Detection of ochratoxin A in tropical wine and grape juice from Brazil

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Abstract

BACKGROUND: Ochratoxin A (OTA) is the main mycotoxin found in grapes, wines and grape juices and is considered one of the most harmful contaminants to human health. In this study, samples of tropical wines and grape juices from different grape varieties grown in Brazil were analysed for their OTA content by high-performance liquid chromatography.

RESULTS: The detection and quantification limits for OTA were 0.01 and 0.03 μg L^{-1} respectively. OTA was detected in 13 (38.24%) of the samples analysed, with concentrations ranging from <0.03 to 0.62 μg L^{-1}. OTA was not detected in any of the grape juice samples. Most of the red wine samples proved to be contaminated with OTA (75%), while only one white wine sample was contaminated. However, the OTA levels detected in all samples were well below the maximum tolerable limit (2 μg L^{-1}) in wine and grape juice established by the European Community and Brazilian legislature.

CONCLUSION: The results of this study indicate a low risk of exposure to OTA by consumption of tropical wines and grape juices from Brazil.
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Keywords: high-performance liquid chromatography; ochratoxin A; wine; grape juice; carcinogen; mycotoxin

INTRODUCTION

Ochratoxin A (OTA) is a secondary toxic metabolite produced by some species of filamentous fungi of the genera *Aspergillus* and *Penicillium* and is often found in a variety of foods and beverages, including wine and grape juice.[1] OTA has exhibited carcinogenic, nephrotoxic, teratogenic and immunotoxic effects in animals,[2] leading to its classification as a possible human renal carcinogen (group 2B).[3] The presence of OTA in grape juice and wine was first reported by Zimmerli and Dick in 1995.[4] Currently, it is considered to be the principal mycotoxin found in grape derivatives.[5]

Since OTA has been reported in various studies as a grape, wine and grape juice contaminant, international regulatory bodies have undertaken risk assessments to determine the effect of the consumption of this mycotoxin on human health. In 2002 a study investigating OTA ingestion in European Union (EU) countries concluded that, after cereals, wine is the principal source of OTA for humans.[6]

Studies show that fungi of the genus *Aspergillus* section *Nigri*, in particular *Aspergillus carbonarius*, are the main culprits in OTA production in grapes.[7] In addition, OTA levels vary depending on the type and region of wine as well as the period of harvest.[2] In fact, studies performed on Brazilian, Argentinian and Chilean grape juices and wines showed that these products contain lower OTA levels than those from Europe.[8] In addition, red wine has higher levels of OTA than white and rosé wine.[9] Similar to what has been observed for wine, red grape juice exhibits higher OTA levels than white grape juice.[10]

OTA contamination in grape juice is of great concern, since children are its main consumers and the consumption of juice is greater than that of wine.[11] Currently, EU legislation permits a maximum of 2 μg L^{-1} OTA in wine and grape juice.[12] In February 2011, Brazilian legislation also established 2 μg L^{-1} as the maximum tolerable limit for OTA in wine, grape juice and grape derivatives.[13]

To date, only a few studies have investigated the presence and levels of OTA in Brazilian grape-derived products; however, more information is necessary to better determine the possible health risks of the consumption of wines and juices produced in this country. Thus, in the present study, we aimed to evaluate the presence and levels of OTA in wines and grape juices from northeastern Brazil using immunoaffinity and high-performance liquid chromatography (HPLC) analysis.

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Table 1. Grape varieties used to prepare wine samples in study

Wine (grape) sample	Period of preparation (month/year)
Red wines	
Syrah Clone 525 RS: IAC-313	11/2009
Syrah Clone 525 RS: 1103-P	11/2009
Tempranillo RS: IAC-313	11/2009
Alicante RS: 1103-P	11/2009
Syrah without maceration	12/2009
Tempranillo RS: 1103-P	11/2009
Marselan Clone 980 RS: 1103-P	11/2009
Petit Verdot 2	11/2009
Petit Verdot 1	06/2009
Tempranillo 1	06/2009
Tempranillo 2	11/2009
Syrah	06/2009
Grenache	09/2009
Cabernet Sauvignon	09/2009
Cabernet Sauvignon RS: IAC-766	12/2009
Carmenère	12/2009
White wines	
Verdejo	07/2009
Viognier 1	06/2009
Viognier 2	11/2009
Chenin Blanc 1	07/2009
Chenin Blanc 2	11/2009
Sauvignon Blanc 1	06/2009
Sauvignon Blanc 2	11/2009

MATERIALS AND METHODS

Study region and sample preparation

Grapes for wine and grape juice samples were obtained from the tropical winemaking region of Brazil (9 °S, 40 °W). A total of 23 types of wine sample (16 red and seven white) were experimentally prepared from red and white grapes using different treatments, such as clones of grape varieties and rootstocks (RSs) (Table 1). The wines, prepared from the varieties Tempranillo, Petit Verdot, Viognier, Chenin Blanc and Sauvignon Blanc, were obtained in two different winemaking periods in 2009 (Table 1). IAC-313 was classified as a high-vigour RS.

Wine was made from the grape varieties listed in Table 1 using the traditional method[14] in 200 L stainless steel vats. Alcoholic and malolactic fermentations were performed at 25 and 18 °C respectively. The wines were then stabilised and bottled.

The grape juice samples were prepared from three grape cultivars grafted onto different RSs, namely Isabel Precoce, Isabel Precoce IAC-313, Isabel Precoce Paulsen 1103, Isabel Precoce IAC-776, Isabel Precoce IAC-572, Isabel Precoce 313 R2, Rúbea Paulsen 1103, Rúbea IAC-766, BRS Cora IAC-572, BRS Cora IAC-776 and BRS Cora IAC-313, for a total of 11 samples. Grape juices samples were prepared in 20 L craft juicers via extraction at 75 °C with flash pasteurisation and subsequent bottling.

OTA analysis in wine and grape juice

OTA levels in the wine and grape juice samples were measured using HPLC with fluorescence detection as described previously.[15]

Solutions and reagents

The dilution solution contained 10 g l^{-1} PEG 8000 and 50 g l^{-1} NaHCO$_3$ in purified water. The washing solution contained 25 g l^{-1} NaCl and 5 g l^{-1} NaHCO$_3$ in purified water. A stock solution of OTA (Sigma-Aldrich, St Louis, MO, USA) was prepared in toluene/acetic acid (99 : 1 v/v) and stored at −15 °C. Concentration was determined using a UV spectrophotometer at 333 nm in accordance with the AOAC method.[16] A working solution was prepared by appropriate dilution in toluene/acetic acid (99 : 1 v/v) for the recovery tests and calibration curve. For the mobile phase, acetonitrile/methanol/aqueous acetic acid (35 : 35:30 v/v/v) was used, followed by vacuum filtration using a 0.45 µm polytetrafluoroethylene (PTFE) regenerated cellulose membrane. Aqueous acetic acid was prepared with a glacial acetic acid solution in purified water (1 : 29 v/v).

Preparation and purification of samples

The samples were initially cooled to 4 °C, then 40 mL of each sample was added to 40 mL of dilution solution and homogenised by agitation for 30 min. This solution was vacuum filtered (2 mL min^{-1}) using a glass microfibre filter membrane, and 40 mL of the filtrate was passed through an immunoaffinity column (Ochraprep, R-Biopharm Rhone Ltd, Glasgow, UK) using a Visiprep™ SPE Vacuum Manifold (Sigma-Aldrich, St Louis, MO, USA) system. The column was washed with 10 mL of washing solution followed by 10 mL of purified water. Methanol (2 mL, three times) was then added to elute OTA from the column. The eluate was evaporated at 50 °C under nitrogen vapour. The dry extract was then reconstituted in 250 µL of mobile phase. A 50 µL aliquot of each standard and sample was used for the subsequent HPLC analysis.

HPLC

Measurement of OTA levels was conducted using a Shimadzu (Kyoto, Japan) liquid chromatography system with a fluorescence detector (LC-10AD) at wavelengths of 333 and 476 nm for excitation and emission respectively. A Shim-pack CLC-ODS RP-18 column (5 µm, 4.6 mm × 250 mm) with a Shim-pack G-ODS precolumn (5 µm, 4 mm × 10 mm) was used at a flow rate of 0.8 mL min^{-1}. Samples and standards (200 µL) were evaporated under nitrogen and the residues were resuspended in 300 µL of boron trifluoride in 140 mL L^{-1} methanol. Samples were then heated at 80 °C for 10 min and allowed to cool to ambient temperature before evaporation and resuspension in 1 mL of mobile phase for injection into the chromatography system. The presence of OTA was determined by the disappearance of the OTA peak and the appearance of a new peak (OTA methyl ester) at longer retention time. OTA levels were calculated from the area of the OTA peaks in samples and standards. Under these conditions the retention time was around 11 min.

Standard addition experiments

The method for determining OTA in wine and grape juice samples was validated by adding OTA to samples known to be negative for OTA (< 0.03 µg L^{-1}). OTA was added to 220 mL of wine at levels of 0.22 and 0.44 µg L^{-1}. The sample was blended and allowed to equilibrate for 1 h at 4 °C. Then five aliquots of 40 mL each were taken and analysed. OTA was added to 100 mL of grape juice at a concentration of 2.78 µg L^{-1}. The sample was blended and cooled to 4 °C, after which two aliquots of 40 mL each were taken and analysed.

Calibration curve

Standard solutions for HPLC calibration were prepared as follows. The working solution (29.613 µg ml^{-1} in toluene/acetic acid) was obtained by evaporating 800 µL of stock solution (111.05 µg ml^{-1} in toluene/acetic acid) in a nitrogen stream followed by resuspension in 3 mL of mobile phase. Seven-point graphs were obtained with 0.6, 5, 10, 20, 30, 60 and 111 µg L^{-1} standards. Calibration graphs were obtained using linear regression of the least squares method and the peak response of each standard injection plotted against OTA concentration. The correlation coefficient was greater than 0.999. The detection limit (DL) was 0.01 µg L^{-1}, calculated as three times the standard deviation of the blank samples divided by the slope of the calibration graph. The quantification limit (QL) was 0.03 µg L^{-1}, calculated as ten times the standard deviation of the blank samples divided by the slope.

Statistical analysis

Statistical analyses were carried out for the samples with OTA levels greater than the QL. Analysis of variance (ANOVA) was performed on the data after square root transformations to meet test assumptions. The Scott and Knott[17] cluster analysis method for grouping means at $P < 0.05$ was used. To test the contrast of interest (contamination of red wines *versus* white wines), the *F* test at $P < 0.05$ was applied. Analyses were performed using Statistics Package R-2.11.1 version (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Validation of OTA measurements in wine and grape juice samples

The results of the validation of the extraction method for OTA in wine and grape juice samples are shown in Table 2. These results are within the standards recommended by the European Commission[18] for contamination values less than 1 µg L^{-1} and between 1 and 10 µg L^{-1} for wine and grape juice respectively.

The calibration curve obtained by linear regression was linear in the range from 0.6 to 111 µg L^{-1}. The correlation coefficient was greater than 0.99, as recommended by Green.[19]

Levels of OTA in wine and grape juice samples

The presence and mean values (minimum and maximum) of OTA found in the wine and grape juice samples are listed in Table 3. OTA was detected in 13 (38.24%) samples analysed at concentrations ranging from 0.03 to 0.62 µg L^{-1}. Of the positive samples, 12 were red and only one was white wine. Most of the red wine samples (75%) had OTA contamination; however, OTA was not detected in any of the grape juice samples. The red wines in which OTA was not detected or in which the concentration was less than the QL (0.03 µg L^{-1}) were obtained from Cabernet Sauvignon, Grenache, Cabernet Sauvignon RS: IAC-766 and Carmenère. These wines were prepared in the same period, which was different from the period of preparation of the positive samples, suggesting that climatic conditions of the harvest period may contribute to the presence of OTA contamination.

When we applied ANOVA to determine the source of variation in OTA levels in the positive samples, the type of grape proved to be significant in affecting OTA levels ($P < 0.05$; Table 4). We confirmed this finding using the Scott and Knott[17] test ($P < 0.05$; Fig. 1). As shown in Fig. 1, the mean OTA levels of the contaminated wines can be separated into statistically distinct groups (a, b, c, d and e). Some wines that were prepared from different grape varieties, such as Petit Verdot and Marselan Clone 980 RS: 1103-P, exhibited the same mean level of contamination. The greatest mean OTA value (0.62 µg L^{-1}) was detected in a red wine sample prepared from the Syrah Clone 525 RS: IAC-313 variety (Fig. 2), and the level of contamination of this wine differed significantly from the rest (Fig. 1). The second most contaminated sample was also a red wine, obtained from the Syrah Clone 525 RS: 1103-P variety. On the other hand, the lowest OTA levels were detected in the Syrah, Tempranillo and Verdejo samples, which were statistically similar.

Unlike the type of grape, the winemaking period did not cause a statistical difference with regard to the OTA content (Table 4).

Table 2. Recovery of OTA from wine and grape juice samples

Type of sample	OTA concentration (µg L^{-1})	Mean OTA detected[a] (µg L^{-1})	Mean recovery[a] (%)	No. of replicates	CV[b] (%)
Wine	0.22	0.16 ± 0.02	70.91 ± 7.95	5	25.07
	0.44	0.33 ± 0.04	74.09 ± 10.33	5	31.18
Grape juice	2.78	2.65 ± 0.05	95.32 ± 1.80	2	2.67

[a] Mean ± standard error.
[b] Coefficient of variation.

Table 3. Presence and mean limits of OTA in red wine, white wine and grape juice samples analysed

Type of sample	Not detected or <QL[a]	Detected[b] (>QL)	Limits[c] (µg L^{-1})
Red wine	4/16 (25%)	12/16 (75%)	0.03 – 0.62
White wine	6/7 (85.7%)	1/7 (14.3%)	0.03
Grape juice	11/11 (100%)	0/11	ND
Total	21/34 (61.76%)	13/34 (38.24%)	0.03 – 0.62

[a] Number and percentage of samples with mean OTA content not detected or less than the QL (0.03 µg L^{-1}).
[b] Number and percentage of samples with mean OTA content greater than the QL.
[c] Least and greatest mean OTA level detected. ND, not detected.

Table 4. ANOVA test for OTA content of contaminated wines

Source of variation	Degrees of freedom	Mean square	F calculated
Grape variety	10	52.867	68.20**
Winemaking period	1	1.315	1.70[ns]
Variety × period	1	2.171	2.80[ns]
Residue	13	0.775	

CV = 8.88%, R^2 = 98%, mean = 123.86, SD = 0.593

** Significant with 95% confidence; [ns] not significant.
CV, coefficient of variation; R^2, coefficient of determination; SD, standard deviation.
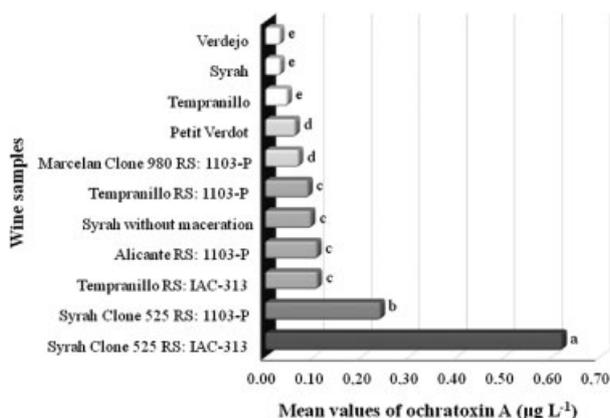
Figure 1. Mean OTA concentration detected in positive samples (>QL). Different letters indicate statistically significant differences by the Scott and Knott[17] test at $P < 0.05$.

The interaction between the grape variety and the winemaking period was also not statistically significant (Table 4).

The contrast between the red and white wines contaminated with OTA was statistically significant ($P < 0.05$), indicating that the mean level of contamination of the positive red wine samples differed significantly from the level detected in the white wines. Our results confirm previous studies that reported a greater occurrence and levels of OTA contamination in red wines compared with white wines. Taken together with Fig. 1, this result suggests that the Verdejo sample, although statistically similar to the Syrah and Tempranillo samples, had the lowest OTA value detected in this study, as it was the only white wine contaminated with OTA.
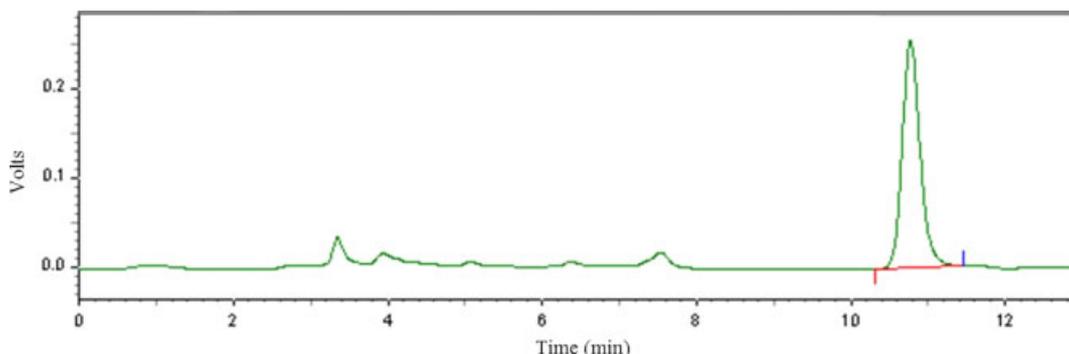
Interestingly, although we detected OTA contamination in some wine samples in this study, the levels detected were low, as they were less than the maximum tolerable limit for this toxin in wine and grape juice established by European[12] and Brazilian[13] legislation.

DISCUSSION

Our results indicate that red wines contain the highest levels of OTA contamination, which is in accordance with previous reports from Europe[9] and Brazil.[20,21] The higher levels of OTA contamination in red wine compared with white wine is probably due to the characteristics of the winemaking process, especially because of the maceration of the grape marc. In addition, the duration of fermentation, which contributes significantly to the reduction of OTA content,[22] is generally shorter for red wine than for white wine, allowing for increased removal of OTA from white wine compared with red wine.

In the process of making white wine, the solid parts of grapes are separated from the must after crushing, allowing minimal further contact between the must and the skin and seeds. However, during the process for making red and rosé wine, it is required that the solid parts ferment together with the must to allow for the extended time necessary for the extraction of colour and aromas. According to Lasram *et al.*,[22] this fermentation phase is when most of the OTA extraction from grapes into wine occurs, suggesting that grape skin is the main source of OTA.

Even though the highest level of OTA contamination observed among the samples tested was 0.62 µg L^{-1}, this value is still less than the maximum limit (2 µg L^{-1}) proposed by the European Commission[12] and by Brazilian legislation.[13] Previously, Shundo *et al.*[21] reported similar results where OTA levels in Brazilian red wines varied from 0.1 to 1.33 µg L^{-1}. However, the authors suggested that, although the OTA content of Brazilian wines was low, small and continuous exposure to this mycotoxin could represent a risk to human health.

The low levels of OTA detected in the wine samples studied may be related to the semi-arid tropical climate of the region, where high temperatures are observed throughout the entire year (average of 27 °C). Lasram *et al.*[23] suggested that, since *A. carbonarius* produces more OTA at lower temperatures (15–20 °C), European climate conditions are more permissive to OTA contamination of grapes than those in regions with a tropical climate, such as Brazil.

We did not detect significant levels of OTA in the grape juice samples tested. Similarly, in a previous study performed in Brazil, none of the 38 samples of grape juice tested was contaminated


Figure 2. Chromatogram of wine sample prepared from Syrah Clone 525 RS: IAC-313 variety.

with OTA.[21] This may be due to the type of grapes used, the geographic origin of the grapes, the climatic conditions and/or the different practices for growing the grapes, which affect the growth of ochratoxigenic fungi and production of OTA. In contrast to what has been observed in Brazilian grape juices, Dachoupakan et al.[24] found OTA in grape juice samples originating from France.

The Scientific Committee on Food[25] has established an acceptable dose for OTA of 5 ng kg^{-1} body weight day^{-1}. Considering that the average wine consumption in Brazil is 5 mL day^{-1}, that the highest level of OTA found in our study was 0.62 μg L^{-1} and that the average body weight of a Brazilian is 70 kg, we can estimate that the maximum daily consumption of OTA in Brazil is 0.88% of the daily tolerable dose. Similarly, considering the mean OTA content of red (0.098 μg L^{-1}) and white (0.004 μg L^{-1}) wine samples in our study, the average daily consumption is estimated to be only 0.14 and 0.005% of the daily tolerable dose respectively.

Further studies investigating OTA contamination in Brazilian wine and grape juice are necessary to gain a better understanding of the factors that significantly contribute to the presence and levels of the toxin in these products. Understanding the critical factors of OTA contamination will allow for the development of better production methods to minimise the risk of exposure to OTA and will facilitate the maintenance of low levels of contamination in grape derivatives.

ACKNOWLEDGEMENTS

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