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Determination of Ochratoxin A in Grapes, Dried Vine Fruits, and Winery Byproducts by High-Performance Liquid Chromatography with Fluorometric Detection (HPLC−FLD) and Immunoaffinity Cleanup

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A liquid chromatographic method for the determination of ochratoxin A in grapes, dried vine fruits, and winery byproducts was developed. A mixture of either acetonitrile/water or acetonitrile/water/methanol was used as an extraction solvent mixture. After immunoaffinity column cleanup, the final extract was analyzed by high-performance liquid chromatography (HPLC) with a fluorometric detector (FLD). Mean recoveries from grapes, grape pomace, and lees samples spiked in the range of 1−200 μg/kg were 78, 86, and 88%, respectively, with a detection limit of 0.1 μg/kg and within-laboratory repeatability ranging from 6 to 15%. Tested on naturally contaminated samples of grapes, grape pomace, and sultanas, the method showed better performances as compared to two other methods also based on immunoaffinity cleanup and HPLC/FLD determination. Ochratoxin A was detected in samples of grape pomace (levels ranging from 34.2 to 456.8 μg/kg) and lees (levels ranging from 48.3 to 602.5 μg/kg) derived from the wine making of red grapes of 2004 and 2005 vintages in southern Italy. After distillation of contaminated grape pomace in a pilot-scale equipment to produce grappa, the toxin remained unchanged in the exhausted pomace and was not detected in any of the distilled fractions (detection limit of 0.02 μg/L).

KEYWORDS: Ochratoxin A; grape; vine fruit; lees; pomace

INTRODUCTION

The occurrence of ochratoxin A has been reported in wine, grape juice, and vine fruits in Europe, North and South America, Africa, and Australia, and risk assessment studies have been performed to estimate the relevant human intake (*1−10*). Accumulation of ochratoxin A in grapes originates in the vineyard and is caused mainly by *Aspergillus carbonarius*, a fungus developing on grape berries especially after veraison (*11*). The period between early veraison and harvesting is considered the critical period for ochratoxin A accumulation in grape berries. Geographical area, meteorological conditions, wounds of berries caused by insects, and cultivar susceptibility are considered the main factors that promote invasion of berries by *A. carbonarius* (*12, 13*).

Several high-performance liquid chromatography (HPLC) methods with fluorometric detection (FLD) have been reported for the determination of ochratoxin A in wine or dried vine fruit, and two of them were successfully validated through collaborative studies, namely, for wine and beer (*14*) and for dried vine fruits (*15*). The method of Visconti et al. (*14*) has been adopted as the official method by the Association of Official Chemists International (AOAC 2001.01), the European Committee for Standardization (CEN) (EN 14133), and the Organisation Internationale de la Vigne et du Vin (OIV 16/2001). The method of MacDonald et al. (*15*) is currently under discussion by CEN (working group TC/275/WG5) for approval as a European standard. Much less effort has been devoted to the analysis of ochratoxin A in grape berries, and no methods have been reported for the analysis of winery byproducts, such as grape pomace and lees. A reliable method for ochratoxin A determination in grape berry is necessary for quality control and research purposes aiming to prevent ochratoxin A contamination in vineyard and to define the efficacy of relevant field control strategies. A good method is also required for ochratoxin A determination in winery byproducts, such as grape pomace or lees, that can be destined to further processing for food/feed or cosmetics use. Grape pomace is the press residue remaining when grapes are processed for wine-making and consists of pressed skins, disrupted cells from the grape pulp, and seeds. Pomace, particularly the red grape skin fractions, is characterized by high contents of phenolic constituents, such as anthocyanins, catechins, flavonol glycosides, phenolic acids, alcohols, and stilbenes; therefore, it represents a valuable source of phenolic antioxidants that may have technological applications as food

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Table 1. Recovery and Repeatability Results from the Analysis of Blank Samples of Grape Berry Puree, Grape Pomace, and Lees Spiked with Ochratoxin A at Different Levels

	grape berry puree		grape pomace		lees	
spiking level (μg/kg)	recovery (%)	RSD$_r$[a] (%)	recovery (%)	RSD$_r$ (%)	recovery (%)	RSD$_r$ (%)
1	75.3	1.6	80.3	3		
10	84.4	1.5	103.1	4		
50	73.9	15.1	86.9	10	90.6	1
100			73.6	7	85.5	7
200					82.6	2
mean of means	77.9		84.1		86.2	

[a] RSD$_r$ = relative standard deviation (n = 3).

additives and possible nutritional benefits. Particular attention is currently dedicated to winery byproducts for obtaining bioactive phenolic compounds with potential application as food antioxidants and preventive agents against cancer and the oxidation of the low-density lipoproteins (*16, 17*). Winery byproducts are also used to extract anthocyanins, which are used as natural food colorants (E 163), also known as enocianina. Grape pomace and lees are also used to produce pomace brandy, such as the Italian grappa and the French marc, although most of the pomace is mainly used as cattle feed or for soil conditioning or it is trucked away to disposal sites (*18*).

The purpose of this study was to develop a chromatographic method with fluorescence detection, which makes use of the immunoaffinity column for determination of ochratoxin A in grape berries, dried vine fruits, and winery byproducts. The method was applied to naturally contaminated samples and compared to two HPLC immunoaffinity-based methods available for wine grapes and dried vine fruits, respectively. The fate of ochratoxin A during distillation of red grape pomace naturally contaminated with a high ochratoxin A level was also investigated.

MATERIALS AND METHODS

Reagents and Materials. Ochratoxin A stock solution (1 mg/mL) was prepared by dissolving the solid standard purchased from Sigma-Aldrich (Milan, Italy) in toluene/acetic acid (99:1, v/v). Ochratoxin A standard solutions for HPLC calibration or spiking purposes were prepared by dissolving adequate amounts of the stock solution, previously evaporated to dryness under nitrogen stream, in the HPLC mobile phase. Acetonitrile, methanol, water (HPLC grade), and glacial acetic acid were purchased from Mallinckrodt Baker (Milan, Italy). Sodium chloride (ACS grade), polyethylene glycol (PEG 8000), and sodium hydrogen carbonate ($NaHCO_3$, ACS grade) were purchased from Sigma-Aldrich. OchraTest immunoaffinity columns were purchased from Vicam (Watertown, MA). No. 4 and GF/A paper and glass microfiber filters were obtained from Whatman (Maidstone, U.K.).

Sample Extraction, Immunoaffinity Cleanup, and HPLC Analysis. A total of 5 g of ground dried pomace or lees was extracted with 30 mL of acetonitrile/water (60:40, v/v) by shaking for 60 min. Slurry samples were prepared for sultanas by blending five parts of dried vine fruits with four parts of water. For grape puree and slurried sultanas, the extraction solvent mixture was acetonitrile/methanol/water (90:90:80, v/v/v) and 26 mL instead of 30 mL was used to extract 5 g of ground grape berries (puree) or 9 g of slurried sultanas. In this way, the 4 mL water contained in the test portion size of grape berries or slurried sultanas were taken into account. After filtration through a filter paper, 6 mL of filtrate was diluted with 44 mL of water solution containing PEG (1%) and $NaHCO_3$ (5%), mixed, and filtered through Whatman GF/A glass microfiber. A 10 mL volume of diluted extract (equivalent to 0.2 g of sample) was cleaned up through an OchraTest immunoaffinity column at a flow rate of about 1 drop per second. The column was washed with 10 mL of water solution containing NaCl (2.5%) and $NaHCO_3$ (0.5%), followed by 10 mL of distilled water at a flow rate of 1−2 drops per second. The eluates were discarded, and ochratoxin A was recovered in a vial after elution with 2 × 1 mL of methanol. The extract was dried under nitrogen stream at ca. 50 °C and reconstituted with 500 μL of the HPLC mobile phase. The reconstituted extract (100 μL, corresponding to 0.04 g of sample) was injected into the HPLC apparatus by a full loop injection system. The HPLC determination and confirmation of ochratoxin A were performed according to the AOAC Official Method 2001.01 (*14*). The HPLC apparatus was an Agilent 1100 series equipped with a G1312A binary pump, a G1313A autosampler, a G1316A column thermostat set at 25 °C, a G1321A spectrofluorometric detector set at 333 nm (λ_{ex}) and 460 nm (λ_{em}), and an Agilent Chemstation G2170AA Windows 2000 operating system (Agilent, Waldbronn, Germany). The separations were performed with a Xterra C_{18} column (150 × 4.6 mm, 5 μm) (Waters, Milford MA) preceded by a Rheodyne guard filter (3 mm, 0.45 μm pore size). The mobile phase was an isocratic mixture of acetonitrile/water/acetic acid (99:99:2, v/v/v) eluted at a flow rate of 1.0 mL/min.

Table 2. Comparison of Ochratoxin A Determination[a] in Red Grape Berry Puree, Red Grape Pomace, Lees, and Sultanas by (A) the Present Method and Methods of (B) Serra et al. (*19*) and (C) MacDonald et al. (*15*)

samples	A this method (μg/kg)	B method[b] (μg/kg)	B/A	C method[c] (μg/kg)	C/A
red berries puree	4.0	2.5	0.63	2.3	0.58
	15.4	11.9	0.77	12.0	0.78
	34.4	23.5	0.68	13.1	0.38
	32.3	22.2	0.69	13.9	0.43
	8.7	5.0	0.58	4.1	0.47
mean ± SEM[d]	19.0 ± 6.2	13.0 ± 4.3*	0.67	9.1 ± 2.4*	0.53
red grape pomace	180. 3	28.9	0.27	87.8	0.49
	112.1	20.7	0.18	64.6	0.57
	148.4	24.9	0.17	87.8	0.59
	168.8	22.5	0.13	80.2	0.47
	138.8	35.6	0.26	103.0	0.74
mean ± SEM	149.7 ± 11.9	26.5 ± 2.6***	0.20	84.7 ± 6.2**	0.64
lees	162.2	40.1	0.25	*e*	*e*
	155.1	38.5	0.25	*e*	*e*
mean ± SEM	158.6 ± 3.6	39.3 ± 0.8**	0.25	*e*	*e*
sultanas	1.2	*e*	*e*	1.0	0.84
	4.2	*e*	*e*	3.7	0.89
mean ± SEM	2.7 ± 1.5	*e*	*e*	2.3 ± 1.3	0.87

[a] Results for pomace and lees are reported on a dry weight basis. [b] Extraction solvent: water solution containing 1% PEG and 5% $NaHCO_3$. [c] Extraction solvent mixture: 0.1 M $MeOH/H_2O/H_3PO_4$ (10:4:1, v/v/v). [d] Mean ± standard error mean. Mean ochratoxin A levels significantly different from A at (*) $p < 0.05$, (**) $p < 0.01$, and (***) $p < 0.001$ (paired t test, one-tailed p values). [e] Not analyzed.

Recovery Experiments. Recovery experiments were performed in triplicate. Ochratoxin-A-free samples of red grape berry puree and pomace, obtained from selected "blank" bunches, were spiked with ochratoxin A at levels of 1, 10, 50, and 100 μg/kg. Because of unavailability of ochratoxin-A-free samples of lees, naturally contaminated samples were used for recovery experiments at spiking levels of 50, 100, and 200 μg/kg, and recoveries were calculated after subtracting the endogenous ochratoxin A content (55.4 μg/kg) determined prior to spiking.

Analysis of Naturally Contaminated Samples. Pressed red grape pomace and wine lees were collected in 2004 and 2005 from two winery industries in southern Italy. In particular, pomace samples (200 g each) were collected from the mass of the tanks (for each tank, 6 tons of pomace was obtained from 50 tons of crushed grapes), while lees samples (100 g each) were obtained from 30 ton tanks after wine decanting. A total of 10 pomace samples and 16 wine lees samples were collected from both 2004 and 2005 vintages. Samples were dried at 50 °C for 48 h and then homogenized by a Sterilmixer blender (International PBI, Milan, Italy) prior to ochratoxin A analysis. To check

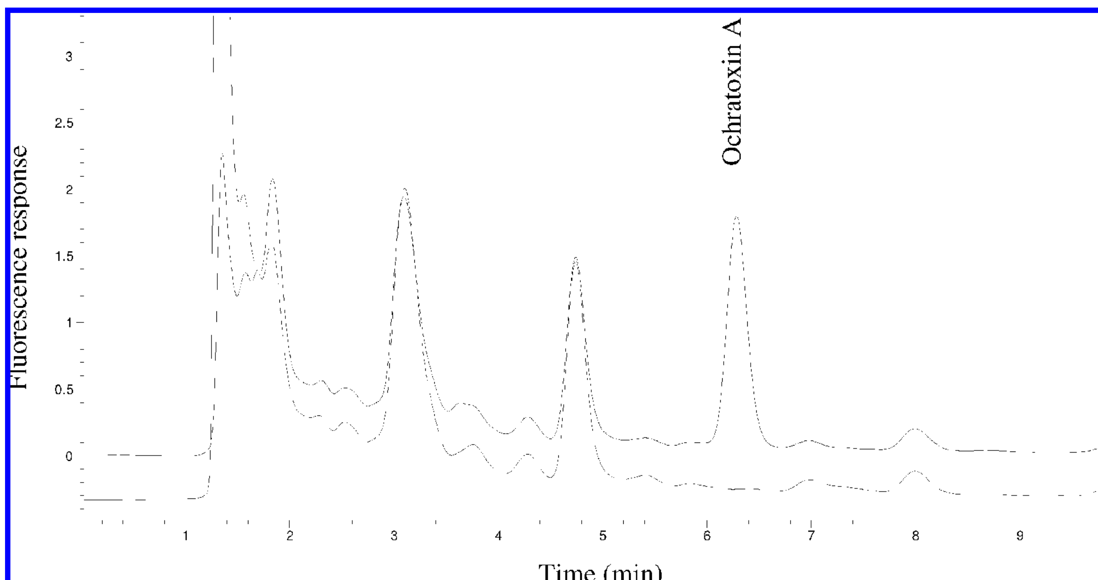

Figure 1. Chromatograms of a sample of grape pomace spiked with 10 μg/kg OTA (—) and blank (—) analyzed with the present method.

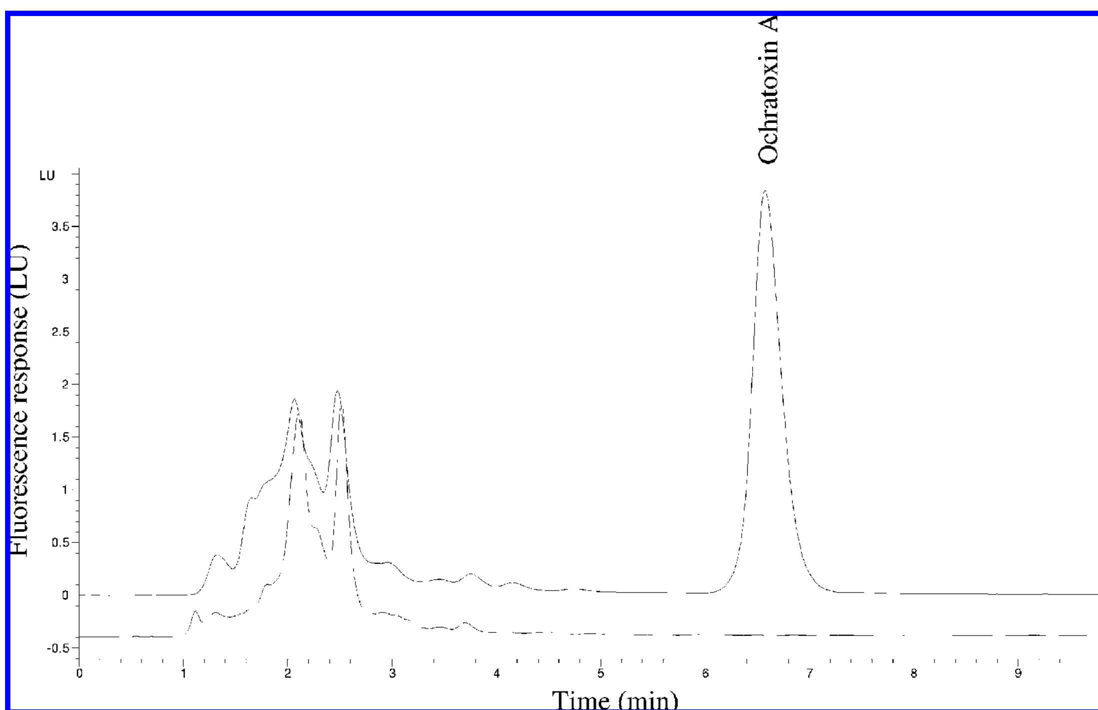

Figure 2. Chromatograms of blank (—) and naturally contaminated (34.4 μg/kg) red grape berries puree (—).

the within-tank variability of ochratoxin A contamination in pomace, tests were performed on all 2005 samples by analyzing separately three subsamples collected from the mass of each tank (one from the middle and two from the external part of the pressed pomace mass). Similarly, within-tank ochratoxin A variability in lees was checked by analyzing three lees subsamples per tank from five samples of 2005 vintage.

Five naturally contaminated samples (20 kg each) of red grape bunches were collected from a field in Apulia, southern Italy. Grape bunches were crushed, and stems were separated and discarded. Aliquots (1 kg) of each sample of crushed berries were homogenized by a Sterilmixer blender.

Two samples (250 g each) of dried vine fruits (sultanas) were purchased from a local market. All samples were analyzed for ochratoxin A by the method described herein. For comparison purposes, five samples of grape berries, five samples of pomace, two samples of lees, and two samples of sultanas were also analyzed by the methods published by Serra et al. (*19*) and MacDonald et al. (*15*) for grape and dried vine fruits, respectively. The paired *t* test (one-tailed *p* value) was used for statistical analysis of the results for method comparison.

Distillation of Grape Pomace. About 25 kg of red grape pomace naturally contaminated with ochratoxin A at level of 849.1 μg/kg (on a dry weight basis) was distilled in a craftsman-like pot still composed of a pot, a condenser, and a receiver. The pot contained 10 L of red wine (15% alcoholic degree) on the bottom, and pomace was suspended in the wine within two grates. The wine was boiled, and hot vapors flowed through pomace before going into the condenser, where they were cooled back to the liquid state that was collected in a receiver. Four distilled fractions were collected for a total volume of 3750 mL. A total of 50 mL of each distilled fraction was evaporated to dryness with a rotavapor, reconstituted with 1 mL of HPLC mobile phase, and injected (100 μL) into the HPLC apparatus. These extracts were directly analyzed by HPLC without immunoaffinity clean up because the chromatogram of these samples were very clean and no interfering peaks eluted at a retention time of ochratoxin A. The limit of detection of ochratoxin A in the distilled fraction was 0.02 μg/L on the basis of a

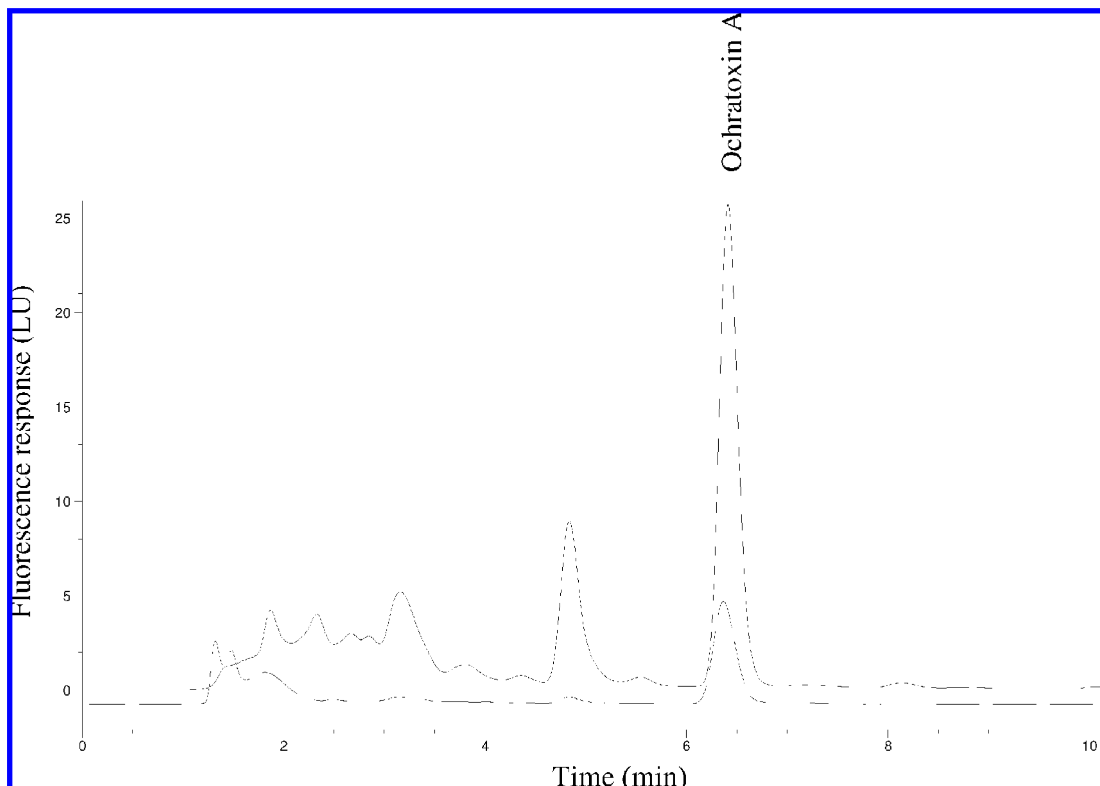

Figure 3. Chromatograms of a sample of red wine lees naturally contaminated with OTA and analyzed with the present method (—) and the method of Serra et al. (*19*) (—).

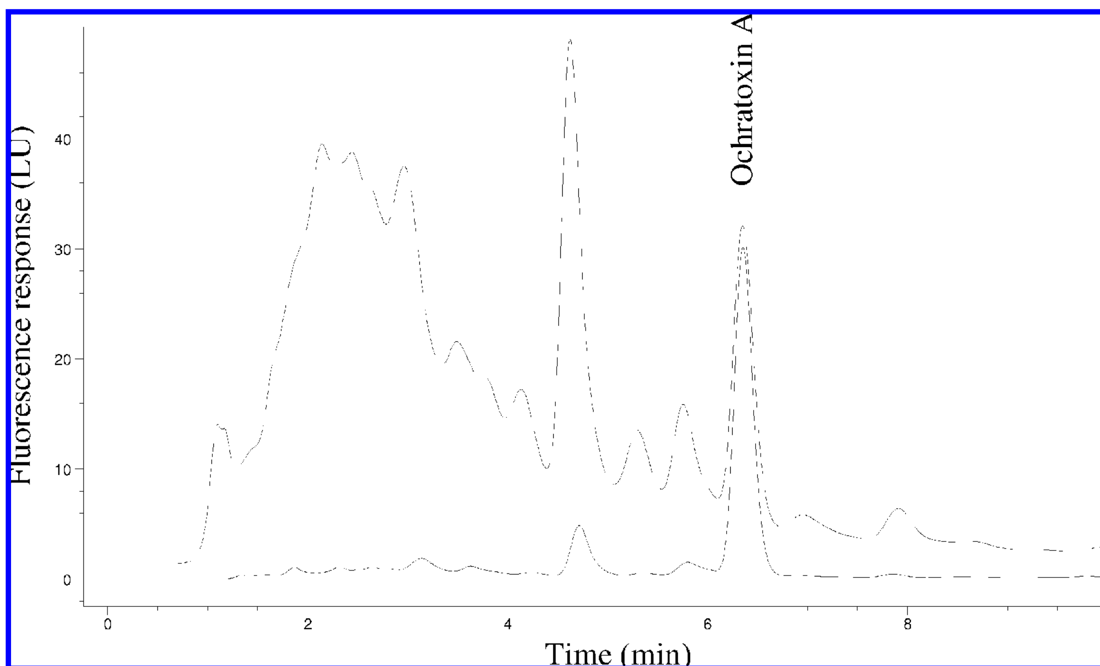

Figure 4. Chromatograms of a sample of grape pomace naturally contaminated with OTA and analyzed with the present method (—) and the method of MacDonald et al. (*15*) (—).

signal-to-noise ratio of 3:1. At the end of the distillation process, the exhausted pomace was dried at 50 °C for 48 h, blended by a Sterilmixer blender, and analyzed for ochratoxin A as described above.

RESULTS AND DISCUSSION

The method described herein used a mixture of acetonitrile/water for the extraction of ochratoxin A from pomace and lees and acetonitrile/methanol/water for grape berries and sultanas. The latter two matrices contain considerable amounts of sugar and could not be extracted by a acetonitrile/water mixture because of the formation of two liquid layers. The addition of methanol to the acetonitrile/water solution prevented the formation of two liquid layers.

The results of recovery experiments (triplicate measurements) of the full analytical procedure carried out on red grape berry puree, pomace, and lees spiked with ochratoxin A at different levels are reported in **Table 1**. The overall average recovery (mean of means) of ochratoxin A from grape puree was 77.9%,

Table 3. Natural Occurrence of Ochratoxin A in Samples of Winery Grape Pomace and Lees Collected in Southern Italy[a]

	2004 vintage		2005 vintage	
	pomace (μg/kg)	lees (μg/kg)	pomace (μg/kg ± SD[b])	lees (μg/kg ± SD[b])
	180.3	83.4	184.3 ± 13.1	217.2
	112.1	118.9	263.2 ± 15.4	318.2
	148.4	138.2	218.8 ± 5.8	241.4
	168.8	141.3	217.7 ± 7.7	250.2
	138.8	145.4	346.6 ± 0.2	255.1
	71.6	162.2	244.3 ± 16.5	377.3
	74.7	135.0	456.8 ± 20.3	353.2
	93.5	125.0	374.8 ± 33.4	435.3
	101.6	147.2	89.9 ± 6.6	362.2
	34.2	231.3	97.6 ± 4.7	602.5
		290.0		279.6
		224.6		243.6 ± 20.3
		131.2		400.2 ± 79.4
		148.3		345.8 ± 10.8
		105.2		517.7 ± 4.8
		48.3		162.7 ± 6.5
mean	112.4	148.5	249.4	335.1
median	106.9	139.8	231.6	332.0

[a] Results are reported on a dry weight basis. [b] Standard deviation ($n = 3$).

with a minimum value at 73.9%, and the relative standard deviation (RSD) ranged from 1.5 to 15.1%. Average recoveries for spiked grape pomace and lees were 84.1 and 86.2%, respectively. Values of RSD ranged from 3 to 10% for pomace and from 1 to 7% for lees. The limit of detection of the method was 0.1 μg/kg on the basis of a signal-to-noise ratio of 3:1. Chromatograms of a blank grape pomace sample and the same sample spiked with 10 μg/kg ochratoxin A are shown in **Figure 1**. Chromatograms of blank and naturally contaminated grape berry puree samples are shown in **Figure 2**.

Recovery experiments from spiked samples is a good way to check for the accuracy of an analytical method for mycotoxins, although spiked samples do not exactly reproduce naturally contaminated samples.

Therefore, samples of red grape berry puree, pomace, lees and sultanas naturally contaminated with ochratoxin A were used to compare the extraction efficiency of the method as compared to two other methods using different extraction solvents (*15, 19*). The latter methods were also based on HPLC/FLD and immunoaffinity cleanup and used $NaHCO_3$/polyethylene glycol (PEG) (*19*) or methanol-aqueous H_3PO_4 (*15*) solutions for ochratoxin A extraction from grapes or dried vine fruits, respectively.

Results of comparative analyses of 14 naturally contaminated samples (5 wine grapes, 2 sultanas, 5 grape pomace, and 2 lees) are reported in **Table 2**. In particular, results obtained with the method of Serra et al. (*19*) for grape berry puree were significantly different ($p < 0.05$) from those obtained with our method, with an average corresponding to 67%, indicating a better extraction efficiency for our method (**Table 2**). For sultanas, the comparison to a method that was fully validated by a international collaborative study (*15*) showed that the difference between the results for these two methods falls within the expected statistical variation. For ochratoxin A determinations in other grape-derived matrices (pomace and lees), a better extraction efficiency was observed with our method as compared to either the MacDonald et al. (*15*) or the Serra et al. (*19*) method (**Table 2**). These results indicate that the ochratoxin A extraction efficiency by either acetonitrile/water (60:40) for grape pomace and lees or acetonitrile/methanol/water (90:90:80) for grapes is better than aqueous solutions of $NaHCO_3$/PEG or methanol/H_3PO_4. **Figure 3** shows the chromatograms of the same sample of contaminated red grape lees extracted with acetonitrile/water (60:40) (this method) and with aqueous solutions of $NaHCO_3$/PEG (*19*). An additional advantage of the method described herein is the applicability to all solid matrices throughout the grape-wine production chain.

A considerable improvement of the sample extract cleanup was obtained by our method with respect to that of MacDonald et al. (*15*), as shown in **Figure 4** (red grape pomace). The good cleanup efficiency of the method can be attributed to the dilution step with $NaHCO_3$/PEG aqueous solution prior to the immunuoaffinity cleanup. In conclusion, $NaHCO_3$/PEG has been shown to give very good results in terms of sample cleanup but poor extraction efficiency for ochratoxin A in all matrices considered in this study.

All samples of winery byproducts analyzed herein were found contaminated by ochratoxin A at levels ranging from 34.2 to 456.8 μg/kg for pomace and from 48.3 to 602.5 μg/kg for lees on a dry weight basis (**Table 3**). The variability of results from triplicate measurements within each tank was low for both pomace and lees samples, with mean coefficient of variations of 5.2 and 7.2% for pomace and lees, respectively (**Table 3**). The low values of within-tank variability indicate that ochratoxin A is homogeneously distributed within pomace after 5−6 days of fermentation and maceration of crushed grape berries. The same applies to lees because of alcoholic fermentation that mix up grape juice within the tank.

Grape pomace and lees are mainly used for cattle feed or soil conditioning, or they are trucked away to disposal sites (*18*). Dietary inclusion of grape pomace has been proposed for chicken because it reduces lipid oxidation of meat during refrigerated storage without altering growth performance and nutrient digestibility in chickens (*20*). Guidance ochratoxin A levels of 50 and 100 μg/kg have been proposed in Europe for complementary and complete feedingstuffs for pigs and poultry, respectively (Commission Recommendation of 17/8/2006). In this preliminary survey, 70% of the tested samples contained ochratoxin A levels higher than 100 μg/kg, therefore being unsuitable for poultry and pig feeding.

The use of grape pomace as a substrate for cultivation of *Pleurotus* spp. has also been proposed (*21*); therefore, the possible carryover of ochratoxin A from naturally contaminated pomace to edible parts of *Pleurotus* should be investigated.

It is well-known that grape pomace is a source of polyphenols with powerful antioxidant capacity (*16, 17, 20*). Several health-promoting products obtained from grape pomace and other plant byproducts are on the market, and a great deal of research efforts is being devoted to testing the putative beneficial effects of grape polyphenols (*22*). Grape pomace is also used to produce color additives, such as grape color extract, grape skin extract (enocianina), and E 163. Grape color extract is a solution of anthocyanin water-soluble pigments extracted from grapes or the relevant powder obtained after dehydration. Enocianina is a purplish-red liquid prepared by concentrating aqueous extracts of fresh deseeded pomace. E 163 is a color additive consisting of a mixture of anthocyanin and anthocyanidin that are mainly extracted from grape pomace. All of these products are at risk of ochratoxin A contamination; therefore, preliminary controls (ochratoxin A analysis) of the grape pomace used for their production are necessary to avoid the risk contamination of the final products. Grape pomace and lees can also be used in wine refermentation to rejuvenate wines, reduce volatile acidity, or correct other wine defects; therefore, ochratoxin A should be

checked in these winery byproducts to avoid the consequent ochratoxin A contamination in wine.

Grape pomace and lees are also used to produce pomace brandy, such as the Italian grappa and the French marc. The fate of ochratoxin A during distillation of grape pomace containing 850 μg/kg OTA was assessed at laboratory scale. The ochratoxin A level in the exhausted pomace after the distillation process remained unchanged, and no toxin was detected in the four alcoholic fractions collected during the distillation process. These results demonstrate that the production of distilled alcoholic beverages, such as grappa or marc, from ochratoxin-A-contaminated pomace can be considered as safe in terms of toxin contamination of the final product, while the exhausted pomace remains unaltered, with the original amount of toxin.

In conclusion, a method has been developed for the analysis of ochratoxin A in wine grape berries, dried vine fruits, and winery byproducts. The use of acetonitrile/water or acetonitrile/methanol/water as extraction solvent and PEG/$NaHCO_3$ as dilution solution prior to immunoaffinity cleanup resulted in high ochratoxin A recoveries and clean chromatograms, respectively. High levels of ochratoxin A were found in samples of winery byproducts collected in 2004 and 2005 vintages in southern Italy. The distillation of highly contaminated grape pomace resulted in the production of ochratoxin-A-free distilled fractions because the toxin remained entirely unmodified in the exhausted pomace. The presence and levels of ochratoxin A in winery byproducts should be checked and avoided when these products are destined to further uses in the food or feed chains.

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