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Evolution of ochratoxin A content from must to wine in Port Wine microvinification

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Abstract To study the evolution of ochratoxin A (OTA) content from must to wine during the making of Port Wine, grapes from the five most common varieties of Port Wine were harvested and combined in equal percentages in order to perform microvinifications. Three sets of assays were studied: a blank (A), where the most common Port Wine-making process was used; in the second (B), a solution of OTA was added to the initial must; in the third (C), the grapes were aspersed with an inoculating solution of OTA-producing fungi. Samples were collected, in duplicate, on four different occasions throughout the process. The influence of the addition of SO_2 to the must was also assessed in each set. The quantification of OTA was based on the standard reference method for wines (European Standard prEN 14133), which includes clean-up via immunoaffinity columns and HPLC with fluorescence detection. The limits of detection were 0.076 µg/l for wine and 0.114 µg/l for must. The method was validated by assessing the precision, accuracy and by obtaining an estimate of the global uncertainty. Overall, the levels of OTA observed during the vinifications dropped by up to 92%, and no grapes used in this work were contaminated naturally.

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Introduction

The wine industry plays an important role in the Portuguese economy, which is one of the reasons to search for good quality standards in Portuguese wines. Besides careful organoleptic characterization, wines are often controlled for trace compounds that may eventually act as contaminants and, therefore, be a source of concern to the consumer. In recent years, there has been a growing awareness of the presence of ochratoxin A (OTA) in foodstuffs. OTA is a mycotoxin (potentially toxic secondary metabolites derived from fungal contamination) produced by strains of *Aspergillus* and *Penicillium*. It is a potent nephrotoxin and hepatoxin with teratogenic, mutagenic and immunosuppressive effects [1], and has been classified as a possible human carcinogenic [2]. Discovered in 1965 by Van der Merwe et al [3], OTA was first reported in wine in 1995 by Zimmerli and Dick [4] and, since then, investigations into the presence of this compound in grapes, grape juices, musts and wines have been triggered in several countries worldwide (France [5], Japan [6], United Kingdom [7], Portugal [8, 9], Italy [10], Chile [11], Greece [12], South Africa [13], Spain [14], Brazil and Argentina [15] and a global survey by Soleas et al [16], amongst many others). *Aspergillus ochraceus* (in tropical climates) and *Penicillium verrucosum* (in temperate and cold climates) are commonly recognized as the main OTA producers [17]. Regarding grapes, the responsibility for OTA contamination has been attributed to black aspergilli (Mostly *A. carbonarius*, and *A. niger* aggregate strains) [18–20]. The presence of this mycotoxin has also been found in other commodities such as cereals, beans, groundnuts, spices, dried fruits, coffee, beer, cheese [21] and even cocoa [22], or products derived from mainly non-ruminant animals [23], such as

milk, pig blood, liver and kidney, and poultry meat [21]. This chain of contamination reaches up to humans, as OTA has been found in human milk [1] and blood [24]. Thus, the main concern is the fact that human exposure to OTA is more likely to be from low level contamination of a wide range of different foods than from high level ingestion of a single food source. Although already regulated for some foods, there is still no legal limit on OTA in grape products. However, the Office International de la Vigne et du Vin (OIV) recommends a maximum level in wine of 2 μg/l [25]. Values reported for wines above this limit are scarce and refer to southern Europe and North Africa regions (Mediterranean climates), that are more prone to contamination than those originating from central Europe [26, 27]. There is no evidence that OTA levels are above 2 μg/l in Portuguese wines (Port Wine and Vinho Verde) [8, 9].

Several techniques for reducing the incidence of OTA have been tried, both preventive and corrective. The content of OTA has been studied in the processing of foods like coffee [28, 29], wheat [30] and cocoa [31]. Focusing on grape products, the conditions and factors required for the development of OTA have also been assessed [32, 33], as well as the response to different treatment strategies [34]. Since corrective measures applied in the final product, such as adsorption with chemical agents [35, 36] and biodegradation [37, 38] are yet to prove practical due to the associated degradation of wine properties, the emphasis is currently on prevention. However, although the fungi that produce OTA, the chemical structure of it, and its stability and toxicity are all known, its appearance and metabolic pathway is still uncertain before and during winemaking. It is not clear how the mycotoxin is distributed between the juice and the skin in the grape, or how vinification affects its content in wines, despite a few studies on this subject. Bellí et al [39] noticed significant grape contamination from setting to harvest; Fernandes et al [40] mentioned a decrease in OTA content during a Vinho Verde vinification trial; Arici et al [41] followed the increase of OTA during the production of grape juice from mouldy grapes.

No data has been published previously on the fate of OTA during a specific vinification like that for Port Wine. Hence, this work intended to study the evolution of OTA content during the making of Port Wine (a fortified wine in which fermentation is arrested at some point) from the must to the wine.

Several analytical methodologies were attempted in order to determine the OTA in the wine, such as immunoaffinity columns (IAC) and HPLC-FD [42, 43], solid-phase extraction (SPE) and reversed phase HPLC-FD [44], SPE and HPLC-PDA or GC-MS [16], liquid–liquid extraction (LLE) and HPLC-FD [45], liquid-phase microextraction (LPME) with HPLC-FD [46], and RP-HPLC-FD with no prior extraction [47]. A comparison of different methods was performed by Leitner et al [48]. For grapes, Serra et al [49] studied several extraction procedures prior to HPLC-FD. In the current work, the quantification of OTA in both musts and wines was based on the standard reference method for wines [50], which includes prior extraction using immunoaffinity columns and determination by HPLC with fluorescence detection. Some adaptations were made and are described later. In order to validate this methodology we evaluated the limits of detection, the precision, the accuracy and estimated the global uncertainty associated with the results, following the rules given in the EURACHEM/CITAC Guide [51].

Experimental

Grape collection

Grapes from the five most representative red Port Wine varieties (Tinta Roriz, Touriga Nacional, Touriga Franca, Tinta Barroca and Tinto Cão) were collected at harvest time and according to the rules commonly employed in viticulture, in Quinta de Santa Bárbara, property of the Centro de Estudos Vitivinícolas do Douro (CEVD), located in the Douro region in the north-east of Portugal (see Fig. 1 for quantities).

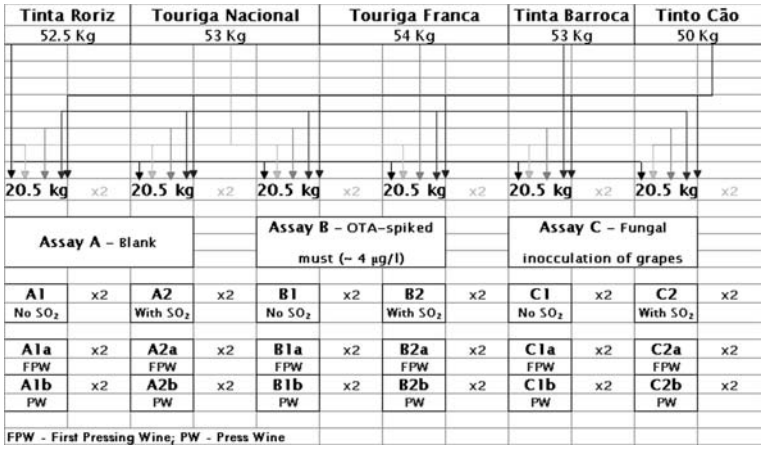

Fig. 1 General scheme for the microvinification assays

Grapes and must contamination

The OTA contamination was derived by either spiking with an alcoholic solution of OTA or inoculating with an *Aspergillus carbonarius* spore suspension.

In the first case, a concentrated 18% ethanol OTA solution was added to the grapes immediately after crushing so that an average OTA concentration of about 4 μg/l was achieved. The inoculation was obtained by spraying the grapes with a spore suspension (10^3 spores/ml) furnished by the MUM culture collection (University of Minho, Braga, Portugal) [52] and then allowing them to incubate in an isolate chamber at 20 °C, until the first signs of damage to the grapes were observed (after five days, in this case).

Vinifications

Microvinification experiments were performed according to the technologies currently employed in Port Wine production, from the whole grape until the clarified wine. First, the grapes are crushed, which produces a combination of two phases: liquid (must) and solid (pomace, comprising the skins and the seeds). When programmed, a solution containing a sulphur dioxide generator is added at this point. Then the mixture is placed in an appropriate stainless steel chamber, where fermentation will take place. For Port Wine, the fermentation is arrested at a given point by the addition of a 77% (v/v) alcohol distillate. This moment is determined by the density of the mixture needed to attain certain properties of the wine (1.04 in this case), using adequate charts. Then, the mixture is manually pressed and the liquid part is collected (first pressing wine). The remaining solid phase is further pressed mechanically to yield the last liquid-phase collection (press wine).

The scheme used in this study for the microvinification assays is displayed in Fig. 1.

The five grape varieties were combined in equal parts to attain a total of 20.5 kg of grapes at the beginning of each assay. Three main sets of assays (A–C) were planned, and two distinct situations were observed in each: with or without the addition of a sulphur dioxide-generating agent. Assay A was the blank, corresponding to the common-used vinification process with no external OTA contamination; Assay B corresponded to the spiking of the must and pomace with the ethanolic OTA solution to obtain approximately 4 μg/l; in Assay C the grapes were inoculated with the spore suspension. This scheme of assays was performed in duplicate (experiments I and II), at an average room temperature of 25 °C

Sampling

Must and wine samples were collected on four distinct occasions during the vinification process: immediately after grape crushing (must); after the beginning of the fermentation, controlled by the temperature and density of the liquid–solid mixture (must); and twice after the fermentation was arrested. These last two samples corresponded to first pressing wine and press wine.

Quantification of OTA

The methodology is based on the reference method for wines, described in the European Standard prEN 14133 [50], comprising clean-up by immunoaffinity columns followed by HPLC quantification. For musts, one modification was made: in the clean-up, the initial ratio of must:dilution solution (PEG) was 25:125 ml [42] (instead of the typical 10:10 ml used for wines). Even so, and due to logistic limitations, some adaptations had to be made to the described procedures:

- The stock solution of OTA was prepared in toluene/acetic acid (99:1, v/v) and the calibration standards, six (0.2, 0.6, 2.0, 6.0, 12.0 and 20.0 μg/l) instead of the proposed five, were obtained from that solution diluted in the mobile phase.
- After OTA clean-up using the immunoaffinity columns, the ethanol was evaporated to dryness on the rotary evaporator (instead of under nitrogen current), and the content was re-dissolved in 1 ml of mobile phase (instead of 250 μl).
- The HPLC injection volume was 118 μl instead of 100 μl.

All samples were injected in duplicate.

Chemicals

The OTA standard was purchased from Sigma (St. Louis, MO, USA). Sodium hydrogen carbonate and sodium chloride p.a. were from Panreac (Barcelona, Spain) and polyethylene glycol 8000 MicroSelect was from Fluka (Buchs, Switzerland). Acetonitrile HPLC grade and methanol p.a. were furnished by Riedel-de Haën (Seelze, Germany) and acetic acid glacial (100%) by Merck (Darmstadt, Germany). Distilled water was produced in our laboratory.

Apparatus

A 12-entry multi-extractor from Whatman (Brentford, UK) with a D-70112 vacuum pump from KNF Neuberger (Freiburg-Munzingen, Germany) were used for the clean-up. The immunoaffinity columns were Ochratest from Vicam (Watertown, MA, USA). A R-1140 rotary evaporator with a B-480 water bath and a B-169 vacuum system from Büchi (Flawil, Switzerland) assisted by a Edwards RV3 high vacuum pump (Crawley, UK) were used to evaporate the cleaned samples to dryness.

The HPLC was a Merck-Hitachi L-7250 (Tokyo, Japan), equipped with a Merck-Hitachi F-1080 fluorescence detector, L-7250 programmable auto-sampler and a Merck-Hitachi D-7000 interface. The data acquisition employed HPLC System Manager (HSM) software, Version 3.1 (Hitachi). A Macherey-Nagel (Düren, Germany) end-capped Superspher reversed-phase column (C_{18}) was used (dimensions 250× 4 I.D. × 4 μm) with a pre-column, at room temperature. The mobile phase was isocratic water:acetonitrile:acetic acid (99:99:2, v/v/v) at 1 ml/min. The runtime was 13 min, with OTA detected at 333 nm (excitation) and 460 nm (emission) wavelengths.

Results and discussion

The evolution of OTA concentration throughout the vinification process (from the must to the wine) commonly-used to make Port Wine was assessed using the aforementioned different approaches. The most significant trends are described, and some comparisons are made between experiments I and II, Assays A–C, and also the replicate samples. The influence of the presence of SO_2 was another feature. These findings may help clarify the sources and main pathways of OTA in vine and wine. Before the quantification, it is essential to know the uncertainty associated with the analytical results. Therefore, the method was validated using the usual parameters, and the global uncertainty (U) was calculated.

Method validation

Linearity, precision and accuracy were studied in order to validate the analytical method. Good linearity was achieved within the calibration range chosen ($R^2 = 0.9993$). The limit of detection (LOD) was calculated according to Miller and Miller [53] and derived from the linearity parameters of the method calibration (the sum of the intercept and three times $s_{y/x} = [S(y_i - y_{icalc})2/(n-2)]^{1/2}$, where y_i represents the experimental values, y_{icalc} is calculated from the calibration curve and n is the number of standards). For the wines, the LOD was 0.076 μg/l, whereas the value was higher (0.114 μg/l) for the musts. This discrepancy is justified by the different dilutions for wines and musts when undergoing immunoaffinity column clean-up, explained previously. The precision was evaluated by determining the repeatability (coefficient of variation of ten independent assays of the OTA standard 6.0 μg/l performed under the same analytical conditions in the same day) and the intermediate precision (coefficient of variation of eight assays of OTA standard 6.0 μg/l performed on different days). The results were 1.22 and 5.89%, respectively. The recoveries for assays performed with six must samples and six wine samples spiked with the 6.0 μg/l OTA standard accounted for the estimation of the accuracy. For musts, the average recovery was 82.4 ± 16.9% and for wines, 92.1 ± 11.7%. These results are within the expected range for similar methodologies and concentration ranges.

Based on these findings, and in complementary calculations, the global uncertainty (U) associated with the results was found [51] and plotted as a function of the calibration range (Fig. 2).

Throughout almost the whole calibration range, U remains below 10%. The values of U increase exponentially when the concentration becomes very low (below 2 μg/l), reaching just over 50% in the neighborhood of the chromatographic LOD (and consequently the LODs for musts and wine, which are derived from it, as previously explained).

OTA from must to wine

As mentioned earlier, two equal experiments (I and II) were performed following the proposed microvinification scheme. Figure 3a,b shows the respective results obtained.

Note that no natural contamination of OTA was observed. That is, for the blank assays (A), no OTA was detected, and therefore, these assays are not presented when the results are displayed. Also, for Assay B, the contamination of OTA by spiking could cover the intended concentration (4 μg/l). As can be seen in the first columns (corresponding to initial must, *IM* or after SO_2, *AS*, when applicable), in experiment I, the contamination varied from 2.24 to 7.38 μg/l, and in experiment II, from 3.36 to 7.91 μg/l. However, if we refer to average values, these are, respectively, 4.70 and 5.44 μg/l. This difference can be explained by the unpredictability of the proportional yield of must to pomace produced after grape crushing, which makes the sampling accuracy quite troublesome. Furthermore, when the spiking solution is mixed, the partition between the solids and the must can be distinct. These problems were also noticed in the inoculation assays (C). However, some contamination was attained under the proposed conditions, although, as expected, at much lower levels than

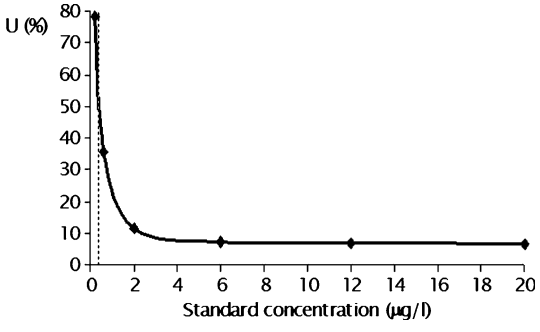

Fig. 2 Global uncertainty (U) as a function of the calibration OTA standards (– – – chromatogaphic LOD)

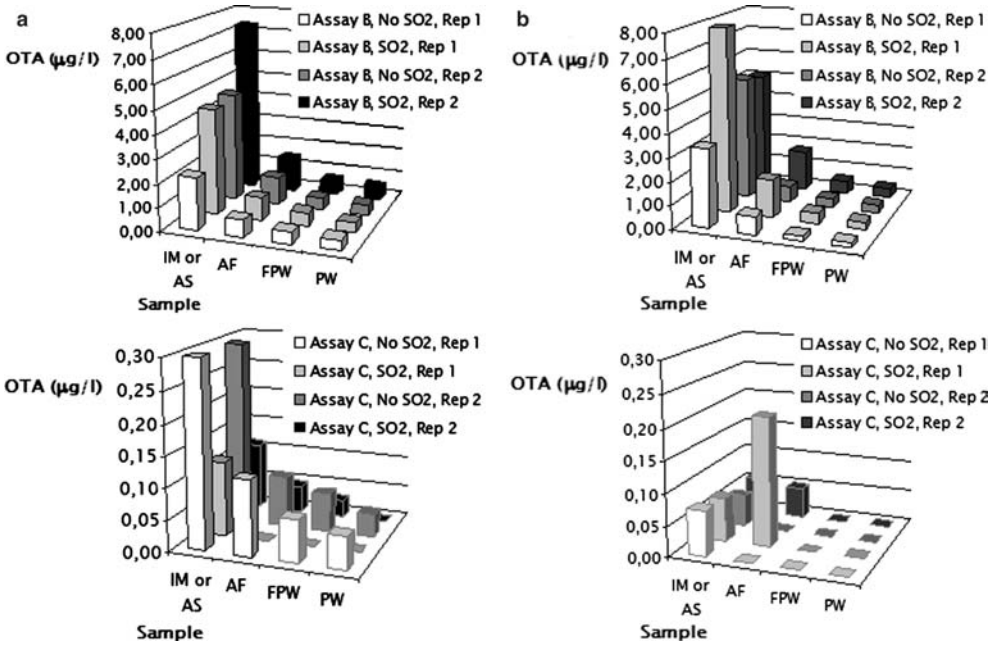

Fig. 3a–b **a** OTA content throughout vinification in experiment I (*IM* initial must, *AS* after SO_2, *AF* after start of fermentation, *FPW* first pressing wine, *PW* press wine, *Rep* replicate). **b** OTA content throughout vinification in experiment II (*IM* initial must, *AS* after SO_2, *AF* after start of fermentation, *FPW* first pressing wine, *PW* press wine, *Rep* replicate). Columns with faint (gray rather than black) outlines indicate values below the LOD

the direct spiking. In fact, in experiment I, the average contamination was 0.23 µg/l (min = 0.11, max = 0.39), whereas for experiment II, the average OTA concentration was even lower: 0.06 µg/l (min = 0.05, max = 0.07). As already known, the conditions are even more difficult to control in terms of fungal contamination. But if these values for Assay C are a good representation of what happens in the vineyards, it is apparent that the contamination (before the vinification) is still well below the limit value of OIV (2 µg/l) for wines.

The general trend for the evolution of OTA concentration throughout the vinification process is clear. Apart from one discrepant value in experiment I, Assay C (perfectly acceptable in light of the global uncertainty values for the concentrations mentioned), OTA levels decreased markedly in all situations, as can be seen in Fig. 3a,b. When summing up and averaging all of the replicates from both experiments, distinguishing only between Assays B and C and plotting the OTA decrease as a function of the four sampling points chosen, this tendency is even more recognisable (Fig. 4).

Clearly, the decrease in OTA is more pronounced from the initial must to after the start of fermentation (averages are 77% for Assay B and 56% for Assay C). This was also the longest period of time between sample collections (it took about 50 h for fermentations to start, on average). From this point to the end of the fermentation (about 25 h in average), where the liquid phase is already considered to be "wine", the reduction of OTA reached 90 and 87% for Assays B and C, respectively, which was then further reinforced after the final pressing to yield a total reduction of 92% for both B and C. This significant reduction can be explained by the partition of OTA between the liquid and the solid phase during vinifications. In fact, there must be extensive adsorption of OTA to the solid parts of the grapes. Fernandes et al [40], who reported a decrease in OTA content from must to wine, noted that the presence of biomass could favor such a trend in the must, which could also be due to an adsorption mechanism onto its surface, explained by the overall negative charge in the cell walls and the acidic nature of OTA [36]. Otteneder and Majerus [27] mentioned that grape juices are usually more contaminated than wines, given the absence of a fermentation process. Given the fact that the blank assays (A) did not present detectable levels of OTA during the evolution from grape to wine, it is safe to conclude that OTA is not present in the initial grapes and, more importantly, is not produced during the process. Current knowledge that the presence of the toxin is due to the grapes or other influences rather than the vinification itself agrees with such findings. Geographic and meteorological conditions, such as high temperature and humidity, are often considered important for OTA development [10, 12]. Relationships to different grape treatments were

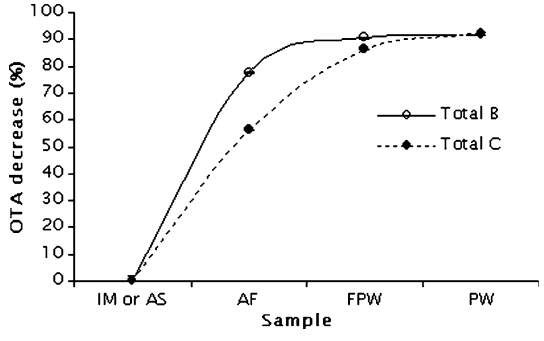

Fig. 4 Overall trend; average of all replicates for Assays B and C (% decrease in OTA)

Table 1 Comparison between overall replicates of assays B and C (SO_2 versus no SO_2)

Samples	Assay B		Assay C	
	OTA content ± SD (μg/l)		OTA content ± SD (μg/l)	
	No SO_2	With SO_2	No SO_2	With SO_2
Initial Must or After SO_2	3.91 ± 1.39	6.22 ± 1.68	0.20 ± 0.16	0.06 ± 0.03
After Fermentation of start	0.86 ± 0.24	0.43 ± 0.35	0.05 ± 0.06	0.01 ± 0.09
First Pressing Wine	0.40 ± 0.14	0.56 ± 0.10	0.03 ± 0.04	0.01 ± 0.01
Press Wine	0.37 ± 0.11	0.45 ± 0.12	0.02 ± 0.03	0.00 ± 0.00

searched for, and Lo Curto et al [34] reported that the use of some synthetic pesticides can reduce OTA levels. However, Battilani and Pietri [32] found discrepant results in grapes from the same locations and undergoing the same treatments. Bau et al [17] stated that the risk of OTA production increases with the ripening of the grapes, which means that good sanitary state is essential to preventing higher wine contamination. Finally, Esteban et al [33] studied the effects of temperature and incubation time on the production of OTA and discovered that for *A. carbonarius* strains at 15–20 °C, five - days was enough to observe contamination, which is in accordance with the findings of this study, although 20 days was the optimum time. Clearly there is still a lot to investigate and improve before reaching sound conclusions on OTA behavior with respect to grape and wine contamination.

To determine the influence of the addition of a SO_2 generator at the beginning of the vinification, the same summing up and averaging of experiments I and II was performed, plotting the concentration of OTA in the must and wine throughout the vinification and separately comparing Assays B and C. Table 1 shows the results.

Apparently, for Assay B, the OTA values are somewhat higher when SO_2 is present. However, the initial difference of about 37% is diluted by the end of the vinification, and can be also attributed to the difficulties of sampling at this stage, mentioned before. For Assay C, the initial ratio is the opposite, with the samples without SO_2 having higher OTA content, with differences $>35\%$. However, given the much lower concentrations involved, the uncertainties of the values could make this difference meaningless. Hence, based on these results, the influence of SO_2 can be considered to be negligible for the conditions used in this study. Overall, although this wine has particular characteristics, such as the high sugar and ethanol (about 19% for red) contents and the nature of the arrest of the vinification process, it seems that these parameters have no influence on the trends and pathways of OTA contamination.

Conclusions

A strong and consistent decrease in OTA concentration was noticed throughout the microvinification process, up to 92% in both direct OTA-spiked and grape fungal inoculation assays (which induced OTA levels of up to 0.35 μg/l within five days). Most of the OTA probably stays in the pomace (solid parts) and the biomass, through adsorption mechanisms. The collected grapes had no intrinsic OTA (the blank assays showed no contamination), which rules out the production of OTA during the vinification process employed for Port Wine production. Tracing the OTA levels in the remaining solids and establishing a thorough mass balance is critical to further clarifying the origins and pathways of OTA contamination from grape to wine. The addition of a SO_2-generating agent did not seem to influence the overall trend. The analytical method established for this study showed good validation parameters. However, near the limits of detection, the global uncertainty associated with the results rose to over 50%. In terms of actual field conditions, and given the proposed maximum limit of 2 μg/l set by the OIV for wine consumption, this work may contribute to reducing any concern over the contamination of the final product, redirecting some of this concern toward the less studied and (we suppose) much more highly contaminated solid residue (and its applications).

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