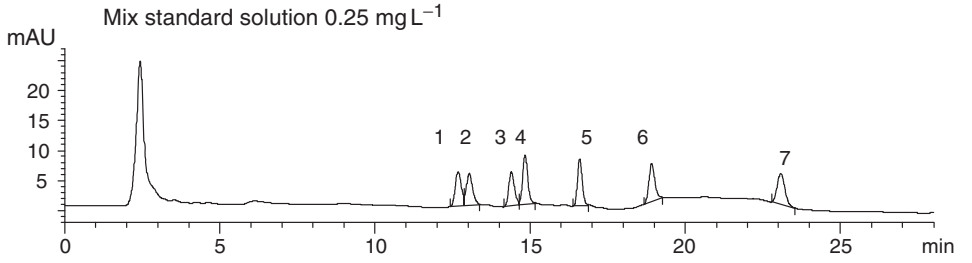

Figure 1. HPLC-UV chromatogram registered at 210 nm for a mixture standard solution (0.25 mg/L in acetonitrile/water 1/1 v/v).
Note: Peaks: 1 – flusilazole, 2 – fenbuconazole, 3 – diniconazole, 4 – tebufenozide, 5 – famoxadone, 6 – trifloxystrobin, 7 – flufenoxuron. (chromatographic conditions as described in Section 2.2).

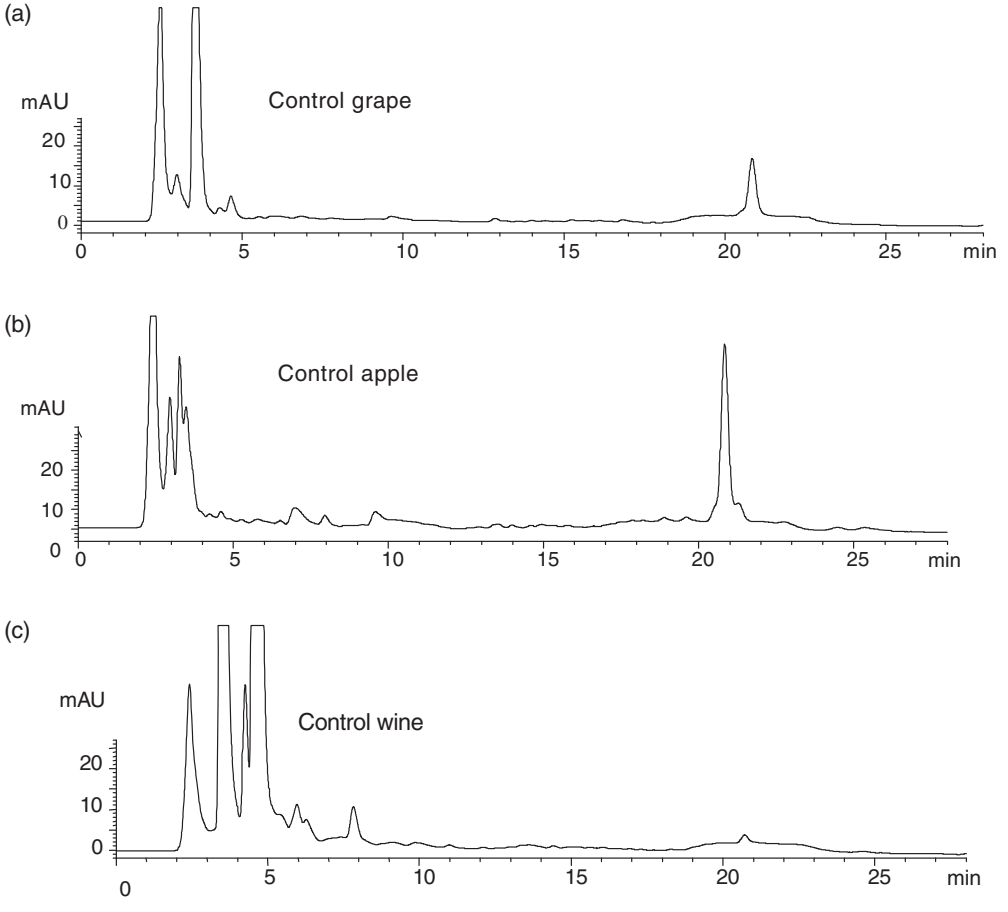

Figure 2. HPLC-UV chromatogram registered at 210 nm for an uncontaminated (control) sample of (a) grape, (b) apple and (c) wine, processed following the experimental procedure described (chromatographic conditions as described in Section 2.2).

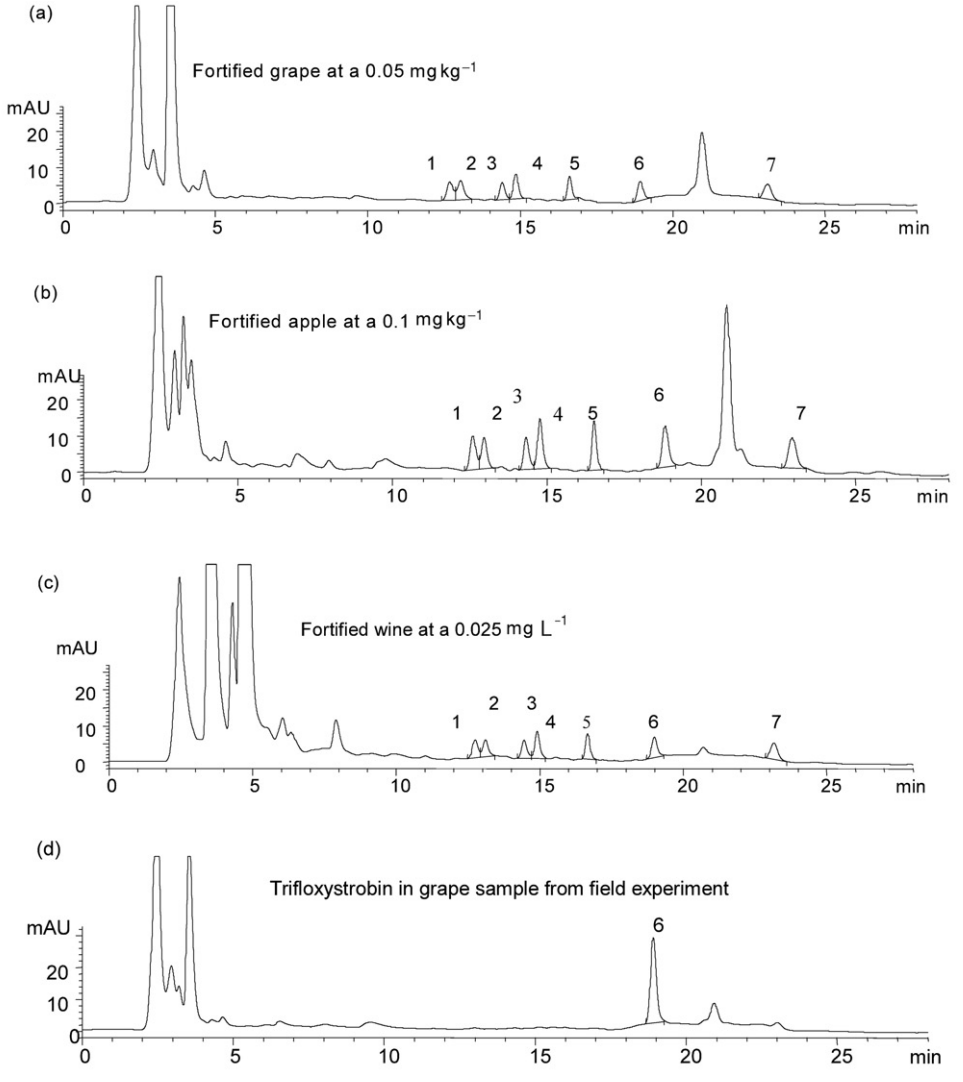

Figure 3. HPLC-UV chromatogram registered at 210 nm for spiked (a) grape at a 0.05 mg kg^{-1}, (b) apple at a 0.10 mg kg^{-1}, (c) wine at a 0.025 mg L^{-1} and (d) for grape sample from field experiment in 28 DAA.
Note: Peaks: 1 – flusilazole, 2 – fenbuconazole, 3 – diniconazole, 4 – tebufenozide, 5 – famoxadone, 6 – trifloxystrobin, 7 – flufenoxuron (chromatographic conditions as described in Section 2.2).

samples extracts (for grape, apple and wine), respectively. The seven compounds are well separated by the developed gradient elution programme and their detection at 210 nm was sensitive and has been approved with minimum absorbance by interfering compounds. The concentrations of the pesticides were determined by comparing the peak heights in the samples with those found in the calibration solutions. In addition to the spiked and treated samples, control samples were analysed as blank samples and standard solutions were injected every seven samples.

3.2 *Method validation*

3.2.1 *Linearity*

Calibration graphs were constructed by plotting peak height versus injected quantity. For obtaining the calibration plots, calibration solutions of pesticides were injected in triplicate at the 0.05–10 mg L^{-1} range (eight levels). The detector's response was linear over the studied range and the least-square linear regression analysis of the data provided excellent correlation for all seven compounds ($R^2 > 0.999$). The results of the regression analysis are given in Table 2.

3.2.2 *Accuracy and precision*

Instrumental precision data was obtained in condition of repeatability (for six injections in the same day) with 0.50 mg L^{-1} standard solution (corresponding to a sample concentration of 0.10 mg kg^{-1} for grape and apple and 0.042 mg L^{-1} for wine). Satisfactory precision was obtained for all the studied compounds with coefficient values in the range 3.0–4.1% (Table 2).

Accuracy data was provided by recovery experiments. Fortified grape, apple and wine samples were analysed to evaluate the effectiveness of the procedure. The mean recovery percentages and the associated standard deviation are given in Table 3. It can be seen that the mean recoveries ranged from 82 to107% for grape, from 85 to 106% for apple and from 87 to 106% for wine and the relative standard deviations (RSDs) were between 5 and 12%. These results are satisfactory for residues analysis [27] and indicate good accuracy and precision of the method.

3.2.3 *LOQs*

The LOQs of the method, calculated as a signal to noise from untreated samples equal to 10, ranged from 0.02 to 0.05 mg kg^{-1} for grape, from 0.02 to 0.10 mg kg^{-1} for apple and from 0.005 to 0.02 mg L^{-1} for wine depending on the final concentration factor for each extract and the sensitivity of each compound. These values of LOQs were also confirmed by including them to the validation procedure and the recovery experiments. The obtained LOQs (Table 2) are clearly below the MRL values of the studied compounds and matrices with the exception of famoxadone in apple where LOQ was at the MRL value. Considering the chromatographic and validation parameters, the developed multiresidue HPLC chromatographic method was able to detect and measure residues in apple, grape and wine in accordance with the fixed MRL values in the European Union (Table 1).

3.2.4 *Uncertainty calculation*

The uncertainty of measurement was obtained by applying the analytical method and was calculated using the bottom-up approach on the basis of in-house validation data according to EURACHEM/CITAC guide [28]. The overall uncertainty (U_{ov}) can be expressed with the equation:

$$U_{ov} = \sqrt{U_{pre}^2 + U_{cal}^2 + U_{rep}^2 + U_{rec}^2},$$

where U_{pre}, U_{cal}, U_{rep}, and U_{rec} represent the contributions of the preparing calibration standards and stock solutions, the contribution of the estimation of the analyte

Table 2. Validation parameters for determination of diniconazole (DNZ), famoxadone (FMX), fenbuconazole (FNB), flufenoxuron (FLF), flusilazole (FLZ), tebufenozide (TBF) and trifloxystrobin (TRF) residues in grape, apple and wine.

			DNZ	FMX	FNB	FLF	FLZ	TBF	TRF
Retention time (min)			14.37	16.63	13.00	23.35	12.63	14.82	19.06
Linear range ($mg\,L^{-1}$)			0.05–10	0.05–10	0.05–10	0.05–10	0.05–10	0.05–10	0.05–10
Coefficient of determination R^2 (calibration curves)						>0.999			
K (slope) $\pm$ CI (95%)			22.65 ± 0.29	28.93 ± 0.40	21.24 ± 0.47	20.71 ± 0.25	23.43 ± 0.36	31.02 ± 0.29	25.86 ± 0.32
y-intercept $\pm$ CI (95%)			0.57 ± 1.42	0.55 ± 1.75	0.15 ± 1.72	0.11 ± 0.85	0.27 ± 1.56	0.21 ± 1.34	0.34 ± 1.15
Instrumental repeatability (at 0.50 mg/L) CV (%), $n=6$			3.5	4.1	3.7	3.0	3.2	3.9	3.6
Limit of quantification	($mg\,kg^{-1}$)	grape	0.03	0.05	0.05	0.02	0.05	0.02	0.03
		apple	0.03	0.02	0.03	0.02	0.03	0.02	0.10
	($mg\,L^{-1}$)	wine	0.02	0.005	0.02	0.005	0.01	0.01	0.005
Expanded uncertainty U	(%)	grape	10.9	12.3	11.1	9.7	10.5	12.1	9.7
		apple	10.2	11.1	11.8	10.2	9.2	11.4	11.7
		wine	10.4	11.7	12.5	9.6	10.6	12.8	11.0

Table 3. Average recoveries (REC %), relative standard deviation (RSD %) from analyses of grape, apple and wine samples fortified at different levels, $n = 5$.

	Fortification level	DNZ		FMX		FNB		FLF		FLZ		TBF		TRF	
		REC	RSD	REC	RSD	REC	RSD	REC	RSD	REC	RSD	REC	RSD	REC	RSD
Grape	0.05 (mg kg^{-1})	107	10	102	11	105	9	103	10	101	11	104	9	91	10
	0.10	89	9	87	10	88	9	95	8	93	9	85	10	87	7
	1.0	92	8	93	9	104	8	88	8	98	7	96	9	102	9
	2.0	95	6	84	7	95	7	99	6	95	9	89	7	82	6
Apple	0.10 (mg kg^{-1})	91	9	92	10	89	9	102	8	85	10	101	9	96	8
	1.0	93	8	85	8	87	10	95	9	89	7	87	9	87	10
	2.0	88	8	92	9	94	7	96	6	93	9	86	8	106	7
Wine	0.025 (mg L^{-1})	106	11	97	10	105	12	105	11	103	10	96	9	104	11
	0.05	98	8	104	9	104	11	91	7	101	9	87	11	97	9
	0.25	94	5	88	8	95	9	103	7	95	11	99	8	88	9

concentration from the calibration curve, the contribution of the intermediate precision expressed as relative standard deviation obtained from repeatability conditions ($n = 6$) in the same day, and the contribution of the accuracy expressed by the recovery percentage obtained from spiking assays. To obtain the expanded uncertainty (U), overall uncertainty must be multiplied by a coverage factor (k). For most purposes, a coverage factor of 2 is recommended, which corresponds to a 95% confidence level. Uncertainty was calculated at 0.50 mg L^{-1}, corresponding to a sample concentration of 0.10 mg kg^{-1} for grape and apple or 0.042 mg L^{-1} for wine, and was expressed as relative standard deviation. Table 2 summarises the results on the expanded uncertainty associated with the quantification of the investigated compounds in apple, grape and wine samples. Calculated U values ranged from 9.2 to 12.8% for the grape, apple and wine matrices. The main contribution of the partial components in the overall uncertainty (U_{ov}), for all matrices and compounds studied, came from the uncertainty associated with accuracy (U_{rec}) assessment.

3.2.5 *Field sample analysis and wine-making*

The percentage of deposit of the pesticides on grapes after the phytosanitary treatment depends on the relationship between application date and sampling date, as well as on the applied concentration and chemical properties of the compounds. The mean residue level in grape samples ($n = 3$) collected three hours after the application (0 DAA) was 0.84 mg kg^{-1} (Table 4). Residues declined to 0.32 and 0.28 mg kg^{-1} after 7 and 14 DAA, respectively, and decreased further to 0.14 mg kg^{-1} at the recommended PHI (28 days). This last concentration is clearly below the established MRL for trifloxystrobin in grape (5 mg kg^{-1}). Therefore, the use of this fungicide should not create limit problems if used following good agriculture practices.

In wine production, it was also important to understand the possible mechanism of the disappearance of trifloxystrobin residues. To study trifloxystrobin residue partitioning during vinification grapes collected at 14 and 28 DAA were used. During wine production without maceration and before fermentation, a part of trifloxystrobin residues around 60% and 50% compared to the concentration in grapes at 14 and 28 DAA, respectively, were removed with the cake and the remaining residues in must were 0.11 and 0.07 mg kg^{-1}. After the must centrifugation, trifloxystrobin residues in the clear must were 0.02–0.03 mg L^{-1}, indicating that trifloxystrobin was absorbed by the suspended solids in the must. At the end of the wine-making process (with and without maceration), trifloxystrobin residues were not detected in the produced wines (Table 4). The residue was eliminated, probably due

Table 4. Trifloxystrobin residue levels on grape (mg kg^{-1}, in parenthesis the relative standard deviation for $n = 3$ samples) of Roditis vine variety at various times after field application (DAA, in days), in must (mg kg^{-1}) and in centrifuged must and wine (mg L^{-1}) after vinification process with and without maceration ($n = 3$).

DAA (days)	Grapes (mg kg^{-1})	Must unprocessed (mg kg^{-1})	Must centrifuged (mg L^{-1})	Wine without maceration (mg L^{-1})	Wine with maceration (mg L^{-1})
0	0.84 (10)	–	–	–	–
7	0.32 (14)	–	–	–	–
14	0.28 (16)	0.11 (12)	0.03 (16)	<LOQ	<LOQ
28	0.14 (17)	0.07 (15)	0.02 (18)	<LOQ	<LOQ

to chemical, physico-chemical or metabolic factors that are presented during vinification such as hydrolysis, absorption capacity and yeast action.

4. Conclusions

This paper described an efficient, reliable and sensitive HPLC–UV method for the simultaneous residues determination of seven widely used pesticides in apple, grape and wine. The proposed method permits the determination of the pesticides, after a simple extraction of the sample (grape, apple, must or wine) following quality control criteria for pesticide residue analysis [27]. The method provides satisfactory accuracy and precision and the obtained LOQs are much lower than the MRLs set by legislation for the studied compounds and matrices, with the exception of famoxadone in apple where LOQ was at the MRL value. The method is suitable for routine analysis of apple, grape and wine samples and was used to evaluate the fate of trifloxystrobin fungicide on grape and wine samples. A field dissipation experiment showed that application of commercial formulation of trifloxystrobin lead to residues on grape clearly below the MRL at the PHI and that after vinification trifloxystrobin was not detected in the produced wine.

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