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# Survey: Ochratoxin A in European special wines

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**Abstract**

The occurrence of Ochratoxin A (OTA) was examined in 121 special wines made using different winemaking techniques and from many European origins. The wine groups with the highest OTA content and occurrence, above 90%, were those were the must was fortified before fermentation (mean: 4.48 μg/l) and those made from grapes dried by means of sun exposure (mean: 2.77 μg/l). Fortified wines with long aging in wooden casks were about 50% contaminated, with OTA levels below 1.00 μg/l. Wines affected by noble rot, late harvest wines and ice wines did not contain OTA. Overall, 19.8% of the wines studied contained OTA levels above the maximum permissible limit for the European Union (2 μg/kg) in wine (excluding liqueur wines).


*Keywords:* Ochratoxin A; Dessert wines; Winemaking; European wines

## 1. Introduction

Ochratoxin A (OTA) is a fungal metabolite with toxic properties, produced by species belonging to the genera *Aspergillus* and *Penicillium* (Ueno et al., 1991; van der Merwe, Steyn, & Fourie, 1965). It has been considered the cause of Balkan endemic nephropathy (BEN) (Marquardt & Frolich, 1992) and it has also been classified by the International Agency for Research on Cancer (IARC, 1993) as a possible human carcinogen (group 2B).

OTA has been found in foodstuffs such as cereals, coffee, cocoa (Pardo, Marín, Ramos, & Sanchis, 2004; Rafai, Bata, & Jakab, 2000; van Egmond & Speijers, 1994), grapes (Abrunhosa, Paterson, Kozakiewicz, Lima, & Venâncio, 2001) and in dried vine fruits (MacDonald et al., 1999; Ostry, Ruprich, & Skarkova, 2002). OTA is present at high levels in red wine (EU Report, 2002), possibly due to the maceration of the must with grape skins, which might favour OTA extraction from skins (Blesa, Soriano, Moltó, & Mañes, 2006). In the case of sweet or special wines, oenological practices are very diverse and may result in different final OTA concentrations (Chiodini, Scherpenisse, & Bergwerff, 2006; Gambuti et al., 2005; Leong, Hocking, & Varelis, 2006; Ratola, Abade, Simões, Venâncio, & Alves, 2005), which are usually higher than those in dry wines (Burdaspal & Legarda, 1999; Pietri, Bertuzzi, Pallaroni, & Piva, 2001; Zimmerli & Dick, 1996). There are several types of sweet wines, defined by their winemaking procedures. Fortified musts (mistelle, Muscat) and fortified wines (Sherry, Port wine) are those in which fermentation is prevented or stopped by adding alcohol to the must or wine, respectively (fortification). Among fortified wines, Fino and Manzanilla (Spain), undergo a secondary fermentation while aging in wooden barrels. The fermentation is achieved by floating yeasts that are alcohol resistant, called Flor yeasts.

Wines from overripe grapes are non-fortified dessert wines. While none of the winemaking processes involves adding grape spirit to halt the fermentation process artificially, all of them require the premature cessation of the fermentation process, leaving behind varying residual sugar levels. The most common cause of fermentation cessation in non-fortified dessert wines is the high sugar content of the fermenting grapes, which naturally drives the alcohol level above 15% by volume. Grapes can be made overripe

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by different techniques, such as by exposure to sunlight (Málaga, Pedro Ximenez, Passito), dehydration in closed chambers of hot or fresh air (Vin de Paille, Vin Santo, Recioto), by the colonisation of grapes by fungus *Botrytis cinerea,* causing noble rot (Sauternes, Montbazillac, Alsace, Loire, Trockenbeerenauslese, Tokaji), by leaving grapes to shrivel in the vineyard, where they may also be occasionally affected by noble rot (Vendage Tardive, Tokaji Late harvest, Spatlese), or by waiting until winter, to produce grape dehydration by ice (ice wine, Vi de Gel, Eiswein).

A wine's origin seems also to be a determinant of its final OTA content (Ottender & Majerus, 2000). Different climatic conditions, due to the latitude and also to variables in local weather, may affect OTA-producing fungi distribution (Battilani et al., 2006; Serra, Lourenço, Alípio, & Venâncio, 2006) and final OTA concentration (López de Cerain, Gónzalez-Peñas, Jiménez, & Bello, 2002).

Despite many surveys on wines from different sources, such as Italy, Spain, Greece, etc (Bellí, Marín, Duaigües, Ramos, & Sanchis, 2004; Pietri et al., 2001; Soufleros, Tricard, & Bouloumpasi, 2003), there is no intensive study on OTA occurrence in sweet or special wines, including wines made from overripe and botrytised grapes.

Currently, several countries have specific regulations for OTA in various commodities, 2 μg OTA/kg being the maximum level allowed for wine, grape must and grapes in the European Union (Commission Regulation (EC) No.1881/2006).

The aim of this study was to assess OTA occurrence in special wines from Europe made using different winemaking techniques.

## 2. Material and methods

### 2.1. Samples

One hundred and twenty one representative special wines from Europe were purchased from Spanish and Portuguese markets and from Italian and Spanish distributors. Wine from all wine-growing zones, according to European regulations, A, B, CI, CII and CIII (Council Regulation (CE) No 1493/1999; Corrigendum (CE) No 1512/2005), which are based on production conditions, soil, region and climate, have been sampled (Fig. 1). Classification of the assayed wines is summarised in Table 1.

### 2.2. Sugar content in wine

Into an Erlenmeyer flask 10 ml of 0.168 M cupric solution (Gab System, Olérdola, Spain), 5 ml of 0.886 M alkaline solution (potassium sodium tartrate, $KNaC_4H_4O_6 \cdot 4H_2O$. Gab System), a small pumice stone and 2 ml of wine,

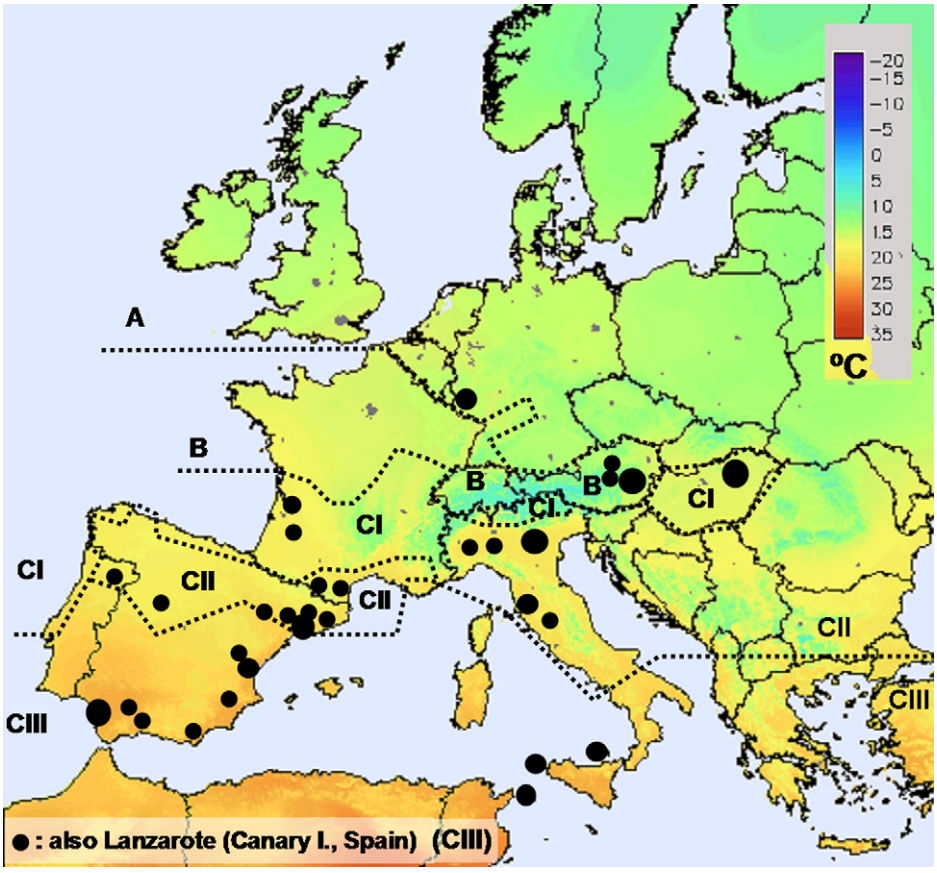


Fig. 1. Map of Europe showing the average temperatures in September and divided by dotted lines into wine-growing zones (A, B, CI, CII and CIII). Black points represent the origin of samples.

Table 1
Classification of the assayed wines according to winemaking type

| Group | | Wines (Number of samples) | Zones |
|---|---|---|---|
| Fortified must/early stopped fermentation | | Mistelle (11), Muscat, (11) | CII, CIII |
| Fortified wines | | Banyuls (1), Marsala (3), Maury (1) | CII, CIII |
| Long aging in wooden casks | | Oloroso (2), Porto (11), Sherry (7) | |
| Fortified wines, 2nd fermentation with Flor yeast, long aging in wooden casks | | Fino (7), Manzanilla (5) | CIII |
| Sparkling wines | | Muscat (1), Cava liquoureux (1) | CII |
| Late harvest | | Fondillón (1), Spätlese (1), Tokaji (4), Others (2) | B, CI, CII, CIII |
| Grapes dehydrated by: | Sun-drying | Fondillón (2), Málaga (5), Malvasía (1), Passito (2), Pedro Ximénez (10), Samos (1), Sherry (1), Other (1) | CIII |
| | Warm chamber | Passito (1), Vin Santo (2) | CII |
| | Fresh chamber | Amarone (2), Amarone Ripasso (1), Garnatxa de l'Empordà (3), Recioto (2) | CII |
| | Noble rot | Auslese (1), Beerenauslese (2), Trockenbeerenauslese (2), Sauternes (4), Montbazillac (1), Tokaji (3) | A, B, CI, CII |
| | Ice | Eiswein (3), Vi de gel (2) | B, CII |

previously diluted if the wine was suspected to be very sweet, were poured. The mixture was boiled for 90 s and cooled. Then, 10 ml of 1.6 M iodide solution, 10 ml mol/l soluble starch and 10 ml of 16% sulphuric acid (all Gab System) were added. Finally, sugar content was quantified through titration with sodium thiosulphate. The end point was determined by the change from dark brown-black to clear beige. The reducing sugar content was calculated according to

Eq. 1 ($r^2 = 0.996$):

$$\text{Sugar content (g/l)} = 31.096 - 1.105 \times \text{thiosulphate volume (ml)} \quad (1)$$

### 2.3. OTA analysis

The OTA extraction method developed by Bezzo, Maggiorotto, and Testa (2000) for wine, using HPLC was followed with some variations. In brief, the pH of 100 ml of wine was adjusted to 7.40 with 4 M NaOH and passed through filter paper number 1. Then the extract was cleaned up by passing the sample through an immunoaffinity column (Ochraprep®, R-Biopharm Rhône Ltd., Glasgow, Scotland) at a flow rate of 2–3 ml/min. The column was then washed with 20 ml double-distilled water and left to dry. OTA was finally eluted from the column with 3 ml methanol:acetic acid (98:2, v:v). The eluted extract was dried under $N_2$ flow at 40 °C and resuspended in 1 ml mobile phase (48% acetonitrile and 52% sodium acetate:acetic acid (19:1)). Separation and OTA quantification was performed using a high performance liquid chromatograph (Waters, Milford, MA, USA) with reverse-phase C18 silica gel column (Waters Spherisorb® 5 μm ODS2 4.6 × 250 nm, Milford, MA) and detected by fluorescence. Excitation and emission wavelengths were set to 230 and 458 nm, respectively. The flow rate of mobile phase was 1 ml/min and the injection volume 25 μl. The retention time of OTA was 13 min (LOD = 0.024 μg OTA/l wine; LOQ = 0.081 μg OTA/l wine), identified according to a standard from Sigma (Steinheim, Germany).

Recovery rates for the method across different sugar contents were calculated in eight sweet wines previously spiked with OTA at 2 μg/l. The original OTA content of the eight wines was also analysed and considered for calculations.

## 3. Results

### 3.1. Correlation between sugar content and recovery rate

Correlation between sugar content and recovery rate was shown to be negative and significant (Fig. 2) (Pearson correlation coefficient ($\rho$) = −0.802, $p = 0.017$). The resultant Eq. (2) ($r^2 = 0.643$) from linear regression between sugar content and recovery percentages was used for calculating the recovery percentage for all wine samples based on their natural sugar content. Then, the real OTA concentration in all wines was calculated based on its recovery percentage: as a result

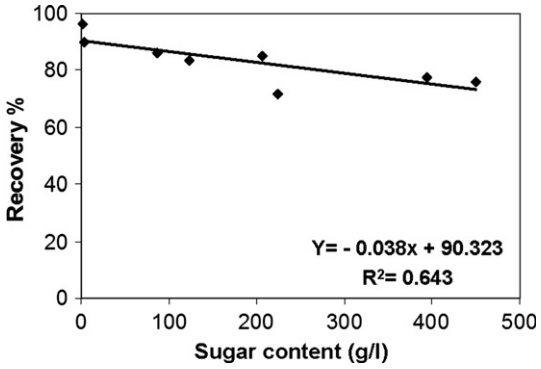


Fig. 2. Recoveries for OTA in special wines according to their sugar content.

$$\text{Recovery percentage} = 90.323 - (0.038 \times \text{Sugar content (g/l)}) \quad (2)$$

Another peak was usually detected at a retention time of 8.2 min in all the analysed samples where OTA was present (Fig. 3).

### 3.2. Occurrence of OTA in special wines

The concentration of OTA found in the wine samples from the five growing zones is presented in Table 2. Means of OTA concentrations were calculated from the average of all samples, zero values included.

The wine groups with highest OTA content and occurrence (91%) were those in which the must had been fortified before fermentation occurred and those made from grapes dried by means of sun exposure (Table 3). Fortified wines with long aging in wooden casks were found to be contaminated in 13 out of 25 samples, with all OTA levels lower than 1.00 μg/l. Among the fortified wines undergoing a second fermentation with Flor yeast, only one of 12 samples was contaminated with OTA (0.08 μg/l), and wines made from noble rot-affected grapes, late harvest wines and ice wines did not have OTA.

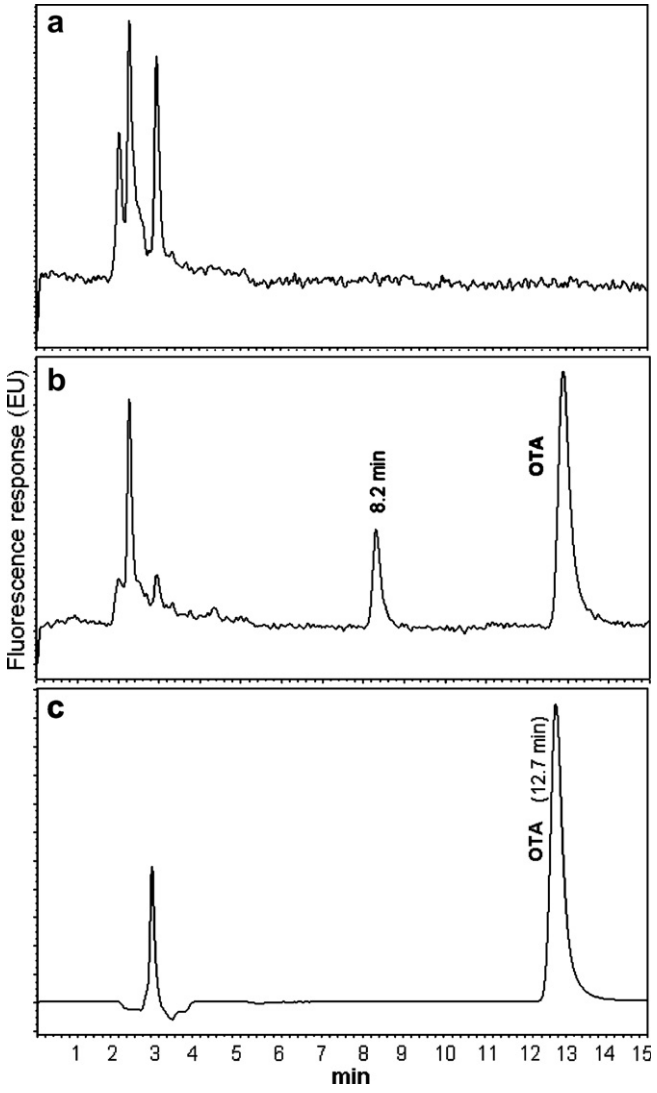


Fig. 3. Chromatograms (HPLC; fluorescence detection) of: (a) wine sample negative for OTA; (b) wine sample positive for OTA (vial concentration: 50.47 μg/l); (c) OTA standard solution 2.24 μg/l.

## 4. Discussion

The extraction and clean-up procedure to analyse OTA from wines was checked for sweet wines and, from our findings, it could be advisable to take into account the interference of sugar in the recovery rate in order not to underestimate the OTA concentration, giving a possible error of up to 30% in some cases.

The unknown peak (8.2 min) found in all positive samples here detected was also reported by Sáez, Medina, Gimeno-Adelantado, Mateo, and Jiménez (2004) in wines, musts and beers at a similar retention time but not identified. Ochratoxin A is sometimes accompanied by the non-chlorinated analogue, ochratoxin B (WHO-IPCS, 1990).

The climatic and geographic differences influence mould development and OTA contamination of grapes, and consequently, the wine. Generally, in the southern wine-growing region sampled, highest OTA occurrences and levels were observed. All special wines analysed from A, B and CI wine-growing zones were negative for OTA. Other studies have reported OTA occurrences, in all types of wines in these regions, from 0 to 50% (Berente et al., 2005; Ottender & Majerus, 2000; Zimmerli & Dick, 1996), which suggests that the winemaking procedure has a stronger effect on OTA content than the origin of this sort of wine.

CII zones are located in warmer regions that may favour fungal development and OTA production by *Aspergillus* section *Nigri*, as reviewed and modelled by Battilani et al. (2006). The 54% of special wines (14 out of 26) from CII zone were positive for OTA with 15% of samples containing OTA concentrations over 2 μg/l. Concentrations found in this study were higher than those previously reported by some authors in table wines (Ottender & Majerus, 2000; Pietri et al., 2001; Zimmerli & Dick, 1996), where occurrences ranged from 53 to 100%, with means up to 0.193 μg OTA/l and maxima between 0.041 and 2.55 μg OTA/l.

The majority of Mediterranean wine-growing regions are included in the CIII zone. Climates are hot and dry during summer and warm and wet in autumn. Sixty percent of wines sampled contained OTA (45 out of 75) with 27% higher than 2 μg OTA/l. Previous authors reported between 80 and 100% of wines from this area were contaminated by OTA with mean values similar to those found in the present study (Ottender & Majerus, 2000; Pietri et al., 2001; Zimmerli & Dick, 1996); however, maximum levels, up to 3.856 μg/l, were much lower than that observed in our study (15.62 μg/l).

Table 2
Occurrence and OTA levels in special wines from five European wine-growing zones

| Wine-growing zones | *n* | Positive samples (%) | OTA (μg/l) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Mean | Range | <LOD-0.10 | 0.10–0.50 | 0.50–1.00 | 1.00–2.00 | >2.00 |
| A | 2 | 0 | <LOD | <LOD | 2 | 0 | 0 | 0 | 0 |
| B | 7 | 0 | <LOD | <LOD | 7 | 0 | 0 | 0 | 0 |
| CI | 11 | 0 | <LOD | <LOD | 11 | 0 | 0 | 0 | 0 |
| CII | 26 | 14 (54) | 2.01 | <LOD-27.79 | 14 | 2 | 4 | 2 | 4 |
| CIII | 75 | 45 (60) | 1.71 | <LOD-15.62 | 29 | 10 | 12 | 4 | 20 |

LOD = <0.024 μg OTA/l.

Table 3
Occurrence and OTA levels in special winemaking

| Group | *n* | Positive samples (%) | (OTA (μg/l) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Mean | Range | <LOD-0.10 | 0.10–0.50 | 0.50–1.00 | 1.00–2.00 | >2.00 |
| Fortified must/early stopped fermentation | 22 | 20 (91) | 4.48 | <LOD-27.79 | 4 | 1 | 4 | 1 | 12 |
| Fortified wines long aging in wooden casks | 25 | 13 (52) | 0.24 | <LOD-0.96 | 12 | 8 | 5 | 0 | 0 |
| Fortified wines, 2nd fermentation with Flor yeast, long aging in wooden casks | 12 | 1 (8) | 0.01 | <LOD-0.08 | 12 | 0 | 0 | 0 | 0 |
| Sparkling wines | 2 | 1 (50) | 0.06 | <LOD-0.13 | 1 | 1 | 0 | 0 | 0 |
| Late harvest | 8 | 0 (0) | n.d. | <LOD | 8 | 0 | 0 | 0 | 0 |
| *Grapes dried by*: | | | | | | | | | |
| Sun | 23 | 21 (91) | 2.77 | <LOD-15.62 | 2 | 2 | 6 | 3 | 10 |
| Warm chamber | 3 | 1 (33) | 0.58 | <LOD-1.74 | 2 | 0 | 0 | 1 | 0 |
| Fresh chamber | 8 | 4 (50) | 1.35 | <LOD-4.79 | 4 | 0 | 1 | 1 | 2 |
| Noble rot | 13 | 0 | n.d. | <LOD | 13 | 0 | 0 | 0 | 0 |
| Ice | 5 | 0 | n.d. | <LOD | 5 | 0 | 0 | 0 | 0 |
| Total | 121 | (50) | 1.49 | <LOD-27.79 | 63 | 12 | 16 | 6 | 24 |

LOD = <0.024 μg OTA/l.

The effect of winemaking procedures on OTA content was also evaluated. Wines from dehydrated grapes obtained through five different techniques were studied in the present survey. In all cases the fermentation progresses slowly, due to the high sugar content of musts, and wines are usually aged in wooden barrels; so variations in OTA content may mainly depend on the dehydrating procedure. Ochratoxin A contamination of dried vine fruits (50–70 μg/l) is usually much higher than that of grapes (Miraglia & Brera, 2002). Twenty one out of the 23 (91%) wines made from sun-dried grapes were positive for OTA and levels were much higher than for other wines made from other dried grapes. Previous surveys have reported occurrences in this type of wine ranging from 57% to 100%, and OTA concentrations were also high, up to 7.3 μg/l, similar to our results (Blesa, Soriano, Moltó, & Mañes, 2004; Hernández, García-Moreno, Durán, Guillén, & Barroso, 2006; JECFA, 2001; Soufleros et al., 2003; Zimmerli & Dick, 1996). Sun-dried grapes are exposed in an open environment, to hot sun-drying during the day and to cool and wet nights for 5–15 days. Under these conditions there are damaged grapes, probably due to harvesting, where fungi can grow and produce OTA (Valero, Marín, Ramos, & Sanchis, 2005). Gómez, Bragulat, Abarca, Mínguez, and Cabañes (2006) found higher *A. carbonarius* occurrences in overripe grapes that were left on the vine or exposed to sun-drying, than at harvesting time.

Wines made from grapes dried in cool and dry chambers contained higher OTA levels than those wines made from grapes dried in hot and dry chambers. This fact could be due to the longer period of time, in the first group of wines, that is necessary for drying these grapes (2–6 months) and the environmental conditions, which are more favourable for fungal development and OTA synthesis. In a study that simulated this process (Gambuti et al., 2005) grapes were left to dry in an aerated room for two months, and wines made from these grapes had OTA levels more than twice as high as wines obtained from grapes at full maturity.

For eisweins (ice wines), noble wines (botrytised grapes) or late harvest wines (usually not botrytised grapes), none

of 26 samples contained ochratoxin A, in agreement with previous results published regarding noble rot-affected wines (Berente et al., 2005; Dumoulin & Riboulet, 2002; Eder, 2005; Kallay & Magyar, 2000; Stander & Steyn, 2002) and ice wines (Eder, 2005). These wines are usually made in Northern European regions or in colder climates that favour noble rot rather than summer rot (*Aspergillus* section *Nigri*). Some studies performed in our laboratory (unpublished data) and also by Abrunhosa, Serra, & Venâncio, 2002 have found that *B. cinerea* isolates are able to degrade OTA spiked in agar medium. Possibly if grape colonisation by *Aspergillus* section *Nigri* occurs, the little toxin produced could be degraded by *B. cinerea*. This theory, however, has not yet been confirmed *in vivo* or *in situ*.

Fortified musts were the most OTA-contaminated of the different wine groups analysed (mean: 4.48 μg/l), OTA being detected in 20 out of the 22 samples (91%). Levels given by some authors are lower than our data but still higher than fortified wines (Blesa et al., 2004; Hernández et al., 2006; Tateo, Bononi, & Lubian, 2000). It is not a surprising finding provided that fermentation is prevented or stopped early and, subsequently the OTA removal during this process reported by many authors (Grazioli, Fumi, & Silva, 2006; Leong et al., 2006; Ottender & Majerus, 2000; Ratola et al., 2005) does not take place. In fortified wines, in contrast, the initial must may be very similar in composition but it is fermented up to a certain alcoholic grade, fortified and next aged in wooden barrels for long periods of time. In addition to OTA reduction by fermentation, either alcoholic or malolactic (Abrunhosa et al., 2002; Grazioli et al., 2006), the oxidative process that occurs during aging, and also the action of Flor yeast, could somehow affect the toxin, but effects are uncertain.

Overall, 20% of wines contained OTA levels above the maximum permissible limit for the European Union, set at 2 μg/kg for wine (excluding liqueur wines) (Commission Regulation (EC) No.1881/2006). Due to the limited amounts of samples in some categories of wines, and considering our findings, it would be interesting to design further studies focusing on some types of wines, in order to confirm our results and to identify the critical points where OTA is reduced.

Summarising, ochratoxin A in special wines is a matter of concern, considering the high levels and occurrence reported in the present study. The origin of the samples is a determinant for fungal development and OTA contamination, more prevalent in Southern regions, as a consequence of the environmental conditions. Winemaking procedures also affect OTA concentration in wines, it being lower in wines with longer or double fermentation and in those made from grapes affected by *B. cinerea*.

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