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From grape to wine: Fate of ochratoxin A during red, *rose*, and white winemaking process and the presence of ochratoxin derivatives in the final products

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ABSTRACT

The presence of ochratoxin A (OTA) in wine is mainly due to the contamination of grapes by *Aspergillus carbonarius* and *A. niger*, still in the vineyard or at stages prior to winemaking. Throughout winemaking process, although there is a reduction in OTA levels, modified mycotoxins may also be formed. In fact, modified mycotoxins are compounds that normally remain undetectable during the conventional analysis used for the parent toxin. In this context, the current study aimed to evaluate the effect of grape variety and winemaking steps on OTA fate as well as the formation of modified ochratoxins. White, *rose* and red wines were prepared from Muscat Italia, Syrah and Touriga Nacional varieties, respectively. OTA was determined during different steps of winemaking by UHPLC-ESI-MS/MS. Identification of ochratoxin derivatives was performed using tandem MS experiments. A reduction of 90.72, 92.44 and 88.15% in OTA levels was observed for white, *rose* and red wines, respectively. Among the sought targets, the following ochratoxin-derived candidates were identified: ochratoxin β, ochratoxin α methyl ester, ochratoxin B methyl ester, ochratoxin A methyl ester, ethylamide ochratoxin A, ochratoxin C and ochratoxin A glucose ester. These results indicate that the formation of ochratoxin derivatives leads to an underestimation of total mycotoxin levels in wine and, therefore, the inclusion of techniques for multi-mycotoxins detection should be considered.

1. Introduction

Moderate consumption of wine has shown beneficial health effects due to the presence of compounds with antioxidant activity. However, components that cause negative effects on human health such as sulfites, toxic metals, pesticide residues and mycotoxins may also be present (Čepo et al., 2018).

Ochratoxin A (OTA) is a mycotoxin produced by some fungal species belonging to genera *Aspergillus* and *Penicillium*. Because OTA has several toxic effects such as carcinogenic, genotoxic, immunotoxic and hepatotoxic (Chen et al., 2018; Luo, Liu, & Li, 2018), it has been classified by the International Cancer Research Agency [IARC] (1993) as a possible carcinogen for humans (group 2B). Moreover, the European Food Safety Authority [EFSA] (2006) established that tolerable weekly intake of OTA is 120 ng/kg body weight.

Among different foods possibly contaminated with fungi and subsequently with OTA, wine is known as the second most important source of OTA considering mean European total dietary intake, only behind cereals (European Commission [EC], 2002). To this end, the European Union has set a maximum tolerable OTA limit of 2 μg/kg in wines (EC, 2006). This same limit was also established by Brazilian legislation (BRASIL, 2011). In addition to the possible toxic effects caused by the ingestion of wines contaminated with OTA, high concentrations of this mycotoxin, still present in grapes, can also impair the fermentative capacity of yeast(s) and result in changes in wine composition, altering taste and color (Bizaj, Curtin, Cadez, & Raspor, 2014).

The presence of this mycotoxin in wine is mainly due to the contamination of grapes still in the vineyard or at stages prior to winemaking by *Aspergillus carbonarius* and *Aspergillus niger*. Factors such as grape variety, damages in grape berries, vineyard location,

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temperature, relative humidity, precipitation, microclimate, harvesting period, pesticide application and good agricultural practices in the vineyard, oenological steps and good manufacturing practices during winemaking will be decisive for OTA levels present in wine (Freire et al., 2017; Quintela, Villarán, López de Armentia, & Elejalde, 2013).

In general, red wines have higher OTA levels in comparison to *rose* and white wines due to differences in applied oenological practices: especially the maceration step (Dachery, Veras, Dal Magro, Manfroi, & Welke, 2017; Lasram et al., 2008). During winemaking, crushing of grape berries and the maceration steps favor OTA release to must, suggesting grape skin is the main source of OTA (Lasram et al., 2008), while its content tends to decrease during fermentation and stabilization steps (Cecchini, Morassut, Garcia-Moruno & Di Stefano, 2006).

The reduction in OTA levels during winemaking has been justified by two main approaches: 1) the partition of OTA between liquid and solid phases, due to the adsorption of OTA in the solid particles of the must and subsequent natural sedimentation (Mariño-Repizo, Gargantini, Manzano, Raba & Ceruttia, 2017) and 2) adsorption or degradation by yeasts and/or lactic acid bacteria (LAB) (Cecchini et al., 2006); with varying degrees of success. However, this reduction is limited and, due to its partial stability, OTA is not completely eliminated throughout the oenological steps and residues of this mycotoxin are still detected in the final product (Cecchini et al., 2006; Cecchini, Morassut, Saiz, & Garcia-Moruno, 2019; Csutorás et al., 2013). A third justification for the reduction may be related to OTA degradation or transformation into modified mycotoxins during fermentation processes (Freire et al., 2019).

Modified mycotoxins cannot be detected when conventional analytical methods are used to quantify the parent mycotoxin due to changes in their structure, polarity and solubility (Berthiler et al., 2013). These forms were first reported by Gareis (1994), who observed that the severity of mycotoxicosis in animals did not correlate with mycotoxin levels detected in the diet. Possibly, the toxin conjugated to another molecule (glucose, amino acids or sulfate) was not detected in the animal feed by the analytical method used and was subsequently hydrolyzed and released into the gastrointestinal tract, thereby increasing animal exposure (Gareis, 1994).

Modified mycotoxins can be formed: 1) in the plant, as part of the detoxification mechanism; 2) during processing, through physical, chemical and biological processes and; 3) animal metabolism (Berthilher et al., 2013; Freire & Sant'Ana, 2018). The most relevant OTA derivatives already identified include ochratoxin B (dechloro analog of OTA), ochratoxin C (ethyl ester of OTA), ochratoxin α (isocoumaric derivative of OTA) and ochratoxin β (dechloro analog of ochratoxin α) (El Khoury & Atoui, 2010). It is possible that these and other derivatives not yet elucidated co-occur with OTA in wines and result in synergistic and/or additive effects to human and animal health. Furthermore, microbial, animal and human metabolism may act on the conversion of modified mycotoxins to parent mycotoxin, increasing OTA bioavailability (Berthiller et al., 2013; Freire & Sant'Ana, 2018).

Since the first discovery of OTA in wines (Zimmerli & Dick, 1996), a number of studies have focused on the reduction of OTA levels during winemaking by degradation and/or adsorption mechanisms (Cecchini et al., 2006, 2019; Csutorás et al., 2013). Nevertheless, the effects of grape variety and different winemaking steps on reduction of OTA levels have not been systematically evaluated. In addition, little information is available regarding the presence of OTA derivatives in wines. Such an investigation is highly relevant to avoid underestimation of the total ochratoxin intake and its possible adverse effects. In this regard, the present study aimed to evaluate the effect of grape variety and winemaking steps on OTA fate as well as the formation of modified ochratoxins. The latter is considered a challenging objective as commercial standards and reference methods are not yet available.

2. Material and methods

2.1. *A. carbonarius* and *A. niger* strains

A. carbonarius 10614 and *A. niger* 10443, isolated from wine grapes and obtained from the Culture Collection of the Department of Food Science/CCDCA-UFLA, were used in the assays. Both strains were previously selected in grape-based medium (Freire, Guerreiro, Pia et al., 2018). A conidia suspension from each strain was prepared individually and their concentration determined in a Neubauer chamber (Sigma-Aldrich, Darmstadt, Germany). The concentration of each suspension was standardized at 10^6 conidia/mL. Following, a mixture containing 50% of suspension from *A. carbonarius* 10614 and 50% of suspension from *A. niger* 10443 was prepared.

2.2. Wine grapes

Grapes were obtained in the wine region of the Vale Submédio São Francisco (Pernambuco, Bahia, Brazil). For each grape variety, a total of 15 kg was collected, to know: Syrah, Touriga Nacional and Muscat Italia. Only healthy grapes with no signs of mechanical damage or fungal growth were used. Grape berries were inoculated by spraying the conidia suspension mixture (*A. carbonarius* and *A. niger*). The final inoculum concentration was 10^4 conidia/g of grapes. As control, grapes were inoculated with sterile distilled water not added of the conidia suspension. Grapes were incubated for 7 days at 25 °C (Freire, Guerreiro, Carames et al., 2018).

2.3. *Saccharomyces cerevisiae* strain

S. cerevisiae 41 PP, used as inoculum in micro-winemaking, has been previously isolated from the viniferous environment (Mendes, Ramírez-Castrillón, Feldberg, Bertoldi, & Valente, 2017). The strain was reactivated in formulated yeast, peptone and dextrose agar (YPD agar) containing 0.5% of yeast extract (Acumedia, Lansing, Michigan, United States of America), 1% of bacteriological peptone (Acumedia, Lansing, MI, USA), 2% of dextrose (Diadema, Brazil) and 2% of agar (Acumedia, Lansing, MI, USA) and incubated for 48 h at 25 °C (Freire et al., 2019). The pre-culture was prepared in grape broth (obtained by macerating the grapes) and agitated on a rotary shaker at 120 rpm for 24 h at 25 °C (Series 25 Shaker/Incubator, New Brunswick Scientific, USA). After, it was inoculated into a new grape broth and incubated on a rotary shaker at 120 rpm for 24 h at 25 °C. This procedure was repeated once more. The final cell concentration in the broth was determined in a Neubauer chamber (Sigma-Aldrich, Darmstadt, Germany). An inoculum containing 10^8 cells/mL was used.

2.4. Micro-winemaking

Micro-winemaking was performed according to Guerra and Baranbé (2005), with adaptations. Red, *rose* and white wines were produced, respectively, from Touriga Nacional (red grape), Syrah (red grape) and Muscat Italia (white grape) grape varieties. After incubation period of grapes with the fungal suspension, the destemming (berry separation from rachis) and the crushing of grapes were performed to obtain the mash. Then, a 0.01% potassium metabisulphite solution (Dinâmica, Diadema, Brazil) was added and after 2 h the mash obtained from each grape variety was inoculated with *S. cerevisiae* strain (10^6 cells/g of must). After inoculation, the mash was divided into 5L-flasks in two repetitions. A control, containing mash prepared from non-contaminated grapes, was also prepared. The primary fermentation was carried out at 25 °C with daily remounting (mixture of liquid and solid parts). For red wine, crush/pressing (pomace separation: skins and seeds from must – must extraction) was performed at the end of primary fermentation. For *rose* and white wines, crush/pressing was carried out 48 h after primary fermentation. The end of primary fermentation was

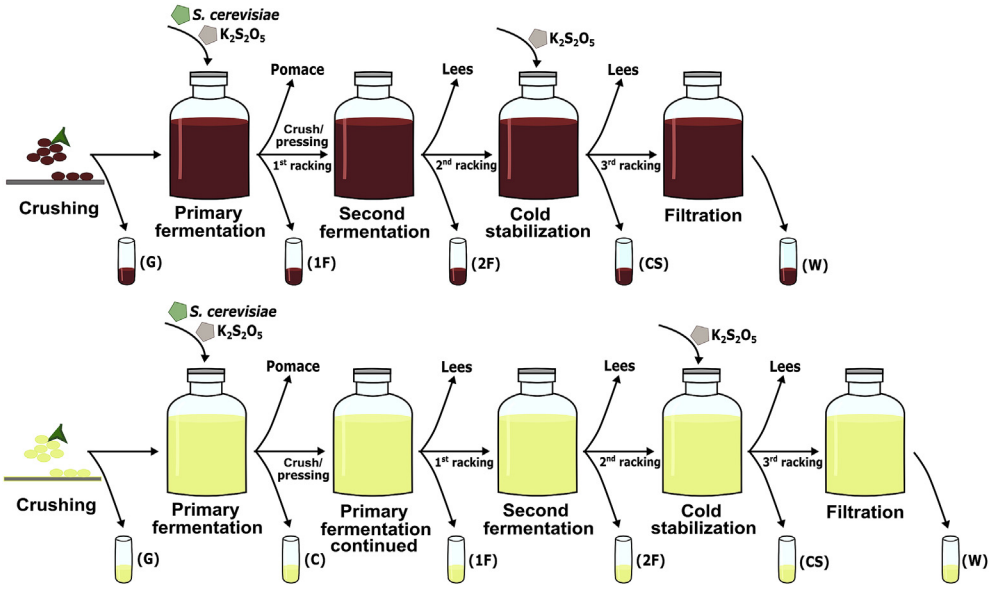


Fig. 1. Different steps for red, *rose*, and white micro-winemaking process evaluated in the present study. Samples were collected in the following steps: G: grape, after crush; C: must, after crush/pressing; 1F: must, at the end of primary fermentation; 2F: must, at the end of second fermentation; CS: must, at the end of cold stabilization; W: wine, after filtration. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

detected by measurement of density (0.992–1.050 g/L) in all wines. Thereafter, a first racking (translocation from one container to another for the separation of solid phases-lees and liquid-must) was performed. The secondary fermentation was carried out at 20 °C for 20 days. Subsequently, a second racking was performed and a 0.005% potassium metabisulphite solution was added, followed by cold stabilization at 4 °C for 30 days. After this period, a third racking and a filtration step for clarification were performed (Fig. 1).

2.5. Determination of OTA concentration

To determine OTA levels throughout red winemaking process, grape samples were collected after destemming and crushing (G) and from the must at the end of primary fermentation (after first racking) (1F); at the end of secondary fermentation (after second racking) (2F); after cold stabilization (after third racking) (CS) and from wine after clarification (filtration) (W). Throughout white and *rose* winemaking process, besides the aforementioned samples (G; 1F; 2F; CS and W), must samples were also collected after crush/pressing (C) performed 48 h after the beginning of primary fermentation.

2.5.1. Sample preparation and extraction

Sample preparation was performed based on the original QuEChERS method and dispersive solid-phase extraction (d-SPE) cleanup, described by Anastassiades, Lehotay, Stajnbaher, and Schenck (2003), with minor modifications. In the first step, 10 g of grapes or 10 mL of wine/must was put into a 50-mL polypropylene tube. Subsequently, 10 mL of acetonitrile (J. T. Baker, Phillipsburg. New Jersey, USA) + 1% formic acid 98% (Sigma-Aldrich, St. Louis, Missouri, USA) was added and tubes were vortexed for 10 min. Then, 4 g of $MgSO_4$ (Sigma-Aldrich, St. Louis, MO, USA) and 1 g of NaCl (Sigma-Aldrich, St. Louis, MO, USA) were added, and the tube was shaken for 5 min before centrifugation carried out at 8000 x *g* for 5 min at 20 °C (model 5810R, Eppendorf, Hamburg, Germany). For cleanup step, an aliquot of 1.5 mL of the supernatant was transferred to a microtube containing $MgSO_4$ and PSA (primary secondary amine) (Sigma-Aldrich, St. Louis, MO, USA), (150:50 mg/mg) and the mixture was vortexed for 5 min, being centrifuged at 5000 x *g* for 3 min at 20 °C (Mini model centrifuge, Gyrozen, Seoul, Republic of Korea). The supernatant was filtered through polytetrafluoroethylene (PTFE) membranes (0.22 μm) (Nova Analítica, São Paulo, Brazil) directly into the vial.

A standard stock solution was previously prepared by dissolving OTA (commercial standard from Sigma-Aldrich, St. Louis, MO, USA) in acetonitrile (100 mg/L). Thereafter, standard OTA solutions were prepared, by dilution in solvent (acetonitrile + 1% formic acid 98%), at concentrations: 1.0, 2.0, 5.0, 10.0, 20.0, 40.0 and 80.0 μg/L. Accordingly, matrix-matched standard OTA solutions of 1.0, 2.0, 5.0, 10.0, 20.0, 40.0 and 80.0 μg/L were obtained by adding an appropriate volume of a sample extract (wine/grape) to each serially diluted standard solution.

2.5.2. OTA quantification by UHPLC-ESI-MS/MS

Chromatographic separation was performed with a Poroshell 120 EC-C18 column (2.7 μm, 2.1 mm × 50 mm) (Agilent Technology, Santa Clara, California, USA, p/n 699775-902) using a mobile phase containing 0.2% formic acid solution (A) and acetonitrile + 0.2% formic acid (B). A linear gradient program was applied, starting with 70% A and 30% B, decreasing linearly the proportion until 5% A and 95% B in 1 min, kept constant until 2.50 min and then returning to the initial proportion. A post run interval of 1.5 min was necessary to re-equilibrate the column for the initial condition. Elution conditions were optimized at a constant flow rate of 0.5 mL/min, injection volume used was 2 μL and column oven temperature was 40 °C. The analyses were performed in a UHPLC system coupled with a 6460 triple quadrupole tandem mass spectrometer with electrospray ionization (ESI) source in the positive mode (Agilent Technology, Santa Clara, CA, USA). The ionization source operation conditions were as follows: gas temperature, 350 °C; gas flow, 11 L/min; nebulizer, 30 psi; sheath gas flow, 12 L/min; sheath gas temperature, 380 °C; capillary voltage, 3.5 kV and nozzle voltage, 0.0 kV. Sample analyses were performed in MRM (multiple reaction monitoring) scan mode, using a dwell time of 200 ms per channel) and MassHunter software workstation, version B.08.00. Two transitions were monitored for OTA to obtain at least three identification points, as recommended by the European Commission Decision 2002/657/EC (EC, 2002). The following optimized mass spectrometric parameters were established: fragmentor, 118 V and collision energy of 9 V for transitions m/z 404.1 → 357.9 (qualifier transition) and 21 V for transitions m/z 404.1 → 238.9 (quantifier transition), which was the high-intensity fragment and, for this reason, chosen for quantitation.

2.5.3. Validation of the analytical method

The analytical method developed for the analysis of OTA in grapes and wine was validated following the recommendations of the

European Commission Decision 2002/657/EC (EC, 2002) and European Commission Regulation 401/2006 (European Commission, 2006), which lay down rules on the use of sampling and analysis methods for the official control of mycotoxins levels in foodstuffs. The parameters evaluated included linearity, sensitivity, selectivity, matrix effect, precision (repeatability and within-laboratory reproducibility), trueness (recovery), limit of quantitation (LoQ) and robustness. The best fit of the analytical curve was achieved using weighted linear regression (1/x), which showed lower precision and accuracy deviations. The matrix effect was determined according to Sapozhnikova and Lehotay (2013), taking into account the slope obtained in analytical curves obtained for OTA prepared in the extract and in the solvent. For OTA identification, two product ions were monitored and the ratio of their abundance was observed. The ion ratios for MRM remained consistent both in matrix and solvent, being within the expected deviation of ± 30%, adding another layer of selectivity to the method.

2.6. Identification of ochratoxin derivatives

The search and identification of the possible elected ochratoxin derivatives present in white, red and *rose* wines (W) was performed using ESI-LTQ-XL Discover (Thermo Scientific, Bremen, Germany) mass spectrometer. Samples were filtered in polyvinylidene fluoride (PVDF) filter membranes (0.22 μm) (Jet Biofil, Guangzhou, China) (Tafuri, Meca, & Ritieni, 2008). An aliquot of 10 μL of the sample was diluted in 490 μL of methanol (J. T. Baker, Phillipsburg, NJ, USA) and homogenized under vortex for 30 s and then added of 1 μL of formic acid (Sigma-Aldrich, Darmstadt, Germany). Next, direct injection of the sample was performed. The following parameters were used: flow rate at 10 μL min^{-1}, spray current at 5 kV, capillary temperature at 280 °C, and sheath gas at 5 arbitrary units. Data were acquired in the positive mode using mass range of 200–750 *m/z* in the survey scan mode, in five replicates.

The identification of elected targets was performed using tandem MS experiments and helium as the collision gas, with energies for collision-induced dissociation (CID) ranging from 18 to 28 eV. The fragmentation analysis profile spectra of MS/MS were analyzed using XCalibur software (v. 2.4, Thermo Scientific, San Jose, CA, USA). The structures of the elected ochratoxin derivatives were proposed using our MS/MS data and by comparison with both literature data and theoretical mass fragmentation obtained with Mass Frontier software (v. 6.0, Thermo Scientific, San Jose, CA).

2.7. Statistical analysis

The analysis of variance (ANOVA), followed by the Tukey test was used to evaluate differences in OTA levels determined throughout winemaking. The significance level was set at 5% ($p < 0.05$) for all analyses performed. The analyses were performed using Sisvar software (version 5.6, 2015, Brazil) (Ferreira, 2011).

3. Results and discussion

3.1. Validation of the analytical method

The optimized method was used to quantify OTA levels in grapes and wines. The parameters used in the method validation are shown in Table 1. Selectivity assays demonstrated the viability of blank samples used in the validation procedure, as no interference signals were observed. The matrix effect observed ranged from 2.7 to 9.1%. Linear correlation coefficients (*r*) of analytical curves were higher than 0.99. Precision (repeatability and within-laboratory reproducibility) (% CV) observed was less than 19.5% and accuracy (expressed as recovery) was determined as 79.3–105.2% for all matrices at all fortification levels tested. LoD and LoQ values observed for both red and white grapes and white wine were 1 μg/kg and 2 μg/kg, respectively; whereas for red wine, the levels determined for the same parameters were, respectively, 0.5 μg/kg and 1 μg/kg. Thus, the analytical methods employed to determine OTA levels in grapes and wines were shown to be in agreement with the validation guides used (EC, 2002; EC, 2006) and, therefore, suitable for the intended purpose.

3.2. Fate of OTA

The highest OTA level produced by strains of *A. carbonarius* and *A. niger* mixture was detected in grapes from Muscat Italia (240.13 μg/kg) variety, followed by Touriga Nacional (12.33 μg/kg) and Syrah (7.97 μg/kg) varieties ($p < 0.05$) (Table 2). These results indicate a direct influence of grape variety on OTA levels produced by the strains tested.

In a previous study, lower OTA levels were detected in Syrah variety, artificially contaminated with *A. niger* (148.04 μg/g) and *A. carbonarius* (93.93 μg/g), in comparison with the values assessed for Touriga Nacional (235.52 μg/g by *A. niger* and 159.99 μg/g by *A. carbonarius*) and Muscat Italia (246.74 μg/g by *A. niger* and 115.21 μg/g by *A. carbonarius*) varieties (Freire, Guerreiro, Carames et al., 2018).

The physicochemical composition and natural metabolites of grapes can affect the metabolites produced by the fungus and the overall regulation of the synthesis of mycotoxins (Kumar, Barad, Sionov, Keller, & Prusky, 2017). A positive correlation between OTA levels produced by *A. niger* and *A. carbonarius* and parameters such as pH, total soluble solids, total glycosides in glucose and total anthocyanin; and a negative correlation with titratable acidity, pectic acid, total phenolic compounds and antioxidant capacity (DPPH and ORAC values) in grapes has been previously established (Freire, Guerreiro, Carames et al., 2018).

At the end of the winemaking, white wine, made from Muscat Italia variety, presented higher OTA levels (22.28 μg/kg), followed by red wine, made from Touriga Nacional variety (1.46 μg/kg), and *rose* wine, made from Syrah variety (< LoQ) ($p < 0.05$). These figures correlate positively with OTA levels detected in grapes.

In addition to the influence of the initial levels of grape contamination, the steps of the winemaking process will also influence the final OTA concentration determined in wine (Fig. 2). However, it is important to highlight that the reduction of free mycotoxin (OTA) levels during winemaking is due in part to physical removal, degradation, transformation into new forms or association with food components and, therefore, it does not necessarily indicate a decrease in total levels of the mycotoxin (free mycotoxin plus modified mycotoxin) in the final product (Humpf & Voss, 2004).

A 90.7% reduction in OTA levels was observed, when grapes (G) and final product (wine – W) were compared for white wine. Throughout winemaking process, the highest reduction of OTA levels occurred in crush/pressing (C) step (76.6%), followed by secondary fermentation (2F) (45.0%). Primary fermentation (1F) also contributed to lower OTA levels (26.9%), while cold stabilization (1.3%) and filtration (0.2%) had little influence ($p < 0.05$).

Throughout *rose* wine-making process, a 92.4% reduction in OTA levels was observed, when grapes (G) and final product (wine – W) were compared. Most of the reduction in OTA levels had already occurred in crush/pressing (C) step (83.4%), followed by primary fermentation (1F) (18.7%). Since initial OTA concentration was lower in grapes and a significant reduction had already occurred in the early steps (C and 1F), OTA levels were below LoQ (1.0 μg/kg), but above LoD (0.5 μg/kg) in secondary fermentation (2F), cold stabilization (CS) and filtration (W) steps. Thus, these steps (2F, CS and W) had little or no influence on reduction of the mycotoxin levels ($p < 0.05$).

During red winemaking process, an 88.2% reduction in OTA levels was observed comparing grapes (G) and final product (wine - W). Since there is no early crush/pressing (C) step (48h) in red wine manufacturing (this step is performed only at the end of primary fermentation [1F] together with a 1st racking), the highest reduction in OTA

Table 1
Parameters used in method validation for quantitation of ochratoxin A in grapes and wine.

Parameters	Criterion[a]	Red wine		White wine		Red grape		White grape	
Range of work (µg/kg)	–	1–80		2–80		2–80		2–80	
Equation of the analytical curve[b]	–	y = 366.8 x + 12.0		y = 258.9 x + 97.5		y = 248.2 x + 26.9		y = 212.3 x + 103.7	
Linearity	–	r = 0.9997		r = 0.9996		r = 0.9987		r = 0.9965	
Specificity	Interferences in blank ≤30% of RL[c]	No interferences		No interferences		No interferences		No interferences	
Matrix effect	–	7.5%		2.7%		4.6%		9.1%	
LOQ	–	1 µg/kg		2 µg/kg		2 µg/kg		2 µg/kg	
Retention time	± 0.1 min	≤0.1 min		≤0.1 min		≤0.1 min		≤0.1 min	
Ion ratio	± 30%	Complies		complies		Complies		Complies	
Robustness	Recovery between 70 and 120 CV ≤ 20%	Complies		Complies		Complies		Complies	
		Analyte concentration	**%CV**	**Analyte concentration**	**%CV**	**Analyte concentration**	**%CV**	**Analyte concentration**	**%CV**
Precision (Repeatability) (n = 5)	%CV ≤ 20%	1 µg/kg	2.2	2 µg/kg	5.4	2 µg/kg	3.9	2 µg/kg	8.3
		10 µg/kg	1.8	10 µg/kg	3.3	10 µg/kg	3.4	10 µg/kg	12.4
		80 µg/kg	3.5	80 µg/kg	1.5	80 µg/kg	1.9	80 µg/kg	16
Precision (within-laboratory reproducibility) (n = 15)	%CV ≤ 30%	1 µg/kg	19.5	2 µg/kg	10.3	2 µg/kg	12.1	2 µg/kg	8.9
		10 µg/kg	4.3	10 µg/kg	2.9	10 µg/kg	6	10 µg/kg	7.7
		80 µg/kg	6.2	80 µg/kg	1.2	80 µg/kg	5.7	80 µg/kg	9.6
Accuracy (n = 15)	% Recovery 70 - 110 (≤20%)	**Analyte concentration**	**% Recovery (%CV)**	**Analyte concentration**	**% Recovery (%CV)**	**Analyte concentration**	**% Recovery (%CV)**	**Analyte concentration**	**% Recovery (%CV)**
		1 µg/kg	102.4 (10.7)	2 µg/kg	105.2 (8.2)	2 µg/kg	101.2 (6.8)	2 µg/kg	99.3 (7.0)
		10 µg/kg	87.0 (4.6)	10 µg/kg	94.2 (3.9)	10 µg/kg	88.4 (8.8)	10 µg/kg	83.4 (8.2)
		80 µg/kg	90.3 (6.8)	80 µg/kg	93.1 (3.4)	80 µg/kg	83.5 (4.0)	80 µg/kg	79.3 (8.7)

[a] Criterion from the European Commission Decision 2002/657/EC and European Commission Regulation EC/401/2006.
[b] All analytical curves are presented using a weighed (1/x) in the linear regression.
[c] RL: reporting limit.

Table 2
Ochratoxin A (OTA) levels (expressed as μg/kg and percentages [%] of reduction) determined throughout red, *rose*, and white winemaking process.

Step	White wine			Red wine			*Rose* wine		
	OTA (μg/kg)[b]		% reduction[a]	OTA (μg/kg)[b]		% reduction[a]	OTA (μg/kg)[b]		% reduction[a]
Grape (G)	240.13^{cD}	± 0.71	–	12.32^{bC}	± 0.64	–	7.97^{aB}	± 0.19	–
Crush/pressing (C)	56.26^{bC}	± 0.28	76.57^{aD}	Na	Na	Na	1.32^{aA}	± 0.03	83.44^{bC}
End of primary fermentation (1F)	41.14^{cB}	± 1.80	26.87^{bB}	4.72^{bB}	± 0.04	61.70^{cC}	1.07^{aA}	± 0.09	18.76^{aB}
End of secondary fermentation (2F)	22.62^{cA}	± 0.74	45.02^{bC}	2.00^{bA}	± 0.09	57.57^{cC}	< LoQ		$> 6.70^{aAc}$
End of cold stabilization (CS)	22.32^{bA}	± 0.26	1.31^{aA}	1.43^{aA}	± 0.04	28.50^{bB}	< LoQ		
After filtration (W)	22.28^{bA}	± 0.55	0.17^{aA}	1.46^{aA}	± 0.00	-1.87^{aA}	< LoQ		
% Total reduction			90.72^{b}			88.15^{a}			92.44^{c}

Na: not available (step not performed).

[a] Reduction in OTA levels, expressed as percentage (%) and calculated in comparison to the previous step. Average values followed by lowercase letters compare OTA reduction (%) determined in a same micro-winemaking step for different wine varieties (white; *rose*; red); uppercase letters compare OTA reduction (%) observed at different micro-winemaking steps (G; C; 1F; 2F; CS; W) for a same wine variety. Different letters show statistically significant difference ($p < 0.05$).

[b] Average values followed by lowercase letters compare OTA levels (μg/kg) determined in a same micro-winemaking step for different wine varieties (white; *rose*; red); uppercase letters compare OTA levels (μg/kg) observed at different micro-winemaking steps (G; C; 1F; 2F; CS; W) for a same wine variety. Different letters show a statistically significant difference ($p < 0.05$).

[c] Below LoQ (Limit of quantification) and above LoD (Limit of detection); LoQ was considered for the calculation of the reduction in OTA levels.

levels occurred in primary (61.7%) and secondary (57.6%) fermentation steps (1F and 2F, respectively). Cold stabilization (CS) also contributed to lower the mycotoxin levels (28.5%), but not filtration step (-1.9%) ($p < 0.05$).

Although all wines showed a remarkable reduction in OTA levels as observed at the end of winemaking, *rose* and white wines had higher reduction rates (92.4% and 90.7%, respectively) in comparison with red wine (88.2%). The absence of the maceration step in *rose* and white wines, due to early crush/pressing (C) step, seems to be the most relevant factor for the reduction of the mycotoxin levels observed throughout the process.

In contrast, several studies (Cecchini et al., 2019; Csutorás et al., 2013; Dachery et al., 2017) have observed a more pronounced reduction of OTA levels in red wines when compared to *rose* and white wines throughout fermentation process. For instance, a reduction of 73, 85, and 90% in white, *rose*, and red wine musts, respectively, was observed by Csutorás et al. (2013) during 90 days of fermentation. It is possible that the interaction between polyphenols (such as anthocyanins) and OTA interferes in the percentage reduction during fermentation. Cecchini et al. (2019) observed a higher reduction in OTA levels in red wines (39–51.6%) in comparison to white wines (around 29%), although the decrease in OTA content observed was not proportional to anthocyanin concentration in wine.

In these studies, the most significant factor in the reduction of OTA levels seems to be a higher presence of particles from maceration step that will act as OTA adsorbents. These particles act as a sponge coated with negative charges interacting with the acidic feature of toxins (Huwig, Freimund, Käppeli, & Dutler, 2001; Ponsone, Chiotta, Combina, Dalcero, & Chulze, 2009). An ionic bonding between OTA and anthocyanins may occur, as well as an esterification reaction between the carboxyl group of the OTA molecule and the hydroxyl group of anthocyanins (Cecchini et al., 2019). However, it is likely that these complexes formed will not be completely removed during winemaking (in racking steps) and will remain in the final product, resulting in masked OTA (Freire & Sant'Ana, 2018). This alteration on OTA structure (binding to must components) alters the characteristics of the molecule, such as structure, polarity and solubility, and makes impossible its quantification by the use of conventional analytical

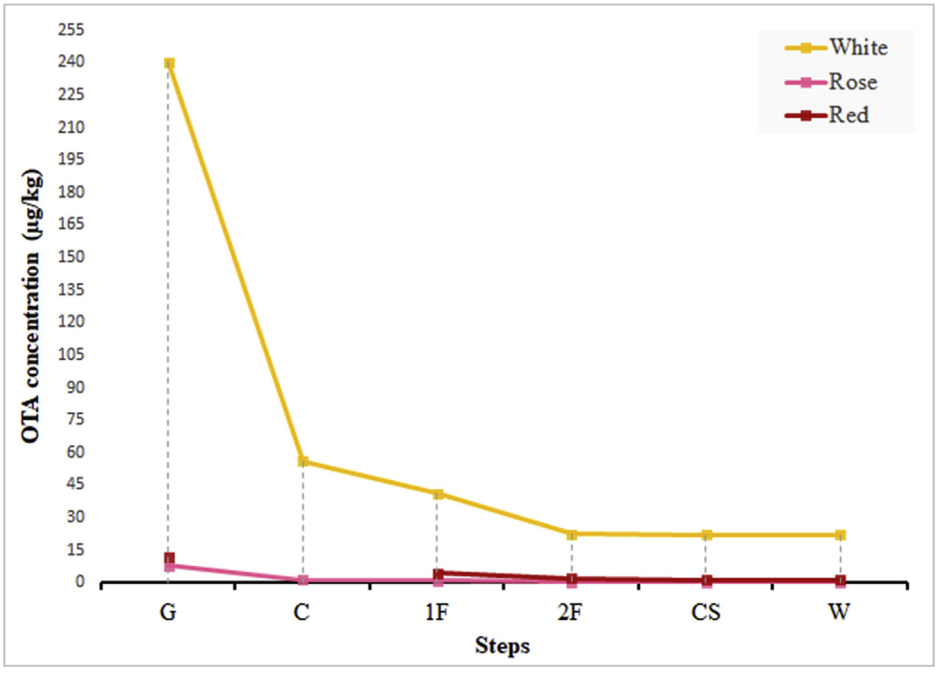


Fig. 2. OTA levels (μg/kg) obtained during red, *rose*, and white micro-winemaking process. G: grape, after crush; C: must, after crush/pressing; 1F: must, at the end of primary fermentation; 2F: must, at the end of second fermentation; CS: must, at the end of cold stabilization; W: wine, after filtration. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

methods, leading to a false reduction of OTA levels and, therefore, underreporting of total mycotoxin levels in wine (Berthiller et al., 2013).

According to our data, by comparison of OTA levels initially present in grapes (G) and those obtained up to primary fermentation step (1F), a reduction of 82.9% was observed in white wine and 86.6% in *rose* wine, whereas this figure corresponded to only 61.7% in red wine. The separation of the pomace in white and *rose* wines occurred after 48h (crush/pressing [C]), while in red wine it only took place at the end of the first fermentation (1F) (7 days), which resulted in a longer contact time between the pomace and the must. In this regard, a higher extraction rate of OTA from seeds (pomace) to must occurred increasing initial levels of OTA in red wines. According to Battilani, Pietri, and Logrieco (2004) an increase of approximately 20% in OTA levels can be observed after the maceration step. The increase of the alcohol concentration in the partially fermented must may favor the extraction of OTA from the solid phase to the liquid phase. However, at later steps, the increase of yeast biomass helps to reduce toxin levels (Lasram et al., 2008). Therefore, when winemaking process was assessed as a whole, the percentages of reduction observed for OTA levels were 90.7, 92.4 and 88.2% for white, *rose* and red wines, respectively.

The results obtained in the present study corroborate the findings of Lasram et al. (2008). According to these authors, after 1 day of the alcoholic fermentation, a significant increase (59.5% of the initial OTA content) in OTA levels was initially observed in red must. However a significant decrease (50.9% of the initial OTA content) in OTA levels was determined in *rose* must. Despite that, at the end of the fermentation process a similar decrease in the initial OTA content was observed in both red (41.0%) and *rose* (44.0%) wines.

In addition to solid-liquid separation (crush/pressing and racking) steps, other factors that occur throughout winemaking process, such as OTA transformation into other metabolites, mycotoxin adsorption by yeasts and components from musts, pomace and lees, will also be responsible for the reduction of OTA levels in wine.

The reductions in OTA levels determined in primary and secondary fermentation steps (1F and 2F, respectively) are closely related to the performance of the present microorganisms. Filamentous fungi species such as *A. niger* and *A. cabonarius* (Bejaoui, Mathieu, Taillandier, & Lebrihi, 2005), lactic bacteria (Abrunhosa et al., 2014), as well as yeasts, including *S. cerevisiae* (Petruzzi et al., 2015), were shown to reduce OTA levels in grape juices and musts (30–80%), growth medium (50–90%) and must (20–76%), respectively. This reduction has been related to the phenomenon of adsorption by cell wall components.

Glucogalactans, glucans, mannoproteins and mannans have been named as responsible for the binding of OTA to yeast cell wall (Chen et al., 2018). Conversely, exopolysaccharides and peptidoglycans appear to be responsible for such binding in LAB (Dalié, Deschamps, & Richard-Forget, 2010). At last, the adsorption phenomenon has been associated with hydrophobic interactions in filamentous fungi (Bejaoui et al., 2005).

However, such ability seems to be variable among strains. Angioni et al. (2007) did not detect OTA residues adsorbed on the cell wall of yeasts tested. The authors suggested that the reduction in OTA levels observed after fermentation was due to OTA degradation to other undetected metabolites. Furthermore, the *S. cerevisiae*-OTA binding may be reversible, with subsequent release of up to 85% of the initially adsorbed OTA (Petruzzi et al., 2015). A slight increase in OTA level was observed in culture medium after being adsorbed by *A. japonicus* and *A. carbonarius* conidia (Bejaoui et al., 2005).

After fermentation, cold stabilization (CS) is performed to remove suspended solids that make the wine cloudy (Gil-Serna, Vázquez, González-Jaén, & Patiño, 2018). This step influenced the reduction of OTA levels only in red wine (28.50%). The present study demonstrated that the percentage reduction in OTA levels throughout the process is cumulative until reaching a maximum reduction (approximately 90%). For white and *rose* wines this maximum reduction was already reached by the end of fermentation. For red wine, the reduction still continued during cold stabilization (CS) due to adsorption of residual mycotoxin by lees and subsequent separation of the liquid and solid phases through racking.

According to other studies (Gentile et al., 2016; Quintela, Villarán, Lopez de Armentia, & Elejalde, 2011) lower OTA levels were observed in commercial *rose* and white wines in comparison with red wines, because the pomace has been separated from the must earlier (anticipated crush/pressing [C]). In contrast, Čepo et al. (2018) determined the highest OTA concentrations (0.24 μg/L) in white wine. The authors justified the higher concentration due to the lack of good winemaking practices. In the present study, three different grape varieties were contaminated with the same amount of inoculum. However, different OTA concentrations were detected in Muscat Italia (240.13 μg/kg) (used to make white wine), Touriga Nacional (used to make red wine) (12.33 μg/kg) and Syrah (used to make *rose* wine) (7.97 μg/kg) grape varieties ($p < 0.05$). Consequently, white wine presented higher OTA levels (22.28 μg/kg), followed by red (1.46 μg/kg) and *rose* (< LoQ) wines ($p < 0.05$). Therefore, the influence of grape variety on final OTA levels in wines may be more relevant than the different steps used in red, *rose*, and white winemaking process.

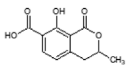
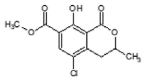
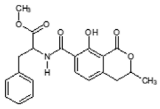
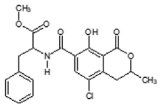
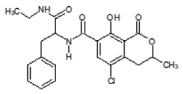
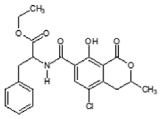
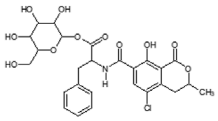
An efficient reduction of OTA levels is due to a set of factors, such as process steps and initial contamination levels. However, if the initial OTA concentration is high, the process steps will not be capable to result in safe levels of OTA. Considering the smallest reduction in OTA level observed in this study (88%), it is proposed that the maximum OTA level in grapes should be less than 16 μg/kg to ensure that the levels of this mycotoxin are below the maximum limit allowed by legislation from several countries (2 μg/kg) (Brazil, 2011; EC, 2006). However, although a recognized safe raw material is used, OTA initially present in grapes can be transformed into other metabolites still on the vine and throughout winemaking, which makes it impossible to determine the real fate of total mycotoxins (free plus modified) during winemaking and final product. Therefore, control of OTA levels in the raw material alone is not sufficient to guarantee the toxicological safety of the end product (Freire & Sant'Ana, 2018).

The best strategy to reduce health risks associated with the ingestion of mycotoxin-contaminated wine is to prevent contamination of the grapes by toxigenic fungi still in the field and throughout the process. Practices such as the use of biological control methods in the vineyard, avoiding the storage of grapes after harvest and commencement of fermentation right after removal of grapes from bunches drastically reduce the probability of growth of toxigenic fungi and, consequently, ochratoxins production. If contamination has already occurred, the following strategies can be used to reduce OTA levels, although its complete elimination is considered impossible: use of selected strains of yeasts and LAB during fermentation, which are able to adsorb and metabolize OTA to less toxic compounds; use of non-contaminated pomace and lees obtained from other processes to act as an adsorbent and other allowed chemical adsorbents, and dilution of contaminated grapes/wine with non-contaminated grapes/wine.

3.3. Formation of ochratoxin derivatives throughout winemaking

The reduction of OTA levels in wines has been justified by several studies (Angioni et al., 2007; Cecchini et al., 2006; Chen et al., 2018; Petruzzi et al., 2015) as a phenomenon of adsorption of mycotoxin onto the yeast cell wall and must components, since ochratoxin derivatives were not detected in musts evaluated by those authors. However, most of these studies only evaluated derivatives ochratoxin α and phenylalanine using HPLC with fluorescence detection (HPLC-FL). In fact, the combined use of HPLC-FL and immunoaffinity column clean-up (IAC) or solid phase extraction (SPE) is considered the most used method for detection of ochratoxin A in several foods (Alcaide & Aguilar, 2008; Cecchini et al., 2019; Petruzzi et al., 2015). Nonetheless, it is known that modified mycotoxins may not be detected by traditional analytical

Table 3
Tandem Mass Spectrometry acquisition parameters for mycotoxin.

Mycotoxin	Structure	Molecular formula	Theoretical Mass	Adduction ion	Precursor ion (m/z)	MS/MS fragmentation
Ochratoxin β		$C_{11}H_{10}O_5$	222.05	$[M + H]^+$	223	163, 177, 205
Ochratoxin α methyl ester		$C_{12}H_{11}ClO_5$	270.03	$[M + Na]^+$	293	187, 233, 247
Ochratoxin B methyl ester		$C_{21}H_{21}NO_6$	383.14	$[M + H]^+$	384	252, 352, 366
Ochratoxin A methyl ester		$C_{21}H_{20}ClNO_6$	417.10	$[M + Na]^+$	440	348, 404, 422
Ethylamide ochratoxin A		$C_{22}H_{23}ClN_2O_5$	430.13	$[M + Na]^+$	453	361, 417, 435
Ochratoxin C		$C_{22}H_{22}ClNO_6$	431.11	$[M + K]^+$	470	342, 434, 452
Ochratoxin A glucose ester[a]		$C_{26}H_{28}ClNO_{11}$	565.14	$[M + H]^+$	566	298, 534, 548

[a] Molecule detected only in red and *rose* wines.

methods used to quantify the parent mycotoxin due to structural and physical modifications of the molecule (Berthiller et al., 2013). Such transformations imply changes in chromatographic parameters and even in extraction efficiency (Freire & Sant'Ana, 2018). Therefore, it is extremely important to search for sensitive and selective techniques capable to elucidate and detect molecules derived from the parent mycotoxin. In this respect, mass spectrometry is an effective strategy for the detection and elucidation of modified mycotoxins in foods due to its versatility and sensitivity (Freire et al., 2019).

Among the sought targets, a number of ochratoxin-derived candidates were identified: ochratoxin β, ochratoxin α methyl ester, ochratoxin B methyl ester, ochratoxin A methyl ester, ethylamide ochratoxin A, ochratoxin C and ochratoxin A glucose ester (Table 3). The putative identification of these compounds occurred in all wines, with the exception of ochratoxin A glucose ester, which was detected only in red and *rose* wines.

These derivatives identified may have been formed by the fungus itself present in the grapes (Freire, Guerreiro, Pia et al., 2018), by the action of the yeast used in the fermentation (Freire et al., 2019) or through reactions with components from both grapes and must (Freire, Guerreiro, Carames et al., 2018).

A. carbonarius and *A. niger* strains isolated from grapes were shown to degrade OTA to ochratoxin α in synthetic grape juice (Bejaoui, Mathieu, Taillandier, & Lebrihi, 2006). These microbial species are also able to produce other derivatives, including ochratoxin β, ochratoxin B, ochratoxin C and methyl esters through their enzymatic complex (Remiro, Irigoyen, González-Peñas, Lizarraga, & López de Cerain, 2013).

The yeast *S. cerevisiae* produces extracellular enzymes such as glucosidase, pectinase and xylanase (Strauss, Jolly, Lambrechts, & van Rensburg, 2001) that can act on the hydrolysis of OTA or its conjugation with components present in the must during fermentation, leading to the formation of several derivatives (Freire et al., 2019).

The formation of ochratoxin derivatives may also be related to the matrix association phenomenon. In this case, acid conditions of the must favor the ionization of the amino group of OTA molecule and an interaction with medium components (Cecchini et al., 2006). Polysaccharides, pectic substances, lignin and proteins present in grapes and must can also bind to OTA through ionic interactions (Cecchini et al., 2019; Valenta, 1998).

Ochratoxin C, ochratoxin B methyl ester and ochratoxin A methyl ester derivatives may have been formed by dechlorination and esterification reactions carried out by the fungus metabolism itself or by the yeast (Freire et al., 2019). Moreover, pH of the must, pectin hydrolysis and the presence of acids may also favor the esterification reaction (methyl and ethyl group addition). These same derivatives have been previously detected in wines (Remiro, González-Peñas, Lizarraga, & López de Cerain, 2012; Remiro et al., 2013). The formation of OTA methyl esters, ochratoxin B, and ochratoxin α in the presence of a strong acid and high methanol concentration has also been observed (Li, Marquardt, & Frohlich, 2000).

The conjugation reactions of OTA and proteins and sugars present in the must may have been responsible for the formation of ethylamide ochratoxin A and ochratoxin A glucose ester due to the high affinity between OTA and proteins (Duarte, Lino, & Pena, 2012) and its binding properties with sugar (Bittner, Cramer, & Humpf, 2013). Ethylamide ochratoxin A has been previously identified in grape-based medium after *A. niger* inoculation (Freire, Guerreiro, Pia et al., 2018) and also in grapes from Syrah variety (Freire, Guerreiro, Carames et al., 2018). This same molecule has been also detected in fermentation broth in the presence of *S. cerevisiae* (Freire et al., 2019).

The putative identification of ochratoxin A glucose ester only in red and *rose* wines, made with red grapes from Touriga Nacional and Syrah varieties, respectively, suggests a possible influence of the grape variety on the formation of modified mycotoxins throughout processing. However, further studies are needed to quantify these compounds and

to evaluate their correlation with physicochemical characteristics of wine grapes.

Although the ochratoxin α derivative was not detected, it was most likely formed during the process and then transformed into ochratoxin α methyl ester. In fact, ochratoxin α is formed due to the hydrolysis of the amide bond by the action of hydrolytic enzymes such as carboxypeptidases, proteases, lipases and ochratoxinase (Abrunhosa, Santos & Venancio, 2006; Dobritzsch, Wang, Schneider, & Yu, 2014; Stander, Bornscheuer, Henke, & Steyn, 2000). Bejaoui et al. (2006) also reported the conversion of ochratoxin α to unidentified derivatives. Yeasts and the fungus itself, still present in grapes, may have been responsible for this conversion due to their extensive enzymatic machinery (Freire et al., 2019).

Although most derivatives, which toxicological properties were previously investigated, present a minor deleterious effect on health (Freire et al., 2019), a major issue is the possible conversion of the modified mycotoxin onto the parent mycotoxin during processing or by human and animal metabolism (Freire & Sant'Ana, 2018). Ochratoxin C has been shown to be converted to OTA throughout storage, increasing OTA levels in wine (Remiro et al., 2012). Furthermore, the co-occurrence of these compounds may act additively or synergistically with OTA, increasing the levels of total mycotoxins in the food and, consequently, the health risk due to the consumption of contaminated wine.

Despite the effort for mycotoxin detoxification, several ochratoxin-derived candidates were detected in wines. In this sense, it is necessary to consider that wine may be contaminated with a mixture of mycotoxins. These results indicate an underestimation of total mycotoxin levels in wine and the need to include techniques for detection and quantification of multi-mycotoxins.

This study contributed significantly to the elucidation of the presence of modified mycotoxins in foods. However, further investigations including the use of naturally contaminated samples and isotopic patterns are needed to establish the fate and the stability of these mycotoxins throughout winemaking process, as well as to estimate possible health risks.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodcont.2020.107167.

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