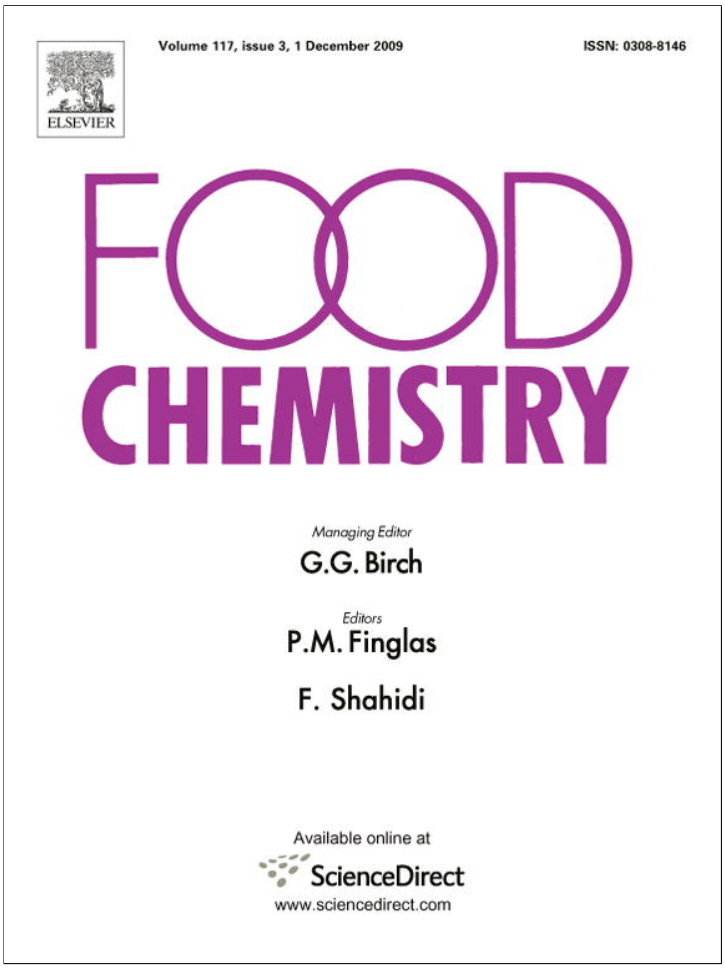

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Analytical Methods

Evolution of tebuconazole residues through the winemaking process of Mencía grapes

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ABSTRACT

The removal of tebuconazole residues during the winemaking process, applied initially to red must elaborated from Mencía variety grapes from A.O.C. Valdeorras (Ourense, N.W. Spain), was studied. Analytical determination of tebuconazole residues in grapes, musts and wines were perfomed by gas chromatography equipped with an ion trap mass spectrometry detector (GC–ITMS). Tebuconazole is retained on the solid matter (cakes and lees) and clarification agent. The eliminated percentage of tebuconazole in the final wine is 86%. The influence of this fungicide on the fermentative activity of *Saccharomyces cerevisiae* yeast and the *Oenococcus oeni* was also studied through *in vitro* assays. Liquid chromatography equipped with triple quadrupole mass spectrometer (LC–MS/MS) was used to determine tebuconazole residues in synthetic must and wine used for *in vitro* assays. No effect on the alcoholic or malolactic fermentation was observed; and no degradation of tebuconazole originated by these microorganisms was registered.

1. Introduction

Grey mold (*Botrytis cinerea*), powdery mildew (*Uncicula necator*) and downy mildew (*Plasmopara viticola*) are the most common fungi encountered in vineyards control (Sala et al., 1996). Fungicides are widely used in the treatment of diseases of grapes for vinification. Tebuconazole is a fungicide widely used in Galicia (N.W. Spain), an important vineyard area which produces white and red wines protected by five Appellation d'Origine (A.O.C) – Rías Baixas, Ribeiro, Valdeorras, Monterrei and Ribeira Sacra.

Although the correct use of fungicides does not cause problems of public concern in health and environmental areas, if inappropriate abusive treatments are applied without respecting safety recommendations, undesirable residues can remain on grapes after harvest and they can be transferred to the wine. Luckily, the winemaking process and the oenological steps carried out contribute to their reduction (Cabras & Angioni, 2000; Cabras et al., 1997, 1999; De Melo et al., 2006; Jiménez, Bernal, del Nozal, Bernal, & Toribio, 2007; Navarro, Barba, Oliva, Navarro, & Pardo, 1999; Navarro et al., 2000; Oliva, Payá, Cámara, & Barba, 2007; Sala et al., 1996). However, several studies are focused on the connection between fungicide residues and stuck and sluggish alcoholic and malolactic fermentations (Cabras & Angioni, 2000; Cabras et al., 1999, 2000; Ruediger, Pardon, Sas, Godden, & Pollnitz, 2005) and therefore the negative effect on the aromatic composition of wines (García et al., 2004; Oliva, Navarro, Barba, Navarro, & Salinas, 1999). As a conclusion, to decrease the risk to the consumer's health and to increase the quality of wine is necessary to know the evolution of fungicide residues and their degradation products during the winemaking process.

No studies have reported the fate of fungicides in Galicia. The first aim of this study is to report the tebuconazole residues through the winemaking process considering red grapes, *Vitis vinifera* var. Mencía, produced in A.O.C Valdeorras (Ourense, N.W. Spain). The second aim is to evaluate, through *in vitro* experiments, the effect of tebuconazole on the behaviour of alcoholic fermentation by *Saccharomyces cerevisiae* yeast and malolactic fermentation by *Oenococcus oeni* lactic bacteria, as well as to determine if yeast and/or bacteria could produce a decrease on tebuconazole pesticide residues in wine.

2. Materials and methods

2.1. Vinification experiments

Red grapes, *Vitis vinifera* var. Mencía, produced in A.O.C. Valdeorras were harvested in September 2007. Two vinification experiments (A and B), by duplicate, were performed at the experimental cellar belonging to Consejo Regulador from A.O.C. Valdeorras. Experiment A, used as a vinification control, consisted of performing the vinification processing with uncontaminated

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crushed and destemmed Mencía grapes. For experiment B, crushed and destemmed grapes were spiked with a pure standard of tebuconazole at level of 3.3 mg/kg. The winemaking process with maceration was identical for all experiments. The selected contamination level is corresponding to around 1.5 maximum residue limit (MRL, 2.0 mg/kg) from tebuconazole established by European legislation to control pesticide levels in grapes from vinification (Commission Regulation, 2008).

2.1.1. Winemaking process

Intact bunches of freshly harvested grapes were weighed into four replicates of 40 kg. Winemaking process, itemised in Fig. 1, was identical for all vinification experiments, as it was commented above. Briefly, each grape sample were crushed and destemmed, introduced into independent metallic vessels of fermentation and supplied with SO_2 (40 mg/L) and the fungicide (only for experiment B). After 24 h, commercial yeast (*S. cerevisiae*) was added. During alcoholic fermentation-maceration (conducted at temperatures below 18–20 °C during 10–11 days), the mixture was re-pressed two times a day, and the temperature and density values were measured in all containers. At the end of the process the wine was strained off, grapes were pressed and the wine-must mixture transferred again to metallic vessels for the malolactic fermentation where commercial lactic bacteria (*O. oeni*) was added). At the end of malolactic fermentation, the wine was racked, supplied with SO_2 and clarified with fresh egg albumin. Finally, wine was bottled.

2.1.2. Sampling

For studying the effect of the different winemaking steps in elimination of tebuconazole, the following samplings (summarized in Fig. 1) were carried out for each vessel such it follows: (1) (crashed grape samples before to start alcoholic fermentation); (2) (wine and pomace samples collected at the end of alcoholic fermentation); (3) (wine samples at the end of malolactic fermentation after racking); and finally, (4) (bottled wine samples after clarification process).

2.2. Chemicals

2.2.1. Tebuconazole extraction and analysis

Tebuconazole [(RS)-1-p-chlorophenyl-4,4-dimethyl-3-(1H-1,2,4-triazol-1-ylmethyl)pentan-3-ol], of certified purity > 98%, was obtained from Riedel-de-Haën (Seelze, Germany). Tebuconazole D6 (100 mg/L, in acetone) from Dr. Ehrenstorfer, was used as internal standard (IS) to correct for variability in chromatographic injection and mass spectrometric detection response. The 3-ethoxy-1,2-propanediol (98%), D-sorbitol (>99%) and L-gulonic acid γ-lactone (>98%), used as analyte protectants, were obtained from Aldrich (Steinheim, Germany). Solvents (residue analysis grade) were acetone, acetonitrile, formic acid (85%), ultra-pure water (Panreac, Barcelona, Spain), dichloromethane (Merck, Darmstadt, Germany), methanol and toluene (Scharlau, Barcelona, Spain). Sodium chloride, anhydrous sodium sulfate and ammonium formiate were purchased from Panreac. Standard solutions of fungicides and analyte protectants were prepared according to a previous work (González-Rodríguez, Rial-Otero, Cancho-Grande, & Simal-Gándara, 2008a).

2.2.2. Assays of in vitro fermentations

Ingredients and culture media (peptone, yeast extract, agar and MRS broth) were obtained from Cultimed (Barcelona, Spain). Glucose PA–ACS, ethanol 96% PA–ACS, glycerol PA–ACS–ISO, malic acid PRS–CODEX, NaCl PRS–CODEX, $CaCl_2$ PA–ACS, H_2SO_4 95–98% PRS–CODEX, NaOH PA–ACS–ISO, 3,5-dinitrosalicylic acid PB and Na–K-tartrate PA–ACS–ISO were obtained from Panreac (Barcelona, Spain).

2.3. Materials and small apparatus

The sorbent material used for solid-phase extraction was Supelclean Envi-Carb II/PSA, 6 mL size (Supelco Corp., Bellefonte, PA, USA). For solid–liquid and liquid–liquid extractions, samples were placed in 50 mL polypropylene screw-capped centrifuge tubes (Sterilin, Staffordshire, UK). Samples initially were vigorously homogenised in an ultrasounds bath (Ultrasons-H, J.P. Selecta, Barcelona, Spain). Centrifugation was performed in a Rotina 35R centrifuge (Hettich Zentrifugen, Tuttlingen, Germany). Organic extracts were evaporated under a stream of nitrogen in Turbo Vap LV evaporator (Caliper Life Sciences, Hopkinton, MA, USA). *In vitro* fermentation assays without agitation were performed in a refrigerated incubator FOC 225E (Velp Scientifica, Milano, Italy). Agitated incubations were done in a thermostatized orbital shaker (Unitron incubator, Infors, Bottmingen, Switzerland). For absorbance measurements a 6505 UV/VIS spectrophotometer (Jenway, Essex, England) was used.

2.4. Extraction procedure

For the extraction of tebuconazole residues in grapes, musts, pomaces and wines, a fungicide multiresidue method, previously developed in our laboratory, was used (Rial-Otero, Cancho-Grande, & Simal-Gándara, 2003); some modifications were performed to improve the purification process and to reduce the matrix contribution (González-Rodríguez et al., 2008a). Briefly, crushed grapes (5 g), must and wine (5 mL) samples were weighed inside a 50 mL polypropylene screw-capped centrifuge tubes. A volume (25 mL) of dichloromethane–acetone (75:25, v/v) was added and the container was vigorously homogenised in an ultrasounds bath for 10 min. Sodium chloride (3 g) and anhydrous sodium sulphate (12 g) were added followed by vigorous shaking for 5 min. After phase partitioning (10 min, 4000 rpm) in a centrifuge, an aliquot of 15 mL of the organic layer was transferred to a 40 mL glass vial and evaporated to dryness under a stream of nitrogen in Turbo Vap LV evaporator and the residue was redissolved in acetonitrile (2 mL). For clean-up, a multi-layer Supelclean Envi Carb-II/PSA SPE cartridge was conditioned with 5 ml of acetonitrile:toluene (3:1, v/v). Acetonitrile extract was loaded and the retained pesticide was eluted weakly, in a 40 mL glass vial, with a volume of 20 ml of acetonitrile:toluene (3:1, v/v). The eluate was evaporated till dryness under a stream of nitrogen and filled up to a final volume of 2 mL with acetone containing the three analyte protectants (3-ethoxy-1,2-propanediol at 10 g/L; and D-sorbitol and L-gulonic acid γ-lactone at 1 g/L; respectively). Use of analyte protectants mixture provided the best results in terms of effective compensation for matrix-induced enhancement effect. Tebuconazole D6 (0.1 mg/L) as internal standard was also added and the acetone extract was finally homogenised by vortex shaking. An aliquot of the final acetone extract was placed via 350 μL glass inserts into 2 mL vials prior to chromatographic analysis.

2.5. Tebuconazole analysis

Residues of this fungicide were determined by gas chromatography-ion trap mass spectrometry (GC–ITMS) in grapes, musts and wines. Liquid chromatography equipped with triple quadrupole mass spectrometer (LC–MS/MS) was instead used to determine tebuconazole residues in synthetic must and wine used for *in vitro* assays.

2.5.1. GC–ITMS analysis

GC analyses were carried out on a Trace GC Thermo Finnigan gas chromatograph (Rodano, Italy) equipped with a PolarisQ ion trap mass selective detector (ITMS), interfaced to a PC computer running the software Xcalibur 1.4, from Thermo Electron Corpora-

tion (Italy). Chromatographic separations were done by using a SPB-5 fused-silica capillary column (30 m × 0.25 mm ID, 0.25 μm film thickness) from Supelco. PTV was used for the 2 μL injection volume into a silcosteel liner (120 × 2 mm id). The temperature programming of the PTV was 85 °C for 0.3 min; 600 °C/min to 270 °C and hold for 2 min; 840 °C/min to 300 °C for 5 min. The carrier gas, helium, operates at 100 kPa in constant pressure mode. Splitless mode was selected for 45 s. The oven temperature was programmed as follows: 80 °C for 3 min; 15 °C/min ramp to 270 °C for 5 min. The transfer line temperature was 270 °C, and the ion-trap manifold temperature was 250 °C. The ion energy for electron impact (EI) was always 70 eV. Mass detection was performed in full scan mode to identify possible degradation products of tebuconazole and in single ion monitoring (SIM) mode to measured tebuconazole residues in samples. The selected ions (m/z) were 163 and 250 for tebuconazole, and 256 for internal standard (tebuconazole D6).

2.5.2. LC–MS/MS analysis

LC analyses were performed with a Thermo Separation Products system equipped with a vacuum membrane degasser, a Spectra Series P200 quaternary pump, an AS1000 autosampler, a column heater and a SN4000 system controller. The LC system was coupled on-line to a TSQ Quantum Access triple quadrupole mass spectrometer (TQMS) (Thermo Fisher Scientific) equipped with an electrospray ionisation (ESI) source and operated in the positive ion mode. The analytical column was a Luna C_8 (150 × 2 mm ID, 5 μm particle size), coupled with a C_8 Security Guard cartridge system (4 × 2 mm ID, 5 μm particle size), both from Phenomenex (Torrance, CA, USA). An aliquot (5 μL) was injected into the column and eluted at 45 °C, with a constant flow-rate of 0.25 mL/min, using as solvents 5 mM ammonium formiate in water (solvent A), 5 mM ammonium formiate in methanol (solvent B) and 0.5% formic acid (solvent C). Elution was performed with a gradient of 35% B (0–2 min), 35–95% B (2–4 min), 95% B (4–9 min), 95–35% B (9–9.1 min) and 35% B (9.1–22 min), using a constant 5% C along all gradient program. A divert valve was placed between the analytical column outlet and the mass spectrometer inlet, and the flow was diverted to waste during the first 5.9 min and the last 8.5 min of the chromatographic run. The MS conditions were as follows: spray voltage 4000 V; capillary temperature 350 °C; sheath gas pressure (N_2) 20 units; auxiliary gas pressure (N_2) 5 units; collision gas (Ar) 1.5 mTorr; and scan time 0.5 s. The mass spectrometer was operated in full scan mode to identify possible degradation products of tebuconazole and in selected reaction monitoring (SRM) mode to quantify tebuconazole residues. The parent mass, the product mass and the collision energy selected were 308 and 314, 70 and 72, and 22 and 30, for tebuconazole and for internal standard, respectively. For instrument control, data acquisition and processing, Xcalibur software version 2.1.0 (Thermo Electron Corporation) was used.

2.6. In vitro assays. Inoculation and fermentation

2.6.1. Microorganisms

2.6.1.1. (A) Yeast inoculation. The commercial dry yeast used was Fermol Arôme Plus (*S. cerevisiae* var. *cerevisiae*) from AEB Group (Brescia, Italy). Before using it as inoculum, the yeast was purified to eliminate unknown coadyuvants that could make difficult the analysis of possible tebuconazole degradation products. It was done by rehydrating the commercial powder as described by the manufacturer, and inoculating it on YPD-agar plates, which were incubated without agitation at 30 °C for 24 h and kept at 5 °C until use. Inoculum was prepared by transferring cells from a sole colony in the plates to YPD liquid broth placed in an Erlenmeyer flask, and incubating it in an orbital shaker (150 rpm, 30 °C). The exponentially growing cells were separated from the supernatant by centrifugation (5,000 rpm/15 min), and washed twice and suspended in an enough volume of sterile water to reach a cell concentration of 2.5 g/L (measured as turbidity in a spectrophotometer at 750 nm).

2.6.1.2. (B) Bacteria inoculation. The commercial freeze dried lactic bacteria used was Uvaferm Alpha (*O. oeni*) from Lallemand S.A. (Saint Simon, France). The bacterium was also purified in the same way as the yeast substituting YPD by MRS (Liofilchem, Roseto degli Abruzzi, Italy) media. Precultures were prepared inoculating MRS liquid broth with a sole colony form the MRS-agar plates, and incubating it without agitation (30 °C) in consecutive batches with increasing ethanol concentrations (0, 10, 40 and 80 g/L) to adapt the microorganism to the alcohol. Cells from exponential cultures with 80 g/L ethanol were then washed twice and suspended in 0.8% of NaCl. The determination of biomass concentration in concentrated inoculum for *in vitro* malolactic fermentation experiments was carried out by optical density measurement at 600 nm ($1x10^9$ cells/mL) (Guilloux-Benatier, Remize, Gal, Guzzo, & Alexandre, 2006).

2.6.2. Alcoholic fermentation assays

Three series of experiments were carried out: in series A′ *S. cerevisiae* was cultured in presence of tebuconazole, in series B′ the yeast grew without the pesticide, and series C′ was a control of the tebuconazole stability in the culture medium. A simple culture medium reproducing the main must characteristics was elaborated with 200 g/L glucose, 7 g/L peptone and 3.5 g/L yeast extract, and sterilized by filtration through 0.22 μm membrane filters (Millipore, Bedford, MA, USA). Part of the culture medium (experiments A′ and C′) was supplemented with a tebuconazole concentrated standard solution in ethanol (400 ppm) to give a final concentration of 2 mg/L in the medium, and the rest (for experiment B′) was supplemented with an equivalent volume of ethanol (which corresponded to a 0.5% of the total volume). These liquid culture media were distributed into 49 mL replications in 100 mL erlenmeyer flasks (12 replications for each experiment A′, B′ and C′). Erlenmeyer flasks of experiments A′ and B′ were inoculated with 1 mL of the yeast suspension, and 1 mL of sterile water was added to the flasks of experiment C′. All flasks were incubated for 13 days in an orbital shaker at 20 °C and 75 rpm. Two samplings of each experiment consisting in the whole culture of a flask were carried out at 0, 3, 4, 6, 9 and 13 days after inoculation. The following analyses were made: biomass, reducing sugars, fermentation metabolites (ethanol and glycerol) and tebuconazole (free and adsorbed to yeast cells). A 15 mL aliquot of the culture medium (in experiments A′ and B′) was centrifuged at 4000 rpm for 20 min. The supernatants were stored at -20 °C for reducing sugars and fermentation metabolites analysis, and the biomass was washed twice to measure the biomass concentration by dry weight. Reducing sugars were assayed by the 3,5-dinitrosalicylic acid (DNS) method (Bernfeld, 1951) with glucose as standard, and ethanol and glycerol were measured by a high-performance liquid chromatograph equipped with a RI-150 Spectra System refractive index detector (Thermo Separation Products) and a column model ION-300 (Transgenomic, San Jose, CA) eluted with 0.0085 M H_2SO_4 at 35 °C and a flow rate of 0.4 mL/min during 40 min. On the other hand, a 30 mL aliquot of the culture medium (experiments A′ and C′) was centrifuged to quantify free tebuconazole and tebuconazole degradation products in the supernatant, while the biomass was washed twice with water, re-suspended in 0.5 M $CaCl_2$, incubated in an orbital shaker for 60 min at 200 rpm to extract the fungicide residues adsorbed on yeast cells, and centrifuged to obtain the supernatant for quantifying the adsorbed fungicide, according to Zadra and co-workers (Zadra, Cardinali, Corte, Fatichenti, &

Marucchini, 2006). Free and adsorbed tebuconazole was directly analysed in LC–TQMS following the instrumental conditions described before. Quantification was performed by external calibration using standards made in their correspondent matrix ($CaCl_2$ and YPD broth medium).

2.6.3. Malolactic fermentation assays

Three series (A″, B″ and C″) were again performed as for *Saccharomyces* fermentation assays, just substituting the yeast by the bacterium in series A″ and B″, reducing the concentration of tebuconazole from 2 to 0.4 mg/L in series A″ and C″, and changing the medium, which consisted on a synthetic wine (pH = 4.9) containing 1 g/L glucose, 7 g/L peptone, 3.5 g/L yeast extract, 5 g/L malic acid and 80 g/L ethanol, sterilized by filtration through 0.22 μm membrane filters and supplemented with tebuconazole as described. These liquid culture media were distributed into 245 mL replications in 500 mL glass bottles (2 replications for each experiment A″ and B″, and 1 replication for experiment C″). Bottles of experiments A″ and B″ were inoculated with 5 mL of the bacteria inoculum, and 5 mL of sterile 0.8% NaCl was added to the bottle of experiment C″. All bottles were closed and incubated without agitation at 25 °C under anaerobic conditions for 31 days. An aliquot of 5 mL from each bottle was taken each 2–3 days. Firstly, biomass was measured by absorbance readings at 600 nm (using the synthetic wine as a blank). The samples were then centrifuged at 4000 rpm for 20 min, and the supernatants were stored at −20 °C for fermentation metabolites (malic and lactic acids) and free tebuconazole and tebuconazole degradation products analysis. Malic and lactic acids were measured by HPLC-RI following the same analytical conditions of ethanol and glycerol analysis described above, and free tebuconazole was directly analysed in LC–TQMS (synthetic wine was used as matrix to make the calibration standards).

3. Results and discussion

3.1. Analytical performance

Determination of pesticide residues in food matrices may be adversely affected by a phenomenon commonly known as the "matrix-induced chromatographic response enhancement effect", contracted to matrix-induced response enhancement, which was first described by Erney and co-workers (Erney, Guillespie, Gilvydis, & Poole, 1993). When pesticides are in absence of matrix during the injection step, poor peaks with low response resulted for analytes susceptible to be degraded and/or absorbed on the GC injector; however, peak intensity and shape of affected compounds improved when they are injected in the presence of a complex matrix (carbohydrates, proteins and/or lipids). This effect is presumably the most discussed matrix effect negatively impacting quantitation accuracy of certain analytes in GC (Hajšlová & Zrostlíková, 2003). The use of analyte protectants, which are compounds that interact with active sites in the GC system, decreases degradation and/or adsorption of co-injected analytes (Anastassiades, Maštovská, & Lehotay, 2003; Maštovská, Lehotay, & Anastassiades, 2005). The mixture of these three analyte protectants (3-ethoxy-1,2-propanediol at 10 g/L; D-sorbitol and L-gulonic acid γ-lactone at 1 g/L; respectively) was previously adopted in the determination of pesticides in leafy vegetables (González-Rodríguez et al., 2008a; González-Rodríguez, Rial-Otero, Cancho-Grande, & Simal-Gándara, 2008b) and the use of fortified blank samples as calibration standards was avoided. In this case, the use of this mixture was also adopted to evaluate the compensation of this effect in the determination of tebuconazole residues in grapes, musts and wines samples.

The performance of the SLE/SPE/GC–ITMS and SLE/SPE/LC–TQMS methods for determining tebuconazole in grapes, musts and wines was assessed by evaluating quality parameters (such as linearity range, precision, limits of detection and quantitation), which experimental values are summarized in Table 1, such it was described as follows. Uncontaminated Mencía crushed grapes, musts and red wine previously fortified for tebuconazole at different levels (ranging from 0.1 to 1.0 mg/kg or mg/L for GC–ITMS/ranging from 0.01 to 0.1 mg/kg or mg/L for LC–TQMS) were treated following the experimental conditions described above. Linearity of each method was established by plotting the relative area of tebuconazole to the tebuconazole D6 (A/A_{is}), versus the concentration of the fungicide (C_i). Acceptable linearity was obtained for each matrix evaluated with a correlation coefficient > 0.99 (Table 1). Absolute recovery value for the whole calibration line was obtained from the ratio between the slope of the calibration line corresponding to the spiked sample and the slope of the calibration line corresponding to the fungicide standards injected directly onto the analytical column (standards ranging from 0.15 to 1.5 mg/L for GC–ITMS and ranging from 0.015 to 0.15 mg/L for LC–TQMS, in acetone, containing the same concentration of internal standard and analyte protectants). Absolute recovery values ranged from 77 to 92%. One-way analysis of variance between mean recovery values obtained with both chromatographic techniques (GC–ITMS and LC–TQMS) for each type of sample (grapes, musts and wines) were performed. The p-values of the F-test (95% probability) in the ANOVA was greater than 0.05 for each type of sample evaluated; then, there is not a statistically significant difference between the mean recoveries for each type of sample obtained with both techniques at the 95.0% confidence level. Moreover, the use of analyte protectants for both chromatographic techniques eliminated practically the differences between calibrations obtained in different samples versus matrix-free solutions. Another aspect to comment is that recoveries in must and wine samples were higher than values obtained in grapes; this fact could be due to the presence of solid particles in grapes (as skins or nuggets), where tebuconazole could be adsorbed and then, the extraction efficiency reduced. Precision of the SLE-SPE procedure, were calculated by analysing, on the same day, five replicates of each type of sample (grape, must and wine) fortified at concentration of 0.4 mg/kg or mg/L for GC–ITMS and 0.05 mg/kg or mg/L for LC–TQMS; precision was expressed as relative standard deviation (RSD%), which was lower than 7% for the three samples evaluated and injected with both chromatographic techniques. Limits of detection (LOD) and quantitation (LOQ) were evaluated following the recommendations of the American Chemical Society (American Chemical Society, 1980); they were calculated from the signal-to-noise ratios obtained by analysing unspiked samples ($n = 7$); thus LOD and LOQ were taken to be the concentrations of tebuconazole resulting in a signal-to-noise ratio of three and ten, respectively. Under these conditions, LOD and LOQ obtained by LC–TQMS were eight times lower than LOD and LOQ obtained by GC–ITMS. For both cases, the SLE/SPE procedure followed of the chromatographic techniques allowed to determine residue levels 40 (for GC–ITMS) and 250 times (for LC–TQMS) than those established in European legislation (Commission Regulation, 2008).

3.2. Decrease of tebuconazole residues during Mencía winemaking process

Red grapes, *Vitis vinifera* var. Mencía, were previously analysed to guarantee the absence of tebuconazole before beginning the vinification experiments. When red grapes were crushed and destemmed, a pure standard of tebuconazole was added at 3.3 mg/kg only in vessels corresponding to experiments B; this contamination level is 1.6 times higher than European maximum residue level (2.0 mg/kg) established to control pesticide levels in grapes for

Table 1
Performance of the optimised method for the determination of tebuconazole in grape, must and wine.

Sample	GC–ITMS					LC–TQMS				
	Linearity[a] (r^2)	Recovery (%)	RSD[b] (%)	LOD[c]	LOQ[c]	Linearity[d] (r^2)	Recovery (%)	RSD[b] (%)	LOD[c]	LOQ[c]
Grape	0.991	83	5	0.028	0.050	0.993	77	2	0.004	0.008
Must	0.991	88	7	0.023	0.041	0.990	83	6	0.003	0.006
Wine	0.991	87	6	0.024	0.042	0.994	92	7	0.003	0.006

[a] Linear range: 0.1–1.0 mg/kg or mg/L ($n = 7$).
[b] Precision, expressed as relative standard deviation (RSD), $n = 5$.
[c] LOD and LOQ, expressed as mg/kg for grape and as mg/L for must and wine, $n = 7$.
[d] Linear range: 0.01–0.1 mg/kg or mg/L ($n = 7$).

vinification (Commission Regulation, 2008). We opted for this contamination level to identify possible new compounds originated by degradation of the initial tebuconazole; moreover, we opted for addition of tebuconazole as pure standard instead of a commercial formulation to eliminate interferences associated to other substances such as additives and/or excipients.

One day after the addition of tebuconazole standard, necessary to provide the liquid–solid partitioning in experiment B, the winemaking process started. Sampling followed in experiments A and B are described in Fig. 1. Residual concentrations of tebuconazole found in the different control stages of vinification experiment B, by duplicate, are shown in Table 2. No tebuconazole residues were detected in the duplicates of vinification experiment A.

Comparing the alcoholic fermentation course in experiments A and B, followed by the evolution of must density, it can be concluded that both fermentation had a regular course (10–15 days). This seems to indicate that this yeast is not negatively affected by tebuconazole in the vinification conditions here assayed, as also reported by other authors with this microorganism and other fungicides like iprodione and fludioxonil (Ochiai et al., 2002) and fenhexamid (Cabras et al., 2001). On the contrary, the maceration-alcoholic fermentation stage was the most influential one in the

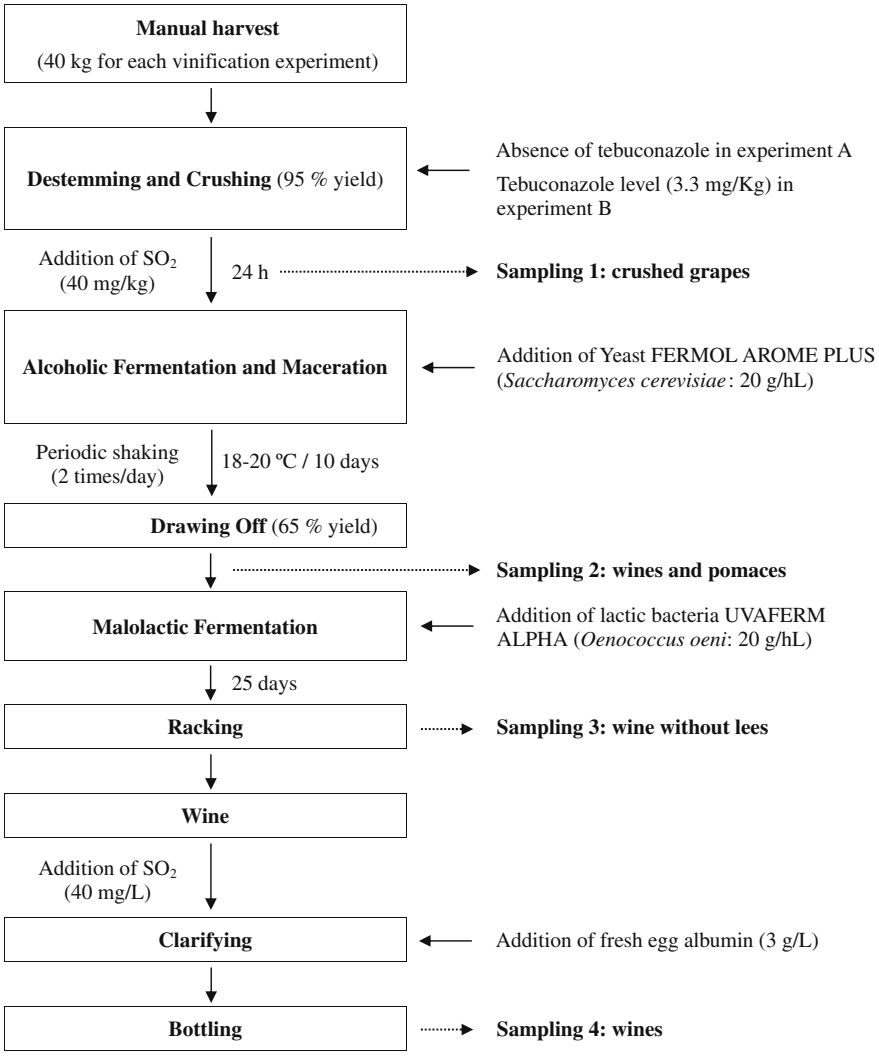

Fig. 1. Flowsheet and sampling for Mencía wine vinification.

Table 2
Average (experiment A) of residual concentrations of tebuconazole ($n = 2$) found in the different control stages of vinification (mg/kg or mg/L ± SD) by GC–ITMS.

Sample 1	Sample 2		Sample 3	Sample 4
Crashed grapes	Must (65%)	Pomace (35%)	Racked wine	Clarified wine
3.49 ± 0.57	0.64 ± 0.15	6.12 ± 0.49	0.59 ± 0.03	0.45 ± 0.01

levels of tebuconazole present in the wine and the pomace. From the initial enrichment of the must to the end of the alcoholic fermentation-maceration, the fungicide was distributed between the fermenting must (the liquid phase) and the pomace (the solid phase). Tebuconazole did not remained completely dissolved in the liquid phase due to its low water solubility (0.032 g/L), unlike other fungicides studied by other authors like metalaxyl (7 g/L) which 64% remains dissolved (Navarro et al., 2000). The remaining amount in the wine was only around 12% of the total amount in comparison to the 88% present in pomace samples (see Table 2); it shows the high affinity of this compound to be adsorbed on the suspended solid matter. For other fungicides, such as quinoxyfen, myclobutanil and tetraconazole (another fungicide of the triazole family), no determinable residues were found at the end of the alcoholic fermentation (Cabras & Angioni, 2000; Cabras et al., 2000).

Malolactic fermentation, followed by thin layer chromatography, had a regular course in all vessels too, also showing the resistance of the bacteria used to the tebuconazole concentration remaining in the wine after the first stage of alcoholic fermentation (0.6 mg/L). Although studies on lactic acidic bacteria inhibition by pesticides (Bordons, Masqué, & Vidal, 1998; Cabras et al., 1994; Haag, Kreiger, & Hammes, 1988) report minimum inhibitory concentrations for various pesticides ranging from as low as 1 to > 30 mg/L, in most cases pesticide residues were found to have little or no effect on malolactic fermentation. Recently, Ruediger and co-workers (Ruediger et al., 2005) evaluated the effect of red wine malolactic fermentation on the fate of seven fungicides (carbendazim, chlorothalonil, fenarimol, metalaxyl, oxadixyl, procymidone and triadimenol); only dicofol has an inhibitory effect on the catabolism of malic acid. On the other hand, the malolactic fermentation stage resulted in no significant reduction on tebuconazole levels (around 0.6 mg/L). Published studies showed that a minimum number of pesticides were degraded or adsorbed by the bacteria during this process (Cabras et al., 1999, 2000; Navarro et al., 2000; Ruediger et al., 2005).

Clarification was performed with fresh egg albumin; it is a clarifying agent used in high prestige red wines. It removes a large number of phenols and mellows wines with a high content in astringent tannins. Compared to other clarifying agents, it offers the advantage of not modifying the sensory qualities (Oliva et al., 2007). Clarification as well as filtration allows achieving the clearness demanded of wines but they also lead to remove other exogenous substances such as pesticide residues. In this case, tebuconazole residues decreased around 25% in this stage with regard to racked wine due to this fungicide was also adsorbed in lees formed along the clarification (see Table 2). Similar results were obtained by Oliva and co-workers (Fernández, Oliva, Barba, & Cámara, 2005; Oliva et al., 2007) who studied the effects of clarification and filtration processes on the removal of other fungicides in the winemaking process of red wine elaborated from Monastrell variety grapes. Fresh egg albumin presented the highest elimination, in the clarified wine, for famoxadone, flunquiconazole and trifloxystrobin (50% for the three target fungicides) (Oliva et al., 2007); and for the blood albumin was observed highest elimination for cyprodinil, pyrimethanil (70%) and fludioxonil, quinoxyfen (30–40%).

At the end of the winemaking process, the residue remaining in the final wine was lower than in grapes, presenting a mean transfer factor from grape to wine of 0.14. These results are in accordance with those published for other fungicides of the triazole family in red grapes winemaking process. As example, penconazole and propiconazole decreased by as much as 90% of the initial value in red wines (Navarro et al., 1999); levels of tetraconazole fell by 100% of the initial value found in grapes (Cabras et al., 1998) and the loss of tebuconazole in the final red wine ranged from 50% to 73% (Cabras et al., 1997; Jiménez, Bernal, del Nozal, Toribio, & Bernal, 2004). For white and rosé wines, the reduction of the contamination levels for tebuconazole is lower as a consequence of the absence of the malolactic fermentation and the minor presence of the matter in suspension that can adsorb the tebuconazole; Cabras et al. (1997) established that tebuconazole residues in wine were ca. 50% that levels found in grapes.

Some fungicides, which are unstable in an acid environment, started to degrade after pressing, and at the end of fermentation new related compounds were present in wines. The results here presented also appear to show the absence of tebuconazole degradation processes in this system since no degradation products could be detected. Jiménez and co-workers (Jiménez et al., 2004) determined the degradation products of bromopropylate, trichlorphon, parathion-methyl and tebuconazole, formed during the winemaking process; degradation products were only identified for the three initial fungicides meanwhile tebuconazole, remained intact. It is necessary to make *in vitro* assays to confirm if tebuconazole is degraded and/or their degradation products were also adsorbed on solid matter.

As a conclusion, tebuconazole showed a continuous decrease through the red winemaking process. Nowadays any European legislation to control maximum residue levels of pesticides in wine exists. In future, according these results, MRL for tebuconazole in red wines could be established as a eight times lower (0.25 mg/L) than MRL established in wine grapes (2 mg/kg).

3.3. Evaluation of in vitro assays

Although the previous winemaking experiments allowed to discard negative effects of tebuconazole on the alcoholic and malolactic fermentation at the concentration assayed, the presence in the must of the solids coming from the grapes during the maceration–alcoholic fermentation stage makes difficult to analyse the real effect of the fungicide on yeast grown and fermenting activity since solids reduce the concentration of soluble tebuconazole by adsorption and interfere in yeast biomass determination. On the other hand, the complex composition of must and wine makes also difficult to detect, identify and quantify possible tebuconazole degradation products produced by the yeast and the bacteria.

Consequently, *in vitro* alcoholic and malolactic fermentation assays were carried out employing liquid synthetic media with a simpler composition than must and wine, but reproducing their main characteristics (sugars, ethanol and malic acid concentration, and pH) and enriched in tebuconazole to evaluate the effect of the fungicide on the main fermentation parameters.

3.3.1. Effect of tebuconazole on alcoholic fermentation

To evaluate whether the presence of tebuconazole can negatively affect yeast growth and alcoholic fermentation of the must by *S. cerevisiae*, experiments in the synthetic medium described in Materials and Methods were carried out using the maximum residue limit of tebuconazole in grapes for vinification (MRL, 2.0 mg/kg). Evolution of sugars consumption, yeast growth and ethanol production, with and without presence of tebuconazole residues, is shown in Fig. 2.

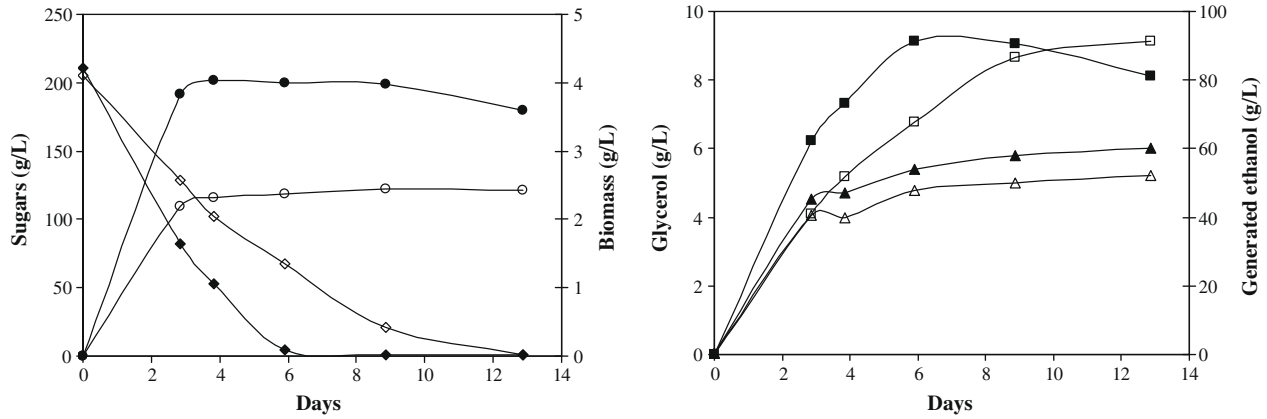

Fig. 2. Time course of sugars consumption (◇, ♦) and biomass (○, ●), glycerol (△, ▲), and ethanol (□, ■) production during alcoholic fermentation of a synthetic medium with *Saccharomyces cerevisiae*, in presence and absence of tebuconazole residues, respectively.

The presence of tebuconazole in the culture medium at a concentration of 2 mg/L caused a decrease of the sugar consumption rate and, consequently, a slower growth and ethanol production rate. Moreover, the maximum biomass reached was clearly lower in the series incubated with tebuconazole, all of that showing the toxic effect of this fungicide for the yeast. These results are in accordance with the general inhibitory effect of azole compounds on the transformation of lanosterol into ergosterol in the cell membranes of the yeasts (Ji et al., 2000). Glycerol production was not affected, while ethanol production was slower than in the control but reached a maximum concentration 4–5 days later that was comparable to the series without the fungicide. Although the final ethanol concentration was lower in the control, this can be explained by the earlier sugars depletion in this series, what causes the stop of the ethanol production and, consequently, a more evident effect of ethanol evaporation as well as the beginning of ethanol consumption as a new substrate.

According to these results the presence of tebuconazole at the concentration assayed has a toxic effect for *S. cerevisiae*, reducing the biomass and slowing the fermentation. Nevertheless, and for practical purposes, it is expected that during red winemaking the levels of soluble tebuconazole in the must are reduced by adsorption on grape solids to a concentration low enough to make these effects irrelevant, as it happened in this work.

There are some works showing the ability of *S. cerevisiae* cells to adsorb inorganic and organic contaminants as fungicides (Aksu & Dönmez, 2003; Gomes, Fragoso, Riger, Panek, & Eleutherio, 2002; Goyal, Jain, & Banerjee, 2003: Martín-Esteban, Fernández, & Cámara, 1997; Razmkhab et al., 2002), which is attributed to the cell wall polysaccharides (Ballou, 1988). To investigate if the tebuconazole concentration in the must during red winemaking is reduced not only by adsorption on grape solids but also by the yeast, pesticide residues were determined both in the fermentation medium (free tebuconazole) and in the yeasts (adsorbed tebuconazole) during the fermentative process (Table 3). In this case the levels of soluble tebuconazole were not modified during the fermentation and the amount of tebuconazole extracted from the cells was not significant. Moreover, degradation products of tebuconazole were not identified. These results indicate that yeast activity during fermentation did not induce any degradation of the pesticide during must fermentation.

3.3.2. Effect on malolactic fermentation

Several studies have directly or indirectly investigated the effect of pesticide residues on the rate of malolactic fermentation. In most cases, pesticide residues were found to have little or no effect on malolactic fermentation, and very few pesticides were degraded or adsorbed by the bacteria during this process (Bordons et al., 1998; Haag et al., 1988). In other cases, the fermentation activity of *O. oeni* was affected by the presence of certain pesticides, azoxystrobin, cyprodinil, fludioxonil, pyrimethanil, (Cabras et al., 1994; Oliva et al., 2007) and dicofol (Ruediger et al., 2005) with a lower degradation of malic acid; however, the amount of these pesticides was not affected significantly during MLF, except in the case of dicofol. For quinoxyfen (Cabras et al., 2000), mepanipyrim (Cabras et al., 1999), carbaryl, carbendazim, chlorothalonil, fenarimol, metalaxyl, oxadixyl, procymidone and triadimenol (Ruediger et al., 2005) no effect on the MLF was observed, and, as before, bacteria do not have any degradative effect on them.

To evaluate whether tebuconazole can negatively affect the malolactic fermentation of wine by *O. oeni* and be adsorbed or degraded by the microorganism, *in vitro* experiments with the wine synthetic medium described in Materials and Methods were carried out using a concentration of 0.6 mg/L of tebuconazole, 80% lower than the maximum residue limit in grapes according to the tebuconazole reduction after alcoholic fermentation in our field experiments. The evolution of malic and lactic acid, as well as tebuconazole residues were analysed during the incubation.

The results in Fig. 3 show that this concentration of tebuconazole did not affect significantly the conversion rate of malic to lactic acid, as well as the final concentration of both acids at the end of the fermentation with respect to the control. As reported by other

Table 3
Tebuconazole residues (mg/L ± SD) during alcoholic fermentation of *Saccharomyces cerevisiae* yeast determined by LC–MS/MS.

Sample	Residues after inoculation					
	0 days	3 days	4 days	6 days	9 days	13 days
Control	2.14 ± 0.04	1.89 ± 0.02	2.01 ± 0.10	1.92 ± 0.04	2.10 ± 0.07	2.01 ± 0.08
Fermentation medium	1.97 ± 0.10	1.97 ± 0.01	1.82 ± 0.13	1.71 ± 0.21	1.92 ± 0.08	1.90 ± 0.05
Yeast cells	n.d.	0.04 ± 0.00	0.05 ± 0.01	0.04 ± 0.00	0.07 ± 0.00	0.07 ± 0.00

n.d.: not detected.

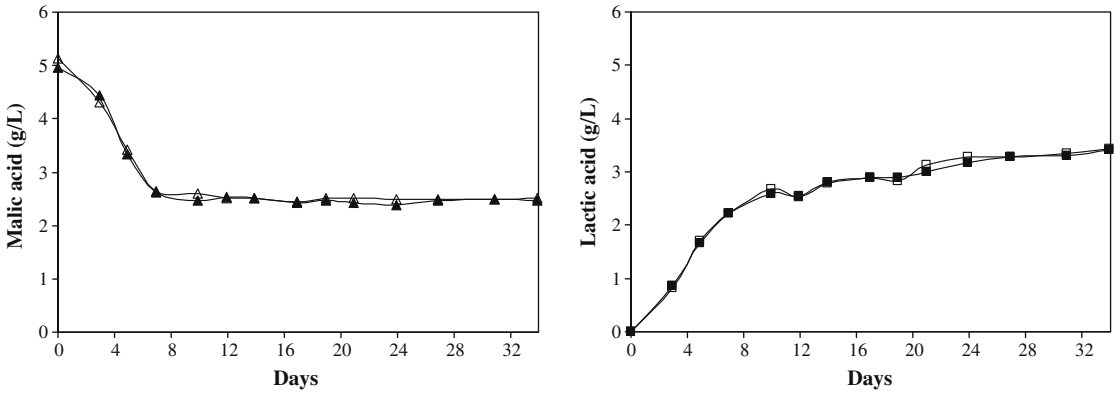

Fig. 3. Time course of malic acid (△, ▲) and generated lactic acid (□, ■) during malolactic fermentation of *Oenococcus oeni* bacteria, in presence and no presence of tebuconazole residues, respectively.

authors (Cabras et al., 1999), there is a remnant malic acid concentration that was not degraded, what is probably due to the lost of cell viability after a long period of exposure to ethanol and lactic acid. Finally, the levels of tebuconazole remained constant during the MLF and no degradation products were detected, what allows concluding that this bacterium does not have any adsorptive or degradative effect on it at the fungicide concentration assayed.

4. Conclusions

The winemaking process followed in A.O.C. Valdeorras for producing red wines allowed to decrease the tebuconazole residues of the initial musts. Tebuconazole presented high affinity to be adsorbed on the suspended solid matter. The eliminated percentage of tebuconazole in the final wine is 86%. In future, according these results, MRL for tebuconazole in red wines could be established as a eight times lower (0.25 mg/L) than MRL established in wine grapes (2 mg/kg). Results of *in vitro* assays showed that the presence of tebuconazole did not affect alcoholic nor malolactic fermentations. At the same time, these two fermentative processes did not affect the amount of tebuconazole either by degradation or by adsorption.

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References

Aksu, Z., & Dönmez, G. (2003). A comparative study on the biosorption of some yeasts for Remazol Blue reactive dye. *Chemosphere, 50*(8), 1075–1083.

American Chemical Society Subcommittee on Environmental Analytical Chemistry (1980). Gidelines for data acquisition and data quality evaluation in environmental chemistry. *Analytical Chemistry, 52*(14), 2242–2249.

Anastassiades, M., Maštovská, K., & Lehotay, S. J. (2003). Evaluation of analyte protectants to improve gas chromatographic analysis of pesticides. *Journal of Chromatography A, 1015*(1–2), 163–184.

Ballou, C. E. (1988). Organization of the *Saccharomyces cerevisiae* cell wall. In D. E. Varner (Ed.), *Self-assembling architecture* (pp. 115–117). New York: Liss.

Bernfeld, P. (1951). Enzymes of starch degradation and enzymes. *Advances in Enzymology, 12*(52), 379–427.

Bordons, A., Masqué, M.C., & Vidal, M. (1998). Isolation and selection of malolactic bacteria and effect of pesticides. In *The management of malolactic fermentation and quality of wine, international symposium*, April 16–17, Verona, Italy, (pp. 51–56).

Cabras, P., & Angioni, A. (2000). Pesticide residues in grapes, wine, and their processing products. *Journal of Agricultural and Food Chemistry, 48*(4), 967–973.

Cabras, P., Angioni, A., Garau, V. L., Melis, M., Pirisi, F. M., Cabitza, F., et al. (1998). Pesticide residues in raisin processing. *Journal of Agricultural and Food Chemistry, 46*(6), 2309–2311.

Cabras, P., Angioni, A., Garau, V. L., Melis, M., Pirisi, F. M., Minelli, E., et al. (1997). Fate of some new fungicides (cyprodinil, fludioxonil, pyrimethanil, tebuconazole) from vine to wine. *Journal of Agricultural and Food Chemistry, 45*(7), 2708–2710.

Cabras, P., Angioni, A., Garau, V. L., Pirisi, F. M., Cabitza, F., Pala, M., et al. (2000). Fate of quinoxyfen residues in grapes, wine and their processing products. *Journal of Agricultural and Food Chemistry, 48*(12), 6128–6131.

Cabras, P., Angioni, A., Garau, V. L., Pirisi, F. M., Cabitza, F., Pala, M., et al. (2001). Fenhexamid residues in grapes and wine. *Food Additives and Contaminants, 18*(7), 625–629.

Cabras, P., Angioni, A., Garau, V. L., Pirisi, F. M., Farris, G. A., Madau, G., et al. (1999). Pesticides in fermentative processes of wine. *Journal of Agricultural and Food Chemistry, 47*(9), 3854–3857.

Cabras, P., Meloni, M., Melis, M., Farris, G. A., Budroni, M., & Satta, T. (1994). Interactions between lactic acid bacteria and fungicides during lactic fermentation. *Journal of Wine Research, 5*(1), 53–59.

Commission Regulation (EC) No 149/2008 of 29 January 2008 amending Regulation (EC) No 396/2005 of the European Parliament and of the Council by establishing Annexes II, III and IV setting maximum residue levels for products covered by Annex I thereto, (OJ L58 of 01/03/2008).

De Melo, S., Caboni, P., Pirisi, F. M., Cabras, P., Alves, A., & Garau, V. L. (2006). Residues of the fungicide famoxadone in grapes and its fate during wine production. *Food Additives and Contaminants, 23*(3), 289–294.

Erney, D. R., Gillespie, A. M., Gilvydis, D. M., & Poole, C. F. (1993). Explanation of the matrix-induced chromatographic response enhancement of organophosphorus pesticides during open tubular column gas chromatography with splitless or hot on-column injection and flame photometric detection. *Journal of Chromatography A, 638*(1), 57–63.

Fernández, M. J., Oliva, J., Barba, A., & Cámara, M. A. (2005). Effects of clarification and filtration processes on the removal of fungicide residues in red wines (Var. Monastrell). *Journal of Agricultural and Food Chemistry, 53*(15), 6156–6161.

García, M. A., Oliva, J., Barba, A., Cámara, M. A., Pardo, F., & Díaz-Plaza, E. M. (2004). Effect of fungicide residues on the aromatic composition of white wine inoculated with three *Saccharomyces cerevisiae* strains. *Journal of Agricultural and Food Chemistry, 52*(5), 1241–1247.

Gomes, D. S., Fragoso, L. C., Riger, C. J., Panek, A. D., & Eleutherio, E. C. A. (2002). Regulation of cadmium uptake by *Saccharomyces cerevisiae*. *Biochimica et Biophysica Acta–General Subjects, 1573*(1), 21–25.

González-Rodríguez, R. M., Rial-Otero, R., Cancho-Grande, B., & Simal-Gándara, J. (2008a). Determination of 23 pesticide residues in leafy vegetables using gas chromatography-ion trap mass spectrometry and analyte protectants. *Journal of Chromatography A, 1196–1197*(1–2), 100–109.

González-Rodríguez, R. M., Rial-Otero, R., Cancho-Grande, B., & Simal-Gándara, J. (2008b). Occurrence of fungicide and insecticide residues in trade samples of leafy vegetables. *Food Chemistry, 107*(3), 1342–1347.

Goyal, N., Jain, S. C., & Banerjee, U. C. (2003). Comparative studies on the microbial adsorption of heavy metals. *Advances in Environmental Research, 7*(2), 311–319.

Guilloux-Benatier, M., Remize, F., Gal, L., Guzzo, J., & Alexandre, H. (2006). Effects of yeast proteolytic activity on *Oenococcus oeni* and malolactic fermentation. *FEMS Microbiology Letters, 263*(2), 183–188.

Haag, V. B., Kreiger, S., & Hammes, W. P. (1988). Inhibition by pesticides of starter cultures for malolactic fermentation in wine. *Wein Wissenschaft, 43*, 261–278.

Hajšlová, J., & Zrostlíková, J. (2003). Matrix effects in (ultra)trace analysis of pesticide residues in food and biotic matrices. *Journal of Chromatography A, 1000*(1–2), 181–197.

Ji, H., Zhang, W., Zhou, Y., Zhang, M., Zhu, J., Song, Y., et al. (2000). A three-dimensional model of lanosterol 14alpha-demethylase of *Candida albicans* and

its interaction with azole antifungals. *Journal of Medicinal Chemistry, 43*(13), 2493–2505.

Jiménez, J. J., Bernal, J. L., del Nozal, M. J., Bernal, J., & Toribio, L. (2007). Persistence and degradation of metalaxyl, lindane, fenvalerate and deltamethrin during the wine making process. *Food Chemistry, 104*(1), 216–223.

Jiménez, J. J., Bernal, J. L., del Nozal, M. J., Toribio, L., & Bernal, J. (2004). Determination of impurities in pesticides and their degradation products formed during the wine-making process by solid-phase extraction and gas chromatography with detection by electron ionization mass spectrometry. II. Bromopropylate, trichlorphon, parathion-methyl and tebuconazole. *Rapid Communications in Mass Spectrometry, 18*(22), 2629–2636.

Martín-Esteban, A., Fernández, P., & Cámara, C. (1997). Baker's yeast biomass (*Saccharomyces cerevisiae*) for selective on-line trace enrichment and liquid chromatography of polar pesticide in water. *Analytical Chemistry, 69*(16), 3267–3271.

Maštovská, K., Lehotay, S. J., & Anastassiades, M. (2005). Combination of analyte protectants to overcome matrix effects in routine GC analysis of pesticide residues in food matrixes. *Analytical Chemistry, 77*(24), 8129–8137.

Navarro, S., Barba, A., Oliva, J., Navarro, G., & Pardo, F. (1999). Evolution of residual levels of six pesticides during elaboration of red wines. Effect of wine-making procedures in their disappearance. *Journal of Agricultural and Food Chemistry, 47*(1), 264–270.

Navarro, S., Oliva, J., Barba, A., Navarro, G., García, M. A., & Zamorano, M. (2000). Evolution of chlorpyrifos, fenarimol, metalaxyl, penconazole, and vinclozolin in red wines elaborated by carbonic maceration of monastrell Grapes. *Journal of Agricultural and Food Chemistry, 48*(8), 3537–3541.

Ochiai, N., Fujimura, M., Oshima, M., Motoyama, T., Ichiishi, A., Yamada-Okabe, H., et al. (2002). Effects of iprodione and fludioxonil on glycerol synthesis and hyphal development in *Candida albicans*. *Bioscience, Biotechnology, and Biochemistry, 66*(10), 2209–2215.

Oliva, J., Navarro, S., Barba, A., Navarro, G., & Salinas, M. R. (1999). Effect of pesticide residues on the aromatic composition of red wines. *Journal of Agricultural and Food Chemistry, 47*(7), 2830–2836.

Oliva, J., Payá, P., Cámara, A., & Barba, A. (2007). Removal of famoxadone, fluquinconazole and trifloxystrobin residues in red wines: effects of clarification and filtration processes. *Journal of Environmental Science and Health Part B: Pesticides Food Contaminants and Agricultural Wastes, 42*(7), 775–781.

Razmkhab, S., López-Toledano, A., Ortega, J. M., Mayen, M., Merida, J., & Medina, M. (2002). Adsorption of phenolic compounds and browning products in white wines by yeasts and their cell walls. *Journal of Agricultural and Food Chemistry, 50*(25), 7432–7437.

Rial-Otero, R., Cancho-Grande, B., & Simal-Gándara, J. (2003). Multiresidue method for fourteen fungicides in white grapes by liquid–liquid and solid phase extraction followed by liquid chromatography-diode array detection. *Journal of Chromatography A, 992*(1–2), 121–131.

Ruediger, G. A., Pardon, K. H., Sas, A. N., Godden, P. W., & Pollnitz, A. P. (2005). Fate of pesticides during the winemaking process in relation to malolactic fermentation. *Journal of Agricultural and Food Chemistry, 53*(8), 3023–3026.

Sala, C., Fort, F., Busto, O., Zamora, F., Arola, L., & Guasch, J. (1996). Fate of some common pesticides during vinification process. *Journal of Agricultural and Food Chemistry, 44*(11), 3668–3671.

Zadra, C., Cardinali, G., Corte, L., Fatichenti, F., & Marucchini, C. (2006). Biodegradation of the fungicide iprodione by *Zygosaccharomyces rouxii* strain DBVPG 6399. *Journal of Agricultural and Food Chemistry, 54*(13), 4734–4739.