Determination of cyprodinil and fludioxonil in the fermentative process of must by high-performance liquid chromatography–diode array detection

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Abstract

BACKGROUND: A quantitative, selective and sensitive high-performance liquid chromatographic method is described for the analysis of new fungicides cyprodinil, fludioxonil and their commercial formulation *Switch* in model solutions of must and wine, as well as samples during alcoholic fermentation. A study of the dissipation of residues was carried out.

RESULTS: The proposed method is based on liquid–liquid extraction (LLE) followed by high-performance liquid chromatography and diode array detection. Dichloromethane was the most appropriate solvent for extracting cyprodinil and fludioxonil in samples. Quality parameters of the proposed method presented good recovery (ca. 97% for almost all compounds) and precision (between 4.8% and 5.4%), and limits of quantification were lower than maximum residue limits (MRLs) in grapes.

CONCLUSIONS: There is no matrix effect in the analysis of cyprodinil and fludioxonil. The application of the fermentative process on cyprodinil and fludioxonil fungicides causes a decrease in the concentrations of these compounds. This decrease is slightly higher, the higher the initial concentration, without observing the appearance of any product in degradation. Fludioxonil shows a higher reduction when the compounds are presented together in *Switch*.

Keywords: fungicides; must; wine; liquid–liquid extraction; HPLC-DAD; matrix effect

INTRODUCTION

The principal parasites of the grapevine are gray mold (*Botrytis cinerea*), downy mildew (*Plasmopara vitícola*) and powdery mildew (*Uncinula necator*). These pests can reduce berry quality.[1] In order to achieve an effective control of this fungus it is necessary to use fungicides at the right stage of growing.[2] The use of these products, particularly when the grape harvest is near, can lead to hazardous residues above the maximum permitted levels in the must obtained. In addition, the presence of pesticides could affect the activity of the yeasts, resulting in a stopped or sluggish fermentation, which is detrimental to the final quality of the product.[3,4] Other studies have dealt with the transformation of pesticides and their dissipation during fermentation.[2,5,6]

Generally, pesticide analyses are carried out by gas chromatography (GC)[7–9] or high-performance liquid chromatography (HPLC) with a diode array detector (DAD); fluorescence detection[10–12] is also used. Routine methods used in pesticide residue analysis in complex matrices are based on extraction and concentration of pesticides prior to chromatographic analysis[13]. Major extraction techniques involve solid-phase extraction[8,14,15] or solid-phase micro-extraction.[10,16] The most commonly used treatment method for liquid samples is liquid–liquid extraction (LLE) with organic solvents. Acetonitrile, hexane, dichloromethane and ethyl acetate are the most commonly used.[6,17–20]

In the analysis of pesticides in enological samples, an essential question is the choice of a methodology for the determination of these compounds both in

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must and wine, no matter their matrix. Since alcoholic fermentation causes changes in the composition of the must, with a reduction of the sugar content and an increase in ethanol, it is important to study the matrix effect which can occur in the fungicide extraction process – an effect which has already been detected by other writers.[21–23] Some researchers[14] have looked at the matrix effect in the determination of fungicides in different types of grapes, while others have worked on the composition of the must itself[21] or of the wine.[17,24]

Recently, some new fungicides were introduced on the market. Cyprodinil (anilinopyrimidine) and fludioxonil (phenylphyrrole) are available together in a commercial formulation called Switch (37.5% and 25%, respectively). The first inhibits the biological synthesis of methionine, one of the principal components of fungal protein synthesis, while fludioxonil stimulates the synthesis of glycerol, which blocks cell growth in the fungus.[25–27] The main aim of this work was to develop a sensitive, selective, precise and simple method for the determination of cyprodinil and fludioxonil residues in the fermentation process of musts by LLE with subsequent HPLC-DAD. Furthermore, a study of the matrix effect on sample treatment and on quantification of the compounds was made. The method was applied in order to evaluate the effect of alcoholic fermentation on the concentration of the target fungicides, which have been applied individually and mixed in the commercial product.

EXPERIMENTAL

Chemicals

Analytical standard pesticides of cyprodinil (4-cyclopropyl-6-methyl-*N*-phenylpyrimidine), fludioxonil [4-(2,2-difluoro-1,3-benzodioxol-4-yl)-1*H*-pyrrole-3-carbonitrile] and Switch were purchased from Riedel-de-Haën (Seelze, Germany). Table 1 shows the chemical characteristics of the compounds studied. Standards were certified at a minimum of >99% pure (Pestanal grade). HPLC-grade methanol, ethanol, acetonitrile and dichloromethane were obtained from Scharlau (Barcelona, Spain). Other reagents used were D(+)-glucose, potassium dihydrogen phosphate, magnesium sulfate, ammonium sulfate and sodium sulfate anhydrous ACS-ISO for analysis from Panreac (Spain). L(+)-Tartaric acid ACS was provided by Sigma Aldrich. Sodium chloride for analysis was purchased from Carlo Erba.

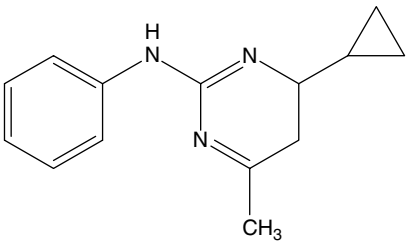
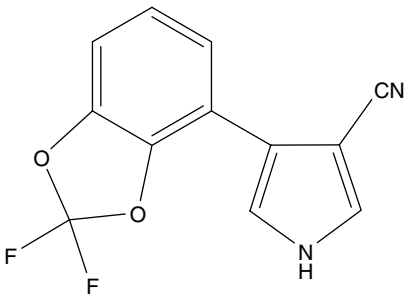
Ultrapure water was obtained in a Milli-Ro plus system together with a Milli-Q system from Millipore (Bedford, MA, USA).

Inoculated yeast VRB *Saccharomyces cerevisiae* was purchased from Lallemand (Australia). Inocula were prepared in 7 g L^{-1} of Difco™ yeast nitrogen base (Difco Laboratories, Detroit, MI, USA) in Milli-Q water.

Stock standard solutions

A stock standard solution (*ca* 1000 mg L^{-1}) of each fungicide was prepared in methanol by weighing approximately 0.0250 g of the analyte into a 25 mL volumetric flask and diluting to volume. An intermediary mixed standard solution was prepared by dilution in methanol of the stock standard solution to give a concentration of *ca* 100 mg L^{-1} for each compound. All standard solutions were stored at −20 °C. Working standard solutions for further studies were prepared by spiking different volumes of the intermediary standard solution in synthetic must and wine (model solutions) in order to obtain a matrix as similar as possible to real wine samples. All working standard solutions were stored in the dark at 4 °C.

Table 1. Chemical characteristics of the compounds studied

Name	Chemical structure	Molecular weight	Molecular formula	K_{ow}[a]	ADI[a] (mg kg^{-1} b.w. daily)	Oral LD[a] (rats; mg kg^{-1})
Cyprodinil		225.3	$C_{14}H_{15}N_3$	4.0	0.04	2800
Fludioxonil		248.2	$C_{12}H_6F_2N_3O_2$	4.1	0.05	>2800

[a] From *The Pesticide Manual* (2000 version, by the British Crop Protection Council, Surrey, UK).

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Synthetic must and wine preparation

The synthetic must solution was prepared in ultrapure water as follows: 202 g L^{-1} D(+)-Glucose, 5 g L^{-1} L(+)-tartaric acid, 5 g L^{-1} potassium dihydrogen phosphate, 2 g L^{-1} magnesium sulfate and 2 g L^{-1} ammonium sulfate. The synthetic wine solution was prepared as follows: ethanol 12% (v/v) and 5 g L^{-1} of L(+)-tartaric acid. The must and wine samples were adjusted to pH = 3.6 with NaOH.

HPLC-DAD system and operating conditions

The separation, identification and quantification of the cyprodinil, fludioxonil and Switch compounds were performed using a Waters HPLC-DAD with two model 515 pumps, an autosampler model 717 plus and a diode-array detector model 996. The reversed-phase column used was a Kromasil C-18 (15 × 0.46 cm) column. The chromatographic conditions used were as follows: eluent A, water; eluent B, acetonitrile; flow rate, 1 mL min^{-1}; gradient, 35–60% B over the first 3.5 min and then was stable for 20 min. Injection volume was 50 µL. The chromatograms were performed at 280 nm and the spectra were recorded from 200 to 350 nm.

Extraction procedure

Extraction of the chosen fungicides from the different matrices which form the basis of this study was performed using the LLE method.

With the best conditions found for fungicide extraction a method was proposed, which has been validated and characterized for model must, must in fermentation and wine samples. The method used for the extraction of the two compounds, cyprodinil and fludioxonil, was as follows: 20 mL of sample (must, must/wine, wine) was placed in a 100 mL extraction funnel, 25 mL of dichloromethane was added and it was shaken for 15 min in orbital agitation. Once the extract was separated out, anhydrous sodium sulfate was added to dry possible remains from the aqueous phase and it was concentrated to dryness in a rotary evaporator. The dry extract was made up to 5 mL with methanol. Two injections were performed for each extract.

RESULTS AND DISCUSSION

Selectivity, precision, recovery and accuracy

Selectivity was checked by injecting extracts of spiked synthetic must and wine samples; it can be deduced from .Figures 1 and 2 that there are no interferences in the extracts of a synthetic must and wine. The proposed conditions generated narrow and reproducible chromatographic peaks. Noise was similar regardless of the matrix: must or wine.

Accuracy was determined as percent recovery. Recovery assays were performed with synthetic must and wine spiked with fungicides at two concentration levels, 1 and 5 mg L^{-1}, for cyprodinil and fludioxonil.

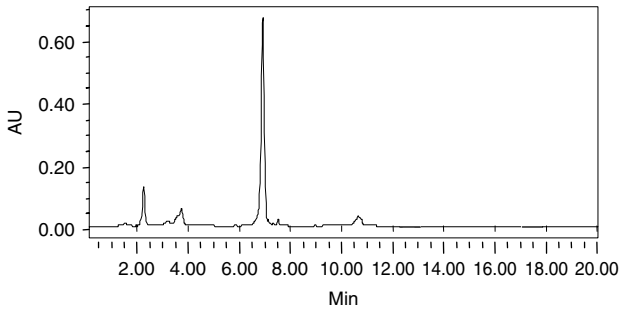

Figure 1. HPLC-DAD chromatogram obtained from non-spiked fermentation sample after LLE.

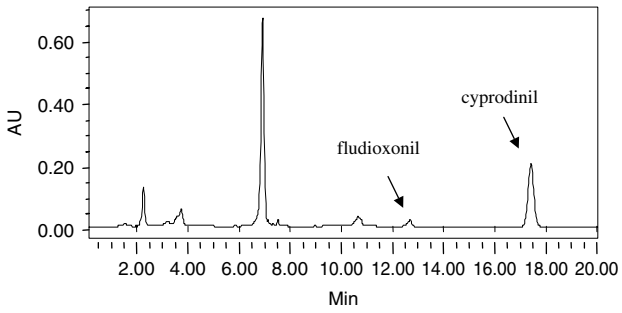

Figure 2. HPLC-DAD chromatogram obtained from fermentation sample spiked with cyprodinil (2 mg L^{-1}) and fludioxonil (1 mg L^{-1}) after LLE.

Table 2. Mean recoveries and precision (both as percentages) for concentrations of 1 and 5 mg L^{-1} after spiking synthetic must and wine samples

	Recovery (RSD, %)	
Pesticide	Must	Wine
Cyprodinil	97.0 (5.3)	99.5 (5.4)
Fludioxonil	97.7 (4.8)	98.8 (5.0)

RSD, relative standard deviation ($n = 6$).

Precision was evaluated in terms of repeatability using two different fortification levels. Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is expressed as an estimate of the relative standard deviation (RSD) of a statistically significant number of samples. Three replica tests were carried out at each concentration level, in accordance with the sample treatment previously developed for each compound. Results showed that the RSD values were lower than 10%, and between 4.8% and 5.4%. The average recoveries ranged from 97.0% to 99.5%. Table 2 shows the mean results obtained for the two types of matrix at the two concentration levels. Analysis of variance (ANOVA) revealed the absence of significant difference ($P < 0.05$) between the two types of sample or matrix for cyprodinil and fludioxonil, and between different concentrations.

Assessment of the matrix effect

The change in composition of the samples during alcoholic fermentation (in the transformation of the grape must into wine) makes it necessary to carry out a study of the possible matrix effect in the process of treating the sample. The possible effect of this change on LLE has been studied. The linear response of the method was studied in the two matrices. The similarity in the calibration curves in both matrices would indicate that there was no matrix effect in the extraction of the compounds. A Student's *t*-test was made to compare the slopes of the lines of regression (synthetic must and wine) obtained for each of the compounds studied, with a significance of 95%. The results showed that there were no significant differences between the slopes in synthetic must and wine for cyprodinil and fludioxonil. Thus it can be concluded that there is no matrix effect in the analysis of cyprodinil and fludioxonil in these matrices.

Calibration curves, limits of detection and limits of quantification

The analytical curves were obtained by spiking synthetic must and wine with seven different concentrations for each analyte, with three replicates. These samples were analyzed by the developed method. Linearity was estimated via linear regression analysis by the least-squares regression method.

The limit of detection (LOD) and limit of quantification (LOQ) parameters were determined by injecting a number of extracts of non-spiked must and wine samples ($n = 6$) and measuring the magnitude of the background analytical response. LOD and LOQ were estimated as the concentration obtained with the average value of noise plus three or ten times the standard deviation, respectively.[28]

Table 3 shows the analytical features of the method for the determination of the fungicides studied in synthetic must and wine. The limits of quantification were always below the maximum residue limits (MRLs).

Analysis of synthetic samples spiked with fungicides during fermentation

Fermentation tests were performed on sterile synthetic must to which different doses of fungicide (doses equal to or above MRLs, which are 2 and 1 mg L^{-1}, respectively, for cyprodinil and fludioxonil in grapes) were added and these were then fermented with the selected strain of yeast, *Saccharomyces cerevisiae*, a strain resistant to the products studied. The musts were spiked with cyprodinil and fludioxonil individually (2 and 4 mg L^{-1} for cyprodinil; 1 and 2 mg L^{-1} for fludioxonil) and Switch (10 mg L^{-1}). The target fungicides were added to different aliquots of 500 mL of synthetic must, sterilized in an autoclave. These must aliquots were inoculated with a yeast population of 10^4 in each flask, with two repetitions for each test.

In order to check the possible degradation of the fungicides in the synthetic must matrix itself, (an aqueous medium at pH 3.6) control samples were prepared in synthetic must which were not subjected to the fermentation process but did maintain the same conditions of pH and temperature: pH 3.6 and a controlled temperature of 28 °C. These control samples were spiked with the same concentration of the compounds as the fermentation samples.

Fermentation proceeded for 7 days. Each test flask was sampled regularly by taking two samples of 25 mL during the entire fermentation process (at 0 and then after 12, 24, 48, 72, 96 and 168 h from the start of fermentation). These samples were centrifuged at 3000 rpm at 3 °C so as to eliminate the remains of fermented yeast. All the tests were duplicated. The samples taken were analyzed according to the described method and optimized in this study. Alcoholic fermentation was controlled by measuring the sugar content.

Wines obtained from fermentations showed these main enological parameters: pH = 3.6, 11% alcohol (v/v), reducing sugars $<2\,g\,L^{-1}$ and tartaric acid 4.6 g L^{-1}. There was no significant difference in the fermentative processes of musts with fungicides compared with the controls.

Tables 4 and 5 reflect the amounts of each compound found both in the control must and in the musts fermented in the different concentrations studied.

The results obtained showed that the matrix must without fermentation (control solutions) caused a slight elimination of the compounds with a decrease in the concentration irrespective of the initial spiked concentration: 5–6% for cyprodinil whether individual or in the product, and 1–4% for fludioxonil in the individual fermentation and 34.7% in the Switch product. In Table 6 the synergic effect is shown for fludioxonil when together with cyprodinil in

Table 3. Analytical features of the method designed for analysis of fludioxonil and cyprodinil in synthetic must and wine

Compound	Matrix	Linear range ($\mu g\,L^{-1}$)	LOD ($\mu g\,L^{-1}$)	LOQ ($\mu g\,L^{-1}$)	Equation[a]	R^2
Cyprodinil	Must	53.6–20 000	42.9	53.6	$A = (256757.8 \pm 7493.9)C + (-8789.6 \pm 76435.3)$	0.996
	Wine	45.9–20 000	34.8	45.9	$A = (251561.2 \pm 10954.0)C + (6539.2 \pm 11727.0)$	0.990
Fludioxonil	Must	35.0–20 000	30.9	35.0	$A = (83978.3 \pm 2445.2)C + (2113.5 \pm 24940.0)$	0.996
	Wine	177.7–20 000	173.4	177.7	$A = (81817.5 \pm 4175.9)C + (-13705.3 \pm 42599.4)$	0.990

[a] *A*, area; *C*, concentration.

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Table 4. Evolution of cyprodinil and fludioxonil content in control and fermented (F) samples with their standard deviation ($n = 4$) for an initial concentration corresponding to the MRL of each compound of 2 mg L^{-1} and 1 mg L^{-1}, respectively: cyprodinil and fludioxonil individually

Time (h)	Cyprodinil (mg L^{-1})		Fludioxonil (mg L^{-1})	
	Control	F	Control	F
0	2.05 ± 0.02	2.05 ± 0.02	1.11 ± 0.05	1.11 ± 0.01
12	2.04 ± 0.03	1.99 ± 0.05	1.13 ± 0.04	1.11 ± 0.02
24	2.03 ± 0.03	2.01 ± 0.03	1.13 ± 0.03	1.06 ± 0.02
48	1.84 ± 0.01	1.92 ± 0.01	1.08 ± 0.06	0.64 ± 0.05
72	1.90 ± 0.07	1.87 ± 0.01	1.07 ± 0.06	0.68 ± 0.07
96	1.84 ± 0.05	1.94 ± 0.03	1.12 ± 0.02	0.56 ± 0.00
168	1.93 ± 0.07	1.69 ± 0.02	1.10 ± 0.01	0.30 ± 0.01

Table 5. Evolution of the cyprodinil and fludioxonil content in control and fermented (F) samples with their standard deviation ($n = 4$) for an initial concentration corresponding to 2 × MRL (4 mg L^{-1} and 2 mg L^{-1}, respectively): cyprodinil and fludioxonil individually

Time (h)	Cyprodinil (mg L^{-1})		Fludioxonil (mg L^{-1})	
	Control	F	Control	F
0	3.88 ± 0.17	3.88 ± 0.17	2.51 ± 0.06	2.51 ± 0.06
12	3.79 ± 0.09	3.79 ± 0.09	2.54 ± 0.04	2.79 ± 0.03
24	3.86 ± 0.01	3.93 ± 0.06	2.67 ± 0.01	2.18 ± 0.09
48	3.83 ± 0.04	3.74 ± 0.06	2.56 ± 0.04	1.40 ± 0.06
72	3.58 ± 0.04	3.21 ± 0.22	2.12 ± 0.17	0.44 ± 0.02
96	3.77 ± 0.13	3.43 ± 0.05	2.38 ± 0.09	0.21 ± 0.11
168	3.68 ± 0.06	3.14 ± 0.12	2.42 ± 0.07	0.19 ± 0.25

Table 6. Synergic effect on fludioxonil in the commercial Switch product in control and fermented (F) samples with their standard deviation ($n = 4$)

Time (h)	Cyprodinil (mg L^{-1})		Fludioxonil (mg L^{-1})	
	Control	F	Control	F
0	3.88 ± 0.02	3.88 ± 0.02	2.59 ± 0.10	2.59 ± 0.10
12	3.84 ± 0.01	3.96 ± 0.02	2.49 ± 0.08	2.27 ± 0.01
24	3.70 ± 0.02	3.57 ± 0.01	2.00 ± 0.07	1.35 ± 0.01
48	3.70 ± 0.01	3.50 ± 0.05	1.96 ± 0.06	1.00 ± 0.01
72	3.78 ± 0.05	3.39 ± 0.01	1.95 ± 0.06	0.67 ± 0.00
96	3.73 ± 0.01	3.22 ± 0.18	1.68 ± 0.17	0.60 ± 0.05
168	3.70 ± 0.02	2.83 ± 0.04	1.69 ± 0.17	0.38 ± 0.01

the commercially available Switch product, as the fludioxonil concentration presents a higher reduction.

For the individual compounds, the fermentative process causes a decrease in the concentrations, depending on the initial concentration. This decrease is slightly higher, the higher the initial concentrations: for cyprodinil between 17% and 19%, respectively, for concentrations of 2.0 and 4.0 mg L^{-1}; for fludioxonil between 73% and 92%, respectively, for concentrations of 1.0 and 2.0 mg L^{-1}. In fermentation with Switch, cyprodinil and fludioxonil decreased by 27% and 85%, respectively.

CONCLUSIONS

LLE is a suitable extraction method for the determination of new phytosanitary products which continue to appear on the market, with high extraction recoveries and values for the limits of detection and quantification which are well below the MRLs imposed by Spanish and European legislation.

Cyprodinil and fludioxonil fungicides do not reveal a matrix effect when the method designed for its determination in must and wine is applied.

The application of the fermentative process on cyprodinil and fludioxonil fungicides causes a greater decrease in the content of these compounds compared to those of the control samples without the appearance of any product in degradation and shows a greater decrease the higher the initial concentration.

In the study of the commercial Switch product a greater decrease can be seen in the concentration for fludioxonil, which shows a synergic effect.

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