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Ochratoxin A Contents in Wine: Comparison of Organically and Conventionally Produced Products

ALESSANDRO M. CHIODINI,† PETER SCHERPENISSE,† AND ALDERT A. BERGWERFF*

Institute for Risk Assessment Sciences, Division Public Health and Food Safety, Utrecht University, PO Box 80175, NL-3508 TD Utrecht, The Netherlands

Ochratoxin A (OTA) content was determined in 44 organically and conventionally produced wines originating from different geographical regions. Wine samples were extracted using a series of C_{18} and mixed-bed solid-phase cartridges and analyzed by HPLC with fluorescence detection. The identity of the mycotoxin was confirmed using liquid chromatography−tandem mass spectrometry. Recoveries were in excess of 90%, intraday precisions were better than 6%, and the interday variation was 15%. Limit of detection was 0.05 μg/L (HPLC). All sampled wines contained OTA below the level permitted by the European Union of 2 μg/L, ranging from not detectable (nd) to 0.75 μg/L for red wines ($n = 26$), from nd to 0.092 μg/L for rosé wines ($n = 2$), and from nd to 0.22 μg/L for white wines ($n = 16$). The concentration of OTA in organically produced wines (nd to 0.72 μg/L, median 0.092 μg/L, $n = 19$) was not significantly different from that in conventional products (nd to 0.75 μg/L, median 0.066 μg/L, $n = 25$) as assessed by a Mann−Whitney statistical test ($p = 0.54$).

KEYWORDS: Mycotoxins; food safety; food quality; organically produced foods; naturally occurring toxicants; biocontaminants

INTRODUCTION

Molds are able to produce relatively small, complex organic compounds as secondary metabolites showing toxicity in humans and animals. Mold-infected food-producing plants may become contaminated with these so-called mycotoxins during their pre- or post-harvest phase. Ochratoxin A (OTA) is a mycotoxin produced by several *Aspergillus* and *Penicillium* subspecies (*1*). It may contaminate important food products, including cereals, coffee and cacao beans, (dried) fruits, oleaginous seeds, and pulses (*2*). This phenylalanine-bound chlorinated dihydro-iso-coumarin toxin residue in contaminated food may show carcinogenic, immunosuppressive, nephrotoxic, and teratogenic properties in consumers. Indeed, the International Agency for Research on Cancer (IARC) has classified OTA as a possible carcinogen for humans (*3*). The mycotoxin might be involved in what is known as Balkan endemic nephropathy, which is a severe chronic renal disease associated with urinary tract tumors in the population of the Balkan region (*4*). Because OTA has been detected in food, including milk, and human blood in western European countries (*2, 5*), governmental authorities have established maximum permitted levels for several foodstuffs in order to minimize public health risks from the intake of this toxic substance.

It is only since 1995 that the occurrence of OTA in wine was recognized (*5*), and early provisional estimates suggested that 15% of the total OTA intake was due to wine consumption (*6*). This level was confirmed at 13% by a co-operative scientific dietary intake assessment study performed by EU Member States (*2*). Mean dietary OTA intake from wine ranges from 0.02 ng/(kg of body weight·day) (Portugal) to 0.86 ng/(kg of body weight·day) (Italy) by the whole population and to 2.94 ng/(kg of body weight·day) (Italy) by specific consumer groups (*2*). In European-produced wines, OTA concentrations have been found from nondetectable up to 15.6 μg/L (*2, 7, 8*) with almost 60% of the tested wines positive (*2*). Of note, red and sweet wines show higher OTA concentrations than rosé wines, which contain higher concentrations than white wines (*2, 9*); however other reports have found no differences (*10, 11*). The incidence of positive samples is greater in wines produced in South Europe compared with those from cooler areas to the north (*2, 9*).

Recently, a maximum permitted level at 2 μg/L for wine was enforced in the EU (*12*). The tolerated maximum concentrations are based on a tolerable daily intake of 5 ng/(kg of body weight·day) suggested by the EU Scientific Committee on Food (*13*). The regulatory safety limit for wine may, however, be adjusted after re-evaluation by the European Food Safety Authority (EFSA) as soon as new research results on the carcinogenicity of OTA are available from a European research project.

The methods for analysis of OTA are reviewed in refs *14* and *15*. To facilitate analysis, isolation and purification of OTA from wines were achieved by, for example, liquid−liquid or solid-phase extraction, liquid- or solid-phase micro-extraction, immunoaffinity chromatography, and the use of molecularly imprinted polymers (*5, 14−18*), although analysis was also

* Corresponding author. Tel: +31 (30) 253 5367. Fax: +31 (30) 253 2365. E-mail address: a.a.bergwerff@iras.uu.nl. Internet: http://www.iras.uu.nl.

† E-mail addresses of co-authors: alessandro.chiodini@tin.it (A. Chiodini); p.scherpenisse@iras.uu.nl (P. Scherpenisse).

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performed without sample cleanup (*19*). Most analytical approaches are based on high-performance liquid chromatography (HPLC) in combination with fluorescence detection. Unambiguous identification of OTA by HPLC may be obtained by ammonia or methyl ester derivatization before fluorescence detection or LC-MS/MS analysis following HPLC analysis (*2*).

With the growing awareness of organic farming, the safety of its food products is increasingly discussed. Regular monitoring systems, however, do not identify the original production system, so only very limited information on the occurrence of contaminants in the sampled food product is available. Hitherto, this issue has not been addressed satisfactorily; only a few studies have investigated the possible difference in levels of naturally occurring toxicants. For example, the incidence and level of the mycotoxin patulin was higher in organically produced apple juice than in the conventional product, but all samples contained less than the proposed limit of 50 μg/L (*20*). In contrast, deoxynivalenol (DON) contamination levels and its incidence in organically produced wheat were lower compared with other wheat products (*21*, *22*). No differences, however, were found for the concentration of DON in rye and of zearalenone in wheat (*22*). The metabolite of aflatoxin B_1 (AFB_1), aflatoxin M_1 (AFM_1), was detected below tolerated levels in milk produced in conventional systems but not detected at all or detected at lower concentrations in organically produced milk (*23*, *24*). On the other hand, the occurrence of zearalenone and derivatives of lysergic acid in organic feed for cattle may become a safety issue for dairy products stemming from organic production sites (*25*). Peanut butter from alternative shops contained higher levels of total aflatoxins and of AFB_1 compared to conventional peanut butter (*24*). With respect to OTA contamination of cereals, it was found that the risk of contamination correlated more with certain management practices, such as the use of home grown seeds and grain handling, than with organic or conventional farming system (*26*).

Several studies have published the results of their determination of OTA in wine (see refs *2* and *27* for a summary), but so far no study has addressed the OTA contents in organically produced wines. From a processing point of view, viticulture is performed in two steps, which are completely different, namely, the culture of the grapes and the winemaking process. Hitherto, organic viticulture has not been not very well regulated, and worldwide regional differences exist whether wines may be labeled as "organic" when (a part of) both phases or only the culturing of grapes is according to organic production standards. In the EU, the only legislative reference point is a general regulation for the organic production of agricultural products (*28*). In practice, differences between organically and conventionally produced wines are, in particular, in the preharvest period and during harvest. Farmers of organic grapes may refuse to use herbicides and will reduce the use of other pesticides and fertilizers compared to their colleagues in the conventional culture of grapes. Herbicides are replaced by cover crops, such as clover, mustard, or vetch, which are grown between the rows of vines. Pests are treated through "natural" means, such as introducing predators or spraying with plant or mineral extracts, natural bacteria, oils, or soaps. Fertilizers are generally compost or manure-based.

The reduced pesticide use, however, may increase the risk of fungal infections and thus may increase the risk of mycotoxin contamination as well. On the other hand, organic farmers tend to, for example, select varieties for cultivation that are more resistant to diseases and take other measures that reduce the risks of disease and spoilage of the product. The aim of this study was therefore to investigate the OTA levels in different wines as products of conventional or organic farming.

Table 1. Recovery of Ochratoxin A from Spiked Red Wine Samples[a]

spiking level (μg/L)	*n*	recovery (%)
0.20	6	103(9)
0.40	6	96(7)
0.60	6	89(7)
1.00	3	82.1(1.8)
2.00 (day 1)	6	105.1(1.0)
2.00 (day 2)	6	94(5)
2.00 (day 3)	6	95(8)

[a] On a single analysis day, the recoveries at 0.20, 0.40, 0.60, 1.0, and 2.0 μg/L were determined. On three different days of analysis, the recovery at 2.0 μg/L was determined. The standard deviation is given in parentheses.

MATERIALS AND METHODS

Reagents and Chemicals. All chemicals used were of analytical grade unless stated otherwise. Acetonitrile, acetic acid, ammonium acetate, ammonium hydroxide 25% (m/v), ethylacetate, methanol (HPLC-grade), and sodium acetate were purchased from J.T. Baker (Deventer, The Netherlands), and propionic acid was purchased from Merck (Darmstadt, Germany). Reversed-phase C_{18} (3 mL, 500 mg) and Oasis MAX (1 mL, 30 mg) columns for solid-phase extraction (SPE) were purchased from J.T. Baker and Waters (Milford, MA), respectively. Water was of Milli-Q quality (Millipore, Bedford, MA).

A stock solution of ochratoxin A (OTA; Sigma-Aldrich, St. Louis, MO) was prepared in methanol at 1.15 mg/mL. The stock solution was used over a period of 6 months and stored in the dark at 4 °C. Bottles of wine in 750-mL, except for one 500-mL, colored glass flasks, were bought in local stores (**Table 1**).

Extraction. Prior to sample processing, C_{18} SPE columns were activated by applying 2 mL of methanol and then conditioned by passing 2 mL of water. Bottles of wine were shaken, and 20-mL samples were loaded into a 75-mL polypropylene reservoir and allowed to flow through the C_{18} SPE cartridge by applying a vacuum at the outlet. The reservoir was washed with 5 mL of water, and finally, the column was washed with 2 mL of water. Each column was dried by centrifugation for 10 min at 3500*g* with an IEC Centra GP8R centrifuge (Needham Heights, IL) followed by elution of the analyte with 3 mL of a mixture of ethylacetate, methanol, and acetic acid (95:5:0.5, v/v/v). The collected eluate was dried under a stream of nitrogen at 37 °C.

Residual material was dissolved in 2 mL of a mixture of 0.02 M ammonium acetate and acetonitrile (75:25, v/v) and loaded on an Oasis MAX SPE column, which was activated with 1 mL of methanol and conditioned with 1 mL of a mixture of 0.02 M ammonium acetate and acetonitrile (75:25, v/v). The column was washed with 1 mL of the same mixture and subsequently with 1 mL of 1.25% (m/v) ammonium hydroxide and finally with 1 mL of a mixture of methanol, water, and acetic acid (30:70:1, v/v/v). The analyte was eluted from the cartridge with three volumes of 1 mL of 6% (v/v) methanol dissolved in acetic acid. Following drying under nitrogen, residual material was reconstituted in 1 mL of aqueous acetonitrile (50:50, v/v) and stored at 4 °C until analysis.

HPLC Analysis. HPLC analysis was achieved according to the method described in ref *19* with modifications. The HPLC system consisted of an autosampler (Triathlon, Spark Holland, Emmen, The Netherlands), an L 7100 pump (Merck Hitachi, Darmstadt, Germany), and a fluorescence detector (FP921, Jasco, Tokyo, Japan) operating at excitation and emission wavelengths of 333 and 460 nm, respectively. Data were collected and processed by a Thermoquest data evaluation system (Chromquest v2.51; San José, CA). Aliquots of 25 μL reconstituted extracts were injected onto a C_{18} Luna analytical HPLC column (150 mm × 4.60 mm, 5 μm; Phenomenex, Torrance, CA). Mobile phase A consisted of 0.5% propionic acid (v/v) mixed in acetonitrile and mobile phase B of 0.5% propionic acid (v/v) mixed in water. Volumes of mobile phases were mixed using a linear gradient as follows: 40% A for 1 min, from 40% to 90% A in 25 min, 90% A

for 5 min, and finally going to 40% A in 1 min at a flow rate of 0.80 mL/min and ambient temperature.

Prior to HPLC analysis, external standard solutions were prepared by appropriate dilution of the OTA stock solution in aqueous acetonitrile (50:50, v/v) to facilitate a ten-point calibration curve between 0.05 and 52 ng/mL corresponding to an OTA concentration range between 0.02 and 2.6 μg/L, respectively.

LC−MS/MS. LC−MS/MS analysis was carried out with an API 3000 LC system (Applied Biosystems) consisting of a binary pump and an autosampler and controlled by Analyst 1.4.1 software package (Perkin-Elmer). The column outlet was coupled to an electrospray ionization (ESI) source operated at an ionization voltage of 5500 V and a drying temperature of 400 °C with the drying gas flow rate set at 700 L/min. The entrance, declustering, and focusing potentials were set at −9, 40, and 180 V, respectively.

To avoid the introduction of high amounts of extracted wine matrix in the mass spectrometer and to improve the selectivity of the chromatography, two HPLC columns in combination with a switching valve were used. Chromatography of 5-μL samples was performed first on a 50 mm × 2.0 mm Luna C_{18} (3 μm) HPLC column (Phenomenex, Aschaffenburg, Germany) eluted isocratically with a mixture of methanol, water, and acetic acid (70:30:0.5, v/v/v) at a flow rate of 0.20 mL/min. This column was connected to a switching valve that wasted the first 5 min of flow-through before it introduced the eluate for 2 min on a second Phenomenex Luna C_{18} HPLC column (250 mm × 2.0 mm, 3 μm). The second column was eluted with linear gradient of acetonitrile and water at an identical flow rate as follows: 50% acetonitrile for 6 min, from 50% to 90% acetonitrile in 5 min, 90% acetonitrile for 6 min. The mobile phase was supplemented with 0.5% (v/v) acetic acid. The mobile phase gradient was applied to elute injected matrix components.

Tandem mass spectrometric analysis was achieved using the multiple reaction-monitoring (MRM) mode. The collision energy (CE) was set separately for each product-ion trace monitored: *m/z* 406 ($[M + H]^+$) → *m/z* 241 (CE 33 V), *m/z* 404 ($[M + H]^+$) → *m/z* 358 (CE 21 V), *m/z* 404 ($[M + H]^+$) → *m/z* 239 (CE 33 V).

Evaluation of Data. Quantification of OTA was performed by measuring peak areas at the retention time of the external OTA standard and by interpolation of the area on a calibration curve. Results were concentration data of OTA corrected for the recovery. Average concentrations were calculated over concentration data of samples containing OTA ≥ LOD. The Shapiro−Wilk statistical test was applied to the data series to test the normal distribution of the results. The Mann−Whitney statistical test was used to test whether the data series of conventional wines and the data series of organic wines were equal.

RESULTS

It was expected that OTA would occur predominantly at concentrations below the maximum tolerated level at 2 μg/L. Establishment of differences between series of concentrations, namely, that in organic versus conventional wines, in a narrow concentration range would need a satisfactory selective and sensitive analytical method. For this reason, samples were processed not only through a C_{18} SPE but also through a mixed bed SPE column containing anion-exchange and reversed phase sorbents, as well to remove possible interferences from the sample matrix. Affinity chromatography was not considered to extract OTA from wine because of the relatively high cost (*18*, *29*) and the risk of interference by wine components (*30*).

The regressed curve of standards showed a squared correlation coefficient (r^2) of better than 0.998. The retention time of OTA was observed at approximately 12 min. Recoveries of OTA from several types of wine, including red, white, and rosé wines, fortified with 2.00 μg/L OTA revealed no matrix-dependent recovery. In addition, no OTA concentration-dependent recovery was observed (**Table 1**). The suitability of the HPLC method was further demonstrated by the spiking of wine in a range of 0.20 to 2.0 μg/L for intraday precision and at 2.0 μg/L for the interday precision (**Table 1**), which were found to be up to 6% (RSD) and 15% (RSD), respectively. At a signal-to-noise ratio (S/N) of 3, the limit of detection was determined at 0.05 μg/L, whereas the limit of quantification (S/N at 10) was found to be 0.16 μg/L.

Table 2. Ochratoxin A Concentrations Determined in Red, Rosé, and White Wines That Were Produced Organically or Conventionally According to the Labels on the Flasks[a]

type of wine	D[b]	E[b]	F[b]	I[b]	ZA[b]	average concentration (min–max), μg/L	median, μg/L
				Red			
conventional			9	4	1	0.18 (nd[c]–0.75)	0.073
organic	1		7	3	1	0.16 (nd[c]–0.72)	0.066
				Rosé			
conventional		1				nd[c]	
organic		1				0.092	0.092
				White			
conventional	4	1	2	1	1	0.059 (nd[c]–0.18)	0.066
organic	1	1	2	2	1	0.081 (nd[c]–0.22)	0.094

[a] The origin of production in terms of geographical region is indicated. Concentration data were corrected for the recovery of OTA from reference samples. Average concentration was calculated over the results of samples containing Ota ≥ Lod. [b] code for region: D, Germany; E, Spain; F, France; I, Italy; ZA, South-Africa; [c] Not detected (OTA concentration was less than 0.05 μg/L).

The sampled wines contained alcohol at a concentration ranging from 11% to 14% according to the labels on the bottles. The region of the origin of each organically produced wine was matched as well as possible with a conventionally produced wine. Five, four, twenty, ten, and four bottles of wine were obtained from the regions of Germany, Spain, France, Italy, and South Africa, respectively (**Table 2**). The red wines were produced between 2001 and 2004, the white between 2003 and 2005, and the rosé wine in 2004. A single white wine was produced in 1997. None of the sampled wines contained OTA at a concentration in excess of the tolerated limit at 2 μg/L. The highest OTA concentrations were found in red wines at 0.75 μg/L (Italy, 2004) and at 0.72 μg/L (France, 2000) for conventional and organic products, respectively. Ten of 25 conventional wines contained OTA below the limit of detection (LOD); this number was 7 out of 19 samples for the organic wines (**Table 3**). Mann−Whitney statistics demonstrated that conventional and organic viticulture produce equal amounts of OTA in wine at $p = 0.54$. This test was executed as the Shapiro−Wilk test showed that the organic and conventional data series were not normally distributed ($p = 0.01$).

LC−MS/MS analysis was performed to confirm the presence of OTA (**Figure 1**). Despite the anionic character of OTA (pK_a for its carboxylic acid and phenolic constituents are at 4.4 and 7.1, respectively), MS analysis was not performed in the negative but in the positive mode, since acidification of the mobile phase was needed to chromatograph OTA on the C_{18} HPLC column. To enhance selectivity, chromatography had to be performed under acidic conditions. Abundant protonated molecular ions $[M + H]^+$ were, therefore, observed at *m/z* 404 and at *m/z* 406 at a ion ratio of 3:1 reflecting the stable isotopes of the chlorine atom in the OTA molecule. The fragmentation of $[M + H]^+$ at *m/z* 404 yielded product ions at *m/z* 386 $[M + H - H_2O]^+$, *m/z* 358 $[M + H - H_2O - CO]^+$, *m/z* 341 $[M + H - HCOOH - NH_3]^+$ and at *m/z* 239 [M + H − phenylalanine]$^+$ in correspondence with those reported by Reinsch et al. (*31*). In contrast to their findings, the most abundant ion in this study was at *m/z* 239, and the corresponding *m/z* 404 → *m/z*

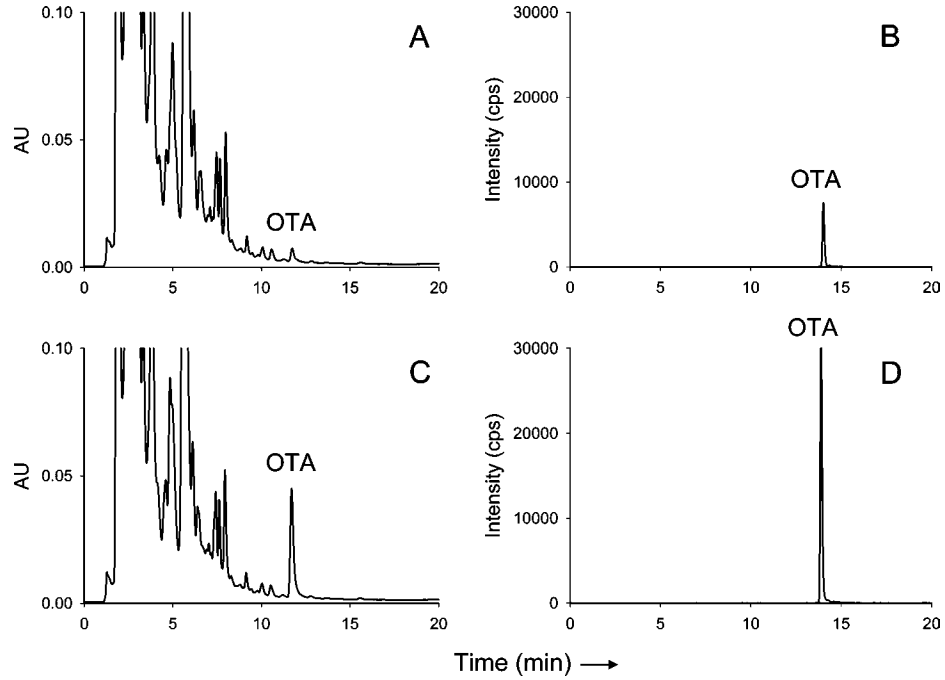

Figure 1. Typical HPLC fluorescence (panels A and C) and LC–MS/MS (panels B and D; MRM trace *m*/*z* 404 → *m*/*z* 239) chromatograms of red wine. The original wine contained 0.22 μg/L OTA (panels A and B) and was also fortified with 2 μg/L OTA (panels C and D). The elution position of OTA is indicated.

Table 3. Ochratoxin A Concentrations (Minimum–Maximum) Determined in Organically or Conventionally Produced Red, Rosé, and White Wines According to Their Regions of Origin

country	*n*	concentration (μg/L)	number of samples at <0.05 μg/L OTA[b]
France			
conventional	11	nd[c]–0.60	4
organic	9	nd[c]–0.72	3
Italy			
conventional	5	nd[c]–0.75	1
organic	5	nd[c]–0.09	3
Germany			
conventional	5	nd[c]–0.07	4
organic	1	nd	1
South Africa			
conventional	2	0.17–0.18	0
organic	2	0.16–0.53	0
Spain			
conventional	2	nd[c]–0.08	1
organic	2	0.09–0.10	0

[a] Concentration data were corrected for the recovery of OTA from reference samples. [b] <0.05 μg/L corresponds to the LOD of the method. [c] Not detected (OTA concentration was less than 0.05 μg/L).

239 trace was used here for LC−MS/MS analysis giving a LOD and a LOQ at 0.002 and 0.007 μg/L, respectively. The detection of, in particular, this *m*/*z* 239 ion was found supportive for the confirmation of OTA, as the loss of a phenylalanine is rather specific. When $[M + H]^+$ at *m*/*z* 406 was selected, corresponding fragments at *m*/*z* 388, *m*/*z* 360, *m*/*z* 343, and *m*/*z* 241 were observed, as expected for the ^{37}Cl isotope.

DISCUSSION

Besides ochratoxin A, wine may contain an ethyl ester derivative of this mycotoxin (*32*). This ochratoxin C (OTC) may occur at as much as 10% of the OTA content. This study applied a sample cleanup procedure with alkaline conditions at a final pH >10, and therefore it was expected that the ethyl ester was predominantly cleaved by saponification before analysis. The sample preparation procedure may thus have converted OTC back to the original OTA, because OTC is probably *de novo* synthesized under the acidic conditions of the wine in the presence of ethanol. After oral intake, OTC is also converted rapidly into OTA (*33*) permitting the analysis of OTA and hydrolyzed OTC as exclusively OTA.

The occurrence and final concentration of OTA in wine is dependent on many factors. Climate conditions, that is, geographical origin of the wine/grapes, and also grape variety and status of the berry were found important for OTA contamination (*2*, *34*). The number of positive samples and the level of OTA contamination is consistently higher in warmer southern regions compared with the cooler northern regions (*2*). Here, the number of samples was too limited to allow a reliable correlation between OTA concentration and region of origin (**Table 3**).

Grape varieties such as Cabernet-Sauvignon, Montepulciano, and Trebbiano were most susceptible toward fungal attack (*34*). Indeed, the wines containing the highest OTA concentrations in this study happened to be made partly from Trebbiano grapes. On the other hand, two wines made partly from the same grape and one from Cabernet-Sauvignon contained OTA below the LOD (results not shown).

Another important factor is control of fungal infection and damage by insects. OTA-producing *Aspergillus* spp. were detected in the beginning of the ripening, and the OTA concentration increased at harvesting of the grapes (*7*, *34*). The reduced use of insecticides and fungicides did not lead to a higher incidence of contamination nor to increased levels of mycotoxin contamination in foodstuffs produced organically (*35*). In contrast, it has been suggested that the use of fungicides in conventional farming may stress molds upon which they start to produce their toxins (*36*). It has also been suggested that fungicides may combat pathogenic molds but not the OTA-producing fungi *per se*, so that by lack of competition the OTA-fungi have better chances to grow and produce toxins (*36*). Good

agricultural, handling, and storage practices are required in organic, as well in conventional, agriculture to minimize the risk of mold growth and mycotoxin contamination. Apparently, this is very well understood by the farmers and producers, as this study showed no significant difference between the organic and conventional products as assessed by a Mann−Whitney statistical test ($p = 0.54$). In addition, no wine was found noncompliant to the maximum permitted level for OTA. This study was, however, not a comprehensive survey of all regions, production methods, and types of wines, and it cannot verify that, currently, all organic or conventional wines are within OTA limits nor that organic and conventional products are always significantly equal with respect to their OTA contents.

ABBREVIATIONS USED

AFB_1, aflatoxin B_1; AFM_1, aflatoxin M_1; CE, collision energy; DON, deoxynivalenol; EFSA, European Food Safety Authority; ESI, electrospray ionization; EU, European Union; HPLC, high-performance liquid chromatography; IARC, International Agency for Research on Cancer; LC−MS/MS, liquid chromatography−tandem mass spectrometry; LOD, limit of detection; LOQ, limit of quantitation; MRM, multireaction monitoring; OTA, ochratoxin A; OTC, ochratoxin C; RSD, relative standard deviation; S/N, signal-to-noise ratio; SPE, solid-phase extraction.

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