OCHRATOXIN A OCCURRENCE IN GREEK DRY AND SWEET WINES

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Abstract

Aims: To assess ochratoxin A (OTA) presence in Greek wines, according to the production area and the technology, as OTA risk is higher in wines from southern Europe. The effect of fining agents was examined in order to investigate OTA reduction.

Methods and results: Dry and sweet wines issued from research programs and trade wines were analyzed, according to the official HPLC method. Greek dry wines do not exceed the European limit of 2 ng/mL. The amount of OTA (in ng/mL) varies between 0 - 1,18 for dry white wines, 0 -1,00 for dry red wines. Among sweet wines, only fortified wines without alcoholic fermentation range between 0,50 -7,64, while other sweet wines range between 0,05 -1,73. Dry yeast reduced OTA by 25 - 60 % according to dosage and duration (1-10 weeks) while the effect of bentonite was 33 - 40 % reduction for a treatment of 3-6 days.

Conclusion: The picture for Greek wine production is positive, even in case of a future lowering of the European limit. OTA risk seems higher in the islands, particularly for some dessert wines. Results from wine treatment with fining agents support the theory of OTA adsorption onto suspended solids and the beneficial effect of yeast presence.

Significance and impact of study: Assessment of OTA danger for Greek wines and information concerning the effect of fining agents contribute to the production of wines free from contaminants and support wine economy.

Key words: Greek wines, HPLC, ochratoxin A, sweet wines, fining agents, OTA reduction, dry yeast, bentonite

Résumé

Objectif : L'évaluation de la présence d'ochratoxine A (OTA) dans les vins grecs, suivant la région de production et la technologie, étant donné que le risque est plus élevé pour les vins de l'Europe du Sud. L'effet d'agents traitants a été examiné afin de suivre la réduction en OTA.

Méthodes et résultats : Des vins secs et des vins doux produits dans le cadre de programmes de recherche et des vins du commerce ont été analysés suivant la méthode analytique officielle. Les vins secs ne dépassent pas la limite européenne de 2 ng/mL. La teneur en OTA (en ng/mL) varie entre 0 -1,18 pour les vins blancs secs et 0 -1,00 pour les vins rouges. Parmi les vins doux, seuls les vins fortifiés qui n'ont pas subi de fermentation alcoolique présentent des teneurs entre 0,50 -7,64, alors que les autres vins doux se situent entre 0,05 -1,73. La levure sèche réduit OTA de 25,4 - 60,6 % suivant la dose et la durée (1-10 semaines) alors que la bentonite a pour effet une réduction de 33,6 - 39,7 % pour un traitement de 3-6 jours.

Conclusion : Les données sont positives pour la production vinicole hellénique, même dans le cas d'une baisse éventuelle de la limite européenne. Le risque de présence d'OTA semble plus élevé dans les îles, particulièrement pour certains vins doux.

Signification et impact de l'étude : L'évaluation du danger OTA pour les vins grecs et les données sur l'effet des agents traitants contribuent à la production de vins dépourvus de contaminants et au soutien de l'économie vinicole.

Mots clefs : vins grecs, HPLC, ochratoxine A, vins doux, agents traitants, réduction OTA, levure sèche, bentonite

manuscript received: 1st June 2007 - revised manuscript received: 31st August 2007

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INTRODUCTION

European wine production represents about 70 % of world production. Wine and grapes have recently been identified as the second most important source of the mycotoxin called Ochratoxin A (OTA). OTA is considered as nephrotoxic, hepatotoxic, immunotoxic and possible carcinogen to humans. It has been detected in the blood of more than 70 % of the people tested in certain countries, indicating wide-spread consumption of contaminated foods (Stander *et al.*, 2002).

Aspergillus carbonarius is the principal source of OTA in fresh grapes and the products made from them: grape juice, wine and dried raisins (Battilani *et al.*, 2006).

A lot of research work has already been done and is going on, worldwide, on the presence of Ochratoxin A in wines (Battilani *et al.*, 2006, Finoli *et al.*, 2004; Hocking *et al.*, 2003; Stander and Steyn, 2002; Tateo *et al.*, 2000). The International Organization for Vine and Wine (OIV) recently issued recommendations on the topic (OIV, 2005). The limit of 2 ng/mL (with the exception of sweet wines) was set by the Commission Regulation (EC) 1881/2006.

Wines produced in hotter and more humid climates (such as those around the Mediterranean region), are more likely to contain higher levels of OTA than wines produced in the cooler regions. Therefore Greek vine growing areas are reported as presenting a high OTA risk (Tjamos *et al.*, 2004; Roset, 2006; Battilani *et al.*, 2006).

In order to confirm this hypothesis, the Wine Institute of Athens investigated the amount of OTA in Greek dry and sweet wines: Wines produced in the frame of research programs and trade wines were analysed.

The wines elaborated at the winery of the Wine Institute are produced in the frame of research programs or as authentic samples destined to the Wine Data Bank of the European Union (Reg. (EC) 2120/2004).

Greek sweet wines were also included as they hold an important part in the Hellenic winemaking sector while legislation is planning in the future to set an upper limit for their OTA content. Generally, sweet wines are classified in three major categories:

- Sweet wine where alcohol of vitivinicultural origin (at least 95 % vol) is added to the fermenting must to stop alcoholic fermentation.

- Natural sweet wine from sun dried grapes whose fermentation stops naturally (under the combined influence of the high amount of sugars and alcohol produced by fermentation), without any addition of alcohol.

- Fortified wine where alcohol of vitivinicultural origin (at least 95 % vol) is added to the must and prevents fermentation (only 1 % vol of alcohol is tolerated to be produced by fermentation).

The presence of OTA in wine is a danger affecting health and wine economy. This is why research very soon included investigation of possible curative treatments on wines contaminated by high concentrations of OTA. Current clarification products used as fining agents (PVPP, potassium caseinate, silica gel, bentonite, etc.) have variable levels of efficiency for reducing contents of OTA: Oenological charcoal is the most efficient (Gambuti *et al.*, 2005) but only at relatively high dosages that cause damage to wine characteristics, while certain silica gel associated with fining with gelatine, only enables a partial reduction. Recent works (Bornet *et al.*, 2005 and 2007) gave promising data for the use of biopolymers of fungal origin (chitin, chitosan and derivatives) for decontamination in enology. More specifically, the use of chitin, chitosan and derivatives seems favorable for stabilization, clarification, removal of heavy metals and for the reduction of OTA in contaminated wines: Treatments with chitosan, chitin-glucan, chitin, and chitin glucan hydrolysate successfully reduced OTA in white, red and sweet wine, thereby improving wine safety. Other works included tests with egg albumin, gelatin and oak chips for red wines, bentonite, potassium caseinate (casein) and PVPP for white wines (Fernandes *et al.*, 2007; Savino *et al.*, 2007).

It was considered of interest to compare two treatments (dry yeast and bentonite) leading to OTA reduction in a naturally contaminated wine, on the basis of previous works (Garcia-Moruno *et al.*, 2005; Gambuti *et al.*, 2005).

MATERIALS AND METHODS

1. Wine samples

The wines analyzed cover the whole country.

The wines (n = 42) elaborated at the pilot-plant scale experimental winery of the Wine Institute of Athens, were produced:

- during research programs aiming at the quality improvement of dessert wines issued from particular vinegrowing districts or the evaluation of the quality of a particular wine, in view of the establishment of a new appellation of origin,

- as samples destined to the Wine Data Bank of the European Union (Reg (EC) 2120/2004).

The winemaking conditions were similar for all the wines produced at the Wine Institute:

White wines: Addition of sulphur dioxide (70 mg/l), static must clarification, addition of dry yeasts *S. cerevisiae*, fermentation at 18-20 °C.

Red wines: Addition of sulphur dioxide (60 mg/l), classic must fermentation with must recycling to keep skins wet, at 28-32 °C, separation from skins after 7 days.

Natural sweet wines: They are produced from sun dried grapes; grapes are vaporized with sulfur dioxide at a dose of 100 mg/kg grapes. Fermentation of these wines issued from sun dried grapes stops naturally, next to the combined action of the high amount of sugars and produced alcohol in the environment; in each case the fermentation is monitored and, if necessary, measures are taken to retard or stop the ongoing fermentation.

All the wines analyzed are issued from vintages 2000 to 2005 with a majority of samples for the years 2004 and 2005. Three sweet wines are issued from vintages 1975, 1985 and 1991.

Trade samples (n=61) were collected in January 2006.

Chemicals: OTA (>98 %) and toluene were purchased from Sigma-Aldrich. All reagents used in this work were of analytical grade. Water was obtained from Milli-Q purification system. HPLC grade solvents acetonitrile and methanol were purchased from LAB-SCAN Analytical Sciences whereas glacial acetic acid was provided by Carlo-Erba Analyticals. Polyethylene Glycol (PEG), sodium chloride, sodium hydrogen carbonate were purchased from Panreac. OchraTest columns were purchased from VICAM. Active dry yeast (ADY) was purchased from Uvaferm CM and Bentonite from KWK Volclay.

2. Analytical determination of OTA

The method of Visconti *et al.* (1999) described in the Compendium of the OIV (2005) was used. Stander and Steyn (2002) give useful details regarding passage of the sample through the immunoaffinity column during application of the aforementioned method.

In order to clean up the samples, solid phase extraction with immunoaffinity (IAC) columns was used according to the aforementioned method: Wine samples (10 mL) were diluted with an equal volume of a solution containing 1 % polyethylene glycol and 5 % $NaHCO_3$. The diluted sample was filtered through fiberglass filter if necessary and afterwards 10 mL were transferred to a syringe fitted to an immunoaffinity column. The sample passed at a flow of one drop per second, the IAC was washed with 5 mL of a cleaning solution (2,5 % NaCl and 0,5 % $NaHCO_3$) and then with an equal volume of water at a flow of one to two drops per second. IAC was dried by blowing air. OTA was eluted in a 4 mL glass vial with 2 mL MeOH at the rate of one drop per second. The eluate was evaporated to dryness at 50 °C with nitrogen and then redissolved immediately in 250 µL of HPLC mobile phase (isocratic mixture: acetonitrile: water: glacial acetic acid, 99:99:2, v/v/v).

High performance liquid chromatography (HPLC) analysis was performed by an HP 1050 chromatography system consisting of a quaternary pump fitted with a thermostatic column compartment, a fluorescence detector and an auto sampler. LC analytical column: Agilent Reversed-phase HPLC column ZORBAX, Eclipse XDB, C8, 5 µ, 4,6 x 150 mm id. The column was protected by a guard column Hypersil ODS, C18, 5 µ, 2,1 x 20 mm id).

An external calibration curve for OTA was prepared daily. Each analysis was performed in duplicate and the values were averaged. Results are expressed in ng/mL.

Average recovery ranged from 90 to 91,5 % for a spiking level of 1,5 ng/mL. The limit of detection was 0,01 ng/mL and the limit of quantification was 0,02 ng/mL.

3. Treatment for OTA reduction

Wine treatment with active dry yeast (ADY), at the doses of 1 g/L and 4 g/L was tested on a red wine naturally contaminated with OTA (grapes were left to rot on the vines) as described by Garcia-Moruno *et al.* (2005). OTA content of the wine was measured at time intervals 1-10 weeks.

The same wine was treated with bentonite, at the dose of 0,8 g/L: OTA content of the wine was determined after 3 and 6 days. Contact duration was based on the usual duration of wine fining with the use of bentonite.

For each agent (ADY and bentonite) concentration, the treatment was performed on two samples and the values were averaged.

4. Statistics

Statistical treatments of data were performed using SPSS (v. 10.0) and Microsoft Excel 2003.

RESULTS AND DISCUSSION

This study included 103 wines, produced either at the Wine Institute or in Greek trade companies (71 dry and 32 sweet wines). An additional sample was issued from grapes left to rot on the vine and it is not included in the overall evaluation. There was no detectable amount of OTA in 40 samples, (38,5 %) and the overall concentrations ranged between <0,01 ng/mL and 7,64 ng/mL with a median value of 0,11 ng/mL.

Among the analyzed dry wines there is no sample exceeding the limit of 2 ng/mL. In contrast, 12,5 % of sweet wines (3,9 % of the total examined wines) exhibit amounts >2 ng/mL.

The OTA content of wines, indicating colour and geographic distribution are given in figure 1 (dry wines) and table 1 (sweet wines). Table 2 presents the distribution of OTA amount according to vintage. In both tables, rosé samples (2 dry and 2 sweet wines) are counted with red wines.

Among dry wines (average=0,14 ng/mL), 56 % of the red wines and 52 % of white wines had undetectable amounts of OTA. Generally the concentrations ranged from < 0,01 - 1,18 ng/mL.

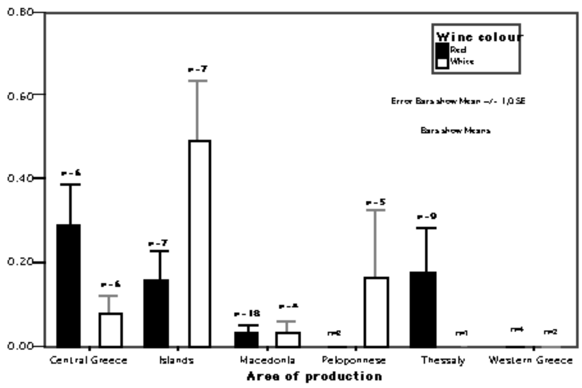

Figure 1 - OTA amount (ng/mL) in dry white (n = 25) and red (n = 46) wines from different regions of Greece

A statistically significant difference is recorded between OTA concentrations of wines produced in the islands and those produced in the mainland (least significant difference at 0,001 level for Macedonia and 0,05 level for Western Greece). There is no differentiation among the other vinegrowing districts. White dry wines produced in the islands exhibit relatively higher OTA content but always lower than the European limit of 2 ng/mL (figure 1 and table 3). The results are in accordance with other authors (Soufleros *et al.*, 2002; Stefanaki *et al.*, 2003) who investigated Greek wines issued from vintages produced before 1999. The OTA content is not influenced by the geographical position of the islands (north or south). It is possible that these relatively higher amounts are caused by the climatic conditions of the islands (a relatively high humidity combined to seasonal winds meltemia).

There is no significant differentiation among dry wines regarding colour and vintage (tables 2 and 3).

Sweet wines were examined separately (tables 1 and 3) from dry wines as the production's procedure and technology are different. Besides, sweet wines are produced in specific vinegrowing districts and they are not equally distributed all over the country.

In the case of sweet wines with addition of alcohol to the fermenting must (n=9, median=0,34), 77,8 % of OTA values do not exceed 0,50 ng/mL while two samples range between 1,50 and 1,73 ng/mL.

Table 1 - OTA amount (ng/mL) in sweet wines (n=32) from production areas in Greece.

n	Colour	OTA range	Average	Median	Standard Deviation
Central Greece					
2	white	0,27 – 0,56			
1	red	0,25			
3	*Total*	0,25 – 0,56	0,36[a]	0,27	0,17
Peloponnese					
3	white	0,15 – 0,86			
6	red	0,11 – 1,73			
9	*Total*	0,15 – 1,73	0,63[a]	0,33	0,62
Islands					
18	white	0,07 – 7,63			
2	red	0,37 – 1,95			
20	*Total*	0,07 – 7,63	1,34[b]	0,49	1,80

n : number of samples; Values with different letters are statistically different (level 0,001)

Table 2 - OTA amount (ng/mL) in 71 dry white and red Greek wines from different vintages.

n	Vintage	OTA range	Average	Median	Standard Deviation
2	2000	0,07 - 0,10	0,09	0,09	0,02
3	2001	0 - 0,27	0,13	0,11	0,14
8	2002	0 - 0,56	0,13	0	0,23
9	2003	0 - 0,87	0,17	0,03	0,28
37	2004	0 - 1,00	0,10	0	0,19
12	2005	0 - 1,18	0,25	0	0,39

n : number of samples

Table 3 - OTA content (ng/mL) in Greek wines, per wine category and colour.

	Category	Colour	n	min	max	Median	Average	Standard Deviation
Sweet wines	Fortified	red	1	1,95	1,95	1,95	1,95	0
		white	7	0,50	7,64	2,66	2,82	2,41
		Total	8	0,50	7,64	2,31	$2,71^{b}$	2,25
	Sweet	red	6	0,11	1,73	0,34	0,73	0,73
		white	3	0,07	0,38	0,34	0,26	0,17
		Total	9	0,07	1,73	0,34	$0,57^{a}$	0,63
	Natural sweet	red	1	0,25	0,25	0,25	0,25	0
		white	12	0,05	1,38	0,40	0,49	0,36
		rosé	2	0,27	0,33	0,30	0,30	0,04
		Total	15	0,05	1,38	0,33	$0,45^{a}$	0,33
	Grand Total		32	0,05	7,64	0,41	1,05	1,5
Dry wines		red	44	0	1,00	0	0,11	0,2
		white	25	0	1,18	0	0,19	0,32
		rosé	2	0		0		0
	Grand Total		71	0	1,18	0	$0,14^{a}$	0,25
GENERAL TOTAL			103	0	7.64	0,11	0,42	0,95

n : number of samples; Values with different letters are statistically different (level 0,001)

The data concerning fortified wines without any fermentation at all (n=8), are very different, as 50 % of the values exceed the limit for dry wines (2 ng/mL), with a median value of 2,31 ng/mL. Fermentation is mentioned to lead to OTA decrease (Battilani *et al.*, 2006), therefore its absence may explain these relatively higher amounts.

The examined wines from sun dried grapes did not exceed 1,38 ng/mL of OTA and 74 % of this type , (n=15, median=0,33) were below 0,50 ng/mL.

The high amounts of OTA in fortified wines (without alcoholic fermentation) have a strong impact on the average value for sweet wines and on the average values for the areas of production as well, as shown in tables 1 and 3. Sweet wines produced in the islands have significantly higher OTA amounts due to climatic and winemaking conditions.

When comparing the obtained levels of OTA between dry and sweet wines, the difference is remarkable: OTA was detected in 100 % of sweet wines, against 45,7 % of dry wines. Once again, these results can be interpreted on basis of the experience that wine treatment with yeast leads to OTA reduction, proportionally with contact time, as reported in the literature (Garcia-Moruno *et al.*, 2005; Battilani *et al.,* 2006), in accordance with the results of this work: The vinification procedure for sweet wines includes short or no contact at all (fortified wines without fermentation) with yeast. Sun drying of grapes also increases the risk of contamination.

However, statistical treatment in table 3 exhibits differentiation of average values for dry wines, wines from sun dried grapes, sweet wines on one hand and fortified wines (without fermentation) on the other.

Treatment with active dry yeast is recommended by OIV (2005) and it was considered of interest to compare this treatment to bentonite, concerning OTA decrease in a naturally contaminated red wine. The results are shown in table 4: both treatments were efficient: 0,8 g/L bentonite decreased the initial OTA amount of 3,15 ng/mL by 33,6-39,7 %, while in a recent work (Fernandes *et al.*, 2007), 0,5 g/L bentonite eliminated 7-17 % of the OTA amount in a white wine (depending on the initial OTA amount: 1,2-2,0 mg/mL). The same work included treatments with other agents, egg albumin (0,1 g/L) and gelatin (0,1 mL/L) for red wines, casein (0,4 g/L) and PVPP (0,16 g/L) for white wines. PVPP had no effect at all, while casein (potassium caseinate) reduced OTA by 13 % in a highly contaminated wine. Egg albumin and gelatin resulted in a reduction between 8 and 34 %, depending on the initial OTA contamination.

In the case of treatment with active dry yeast, in agreement with international references (Garcia-Moruno *et al.*, 2005), in the present work 25,4-38,7 % of OTA was eliminated for the treatment with 1 g/L ADY and 48,9-60,6 % when the dosage was increased to 4 g/L.

Other agents were successfully used for OTA reduction. Recently (Bornet *et al.*, 2007) treated a wine enriched in OTA with chitin-glucan, chitin, chitin glucan hydrolysate and chitosan at doses of 2 and 5 g/L. After two days, the levels of OTA in wines were analyzed by HPLC and OTA was reduced by 56,7-83,4 % in red wine, by 53,4-64,5 % in white wine and 26,1-43,5 % in sweet wine.

Another work concerns the possible use of oak wood fragments in red wines (Savino *et al.*, 2007) for reduction of OTA contamination. The effectiveness of the treatment depended upon the quantity of the oak wood fragments

Table 4 - Influence of active dry yeast (ADY) and bentonite treatment on the amount (ng/mL) of ochratoxin A in a naturally contaminated red wine.

Control wine	+1 g/l ADY	% Reduction	+4 g/l ADY	% Reduction	Treatment's duration	+0,8 g/l Bentonite	% Reduction	Treatment's duration
3,15	2,35	25,40	1,61	48,89	1 week	2,09	33,65	3 days
	2,32	26,35	1,60	49,21	2 weeks	1,90	39,68	6 days
	2,04	35,23	1,24	60,63	8 weeks	-	-	-
	1,93	38,73	1,24	60,63	10 weeks	-	-	

(chips and powder) used; best results were obtained with powder.

CONCLUSIONS

With reference to the European Regulation limit of 2 ng/mL, the results lead to the conclusion that there is no apparent problem associated with the level of OTA in Greek dry wines. There is a positive picture for the Greek wine production and a further lowering of the European limit down to 1 ng/mL, will not create a problem for most dry Greek bottled wines.

We need to be increasingly cautious in the case of wines produced in the islands where weather conditions and the types of wines traditionally produced, lead to a higher risk for presence of the contaminant OTA.

Among sweet wines, the category exhibiting increased levels was that of fortified wines where traditional winemaking does not include any kind of fermentation. Wines' colour or vintage do not appear to influence the OTA concentrations. Treatments with active dry yeast or bentonite reduced successfully OTA content of contaminated wines in accordance with previously reported works. Data collected from the trials of the present work support the theory of OTA adsorption onto suspended solids as well as the beneficial effect of yeast presence for a reduced OTA amount. Innovative substances appear to be promising such as chitin and chitosan derivatives and they may open new ways in enology for a healthier wine.

Acknowledgements : The authors would like to thank Dr Argyris TSAKIRIS, Professor at the TEI of Athens, section of Enology and Beverage Technology for his assistance in the statistical treatment of data.

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