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# Ochratoxin A occurrence in experimental wines in relationship with different pesticide treatments on grapes

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**Abstract**

A reversed-phase HPLC method was utilized for Ocrhratoxin A (OTA) determination in 23 white and red wine samples produced in the year 2000. All were come from some vineyards, treated with different pesticides, located in three Italian regions. Analytical methods included commercial immunoaffinity columns and a HPLC system equipped with a RF detector. The sensitivity of the analytical method was 0.01 ng $ml^{-1}$. Values of OTA found in wine samples show that OTA is more frequently detected in red than in white wines. In fact, all red wine samples were contaminated. The OTA levels reported in this work are comparable with those reported for mycotoxin in red wine. Moreover, the different contents of Ochratoxin A in the wines can be considered an efficiency index of pesticides used.
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## 1. Introduction

Mycotoxins are a class of highly toxic chemical compounds produced, under particular environmental conditions, by several moulds developing in many foodstuffs. Their presence depends on several factors, such as: fungal strain, climate and geographical conditions, cultivation technique and foodstuff conservation (Capuano, Dugo, & Restani, 1999). Mycotoxins may occur in various vegetal products, such as as cereals, dried fruits, coffee beans, cocoa and beverages, such as beer and wine (Blanc, Pittet, Munozbox, & Viani, 1998; Pittet, Tornare, Huggett, & Viani, 1996; Solfrizzi, Avvantaggiato, & Visconti, 1998). Among mycotoxins, very important are the ochratoxins. This term indicates a group of metabolites, having similar chemical structures, produced by strains of the genus *Aspergillus* (*A. ochraceus*) and *Penicillium* (*P. ferrucosum*) (Blank, Hohler, & Wolffram, 1999; Capuano et al., 1999; Hohler, 1998).

The most studied, both for its diffusion and toxicological importance, is Ochratoxin A (OTA), i.e. R-N-[(5-chloro-3,4-dihydro-8-hydroxy-3-methyl-1-oxo-1H-2-benzopyran-7-yl)carbonyl]-phenylalanine. It has an oral LD50 level of 20 mg/kg in rat and pig (Cerutti, 1992). OTA is highly toxic and causes severe animal and human intoxications, for example ''Porcine nephropathy'' and ''Balcan Endemic nephropathy'' (Castegnaro, Plestina, Dirheimer, Chernozemsky, & Bartsch, 1991; Creppy, 1999; Creppy, 1998).

OTA exhibits nephrotoxic and teratogen activites and, moreover, suppressive actions on the immune system, causing a diminution of immune globulin level and of other humoral factors both in mice and chicken and a reduction of cell immune responses (Castegnaro et al., 1998; Pohl-Leszkowicz, Pinelli, Bartsch, Mohr, & Castegnaro, 1998).

Presence of OTA, at levels ranging from 0.1 to 40 ng/ml in biological fluids, demonstrates that 50% of the population of Italy enclosed, is exposed to this contamination (Scott et al., 1998; Ueno, Maki, Lin, Furuya, Sugiura, & Kawamura, 1998; WHO, 1996). Codex Alimentarius Commission, based on limited data, suggests that 15% of the total intake of OTA in humans is due to wine consumption (Codex Alimentarius Commission, 1998).

Recent carcinogenic studies on two species of rats suggest a tolerable intake (TDI) for OTA ranging from

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0.2 to 5 ng/kg body weight/daily. The WHO/FAO/JECFA proposes that a provisional tolerable weekly intake (PTWI) value, on a renal damage basis, is 100 ng/kg body weight (European Commission, 1996, 1997; JECFA, Evaluation of Certain Food Additives and Contaminants, 1995; Olsen, Thorup, Knudsen, Larsen, Hald, Olsen, 1991; Scientific Committee on Food Opinion on Ochratoxin A, 1998; WHO, 1996; WHO/IARC, 1993).

Recently, more attention has been focused on Ochratoxin A levels in commonly consumed foods, especially fruits and cereals (MacDonald et al., 1999; Scudamore, Nawaz, & Hetmanski, 1998a, 1998b) and on fermentation products such as beer (Degelmann, Becker, Herderich, & Humpf, 1999; Nakajima, Tsubouchi, & Miyabe, 1999; Visconti, Pascale, & Centonze, 2000) and wine (Burdaspal & Legarda, 1999; Festas, Herbert, Santos, Cabral, & Barros, 2000; Leitner et al., 2002; Majerus & Otteneder, 1996; Ospital, Cazabeil, Betbeder, Tricard, Creppy, & Medina, 1998; Otteneder & Majerus, 2000; Soleas, Yan, & Goldberg, 2001; Visconti, Pascale, & Centonze, 1999; Zimmerli & Dick, 1996).

As far as wine is concerned, available data regarding the presence of OTA are very discordant (Burdaspal & Legarda, 1999; Majerus 1996; Ospital et al., 1998; Otteneder & Majerus, 2000; Soleas et al. 2001; Zimmerli & Dick, 1996). Some authors report a high toxin concentration (up to 7.0 ng/ml) with considerable levels of contamination (incidence up to 92%) in red wine produced in southern regions of Europe and in North Africa. Other authors reported OTA contamination levels ranging from 3.9% for white wines to 16.6% for red wines, even if these data were obtained by different analytical methods. Generally red wines contain a greater amount of OTA than the white or rosè ones. These differences can be attributed to climatic factors, grape cultivation and storage conditions, apart from wine-making techniques.

The content of OTA in 23 samples of red and white wine, produced in the year 2000 in three experimental viticultures, situated in three different Italian regions treated with different pesticides (Tables 1–3), is reported in this work.

Table 1
Pesticide treatments on "Inzolia" and "Carricante" (Sicily) varieties of grapes

| Wine samples | No. of pesticide and treatments during grape ripening | |
|---|---|---|
| Sicily 1 | **1** Sulfur 80 Pb | **6** Quinoxyfen250 SC |
| Sicily 3 | **1** Sulfur 80 Pb | **6** Fenarimol 12 SC |
| Sicily 4 | **1** Sulfur 80 Pb | **6** Azoxystrobin 250 SC |
| Sicily 5 | **1** Dinocap 350 EC | **6** Penconazole 100 EC |
| Sicily 8 | **1** Sulfur 80 Pb | **6** Sulfur 80 Pb |
| Sicily 9 | **1** Sulfur 80 Pb | **6** Dinocap350EC |
| Sicily 10 | **1** Sulfur Powder | **6** Sulfur Powder |
| Sicily 11 | **1** Water | **6** Water |

Table 2
Pesticide treatments on "Fiano di Avellino" (Campania) variety of grapes

| Wine samples | No. of pesticide and treatments during grape ripening | |
|---|---|---|
| Campania 1 | **2** Sulfur 80 Pb | **6** Quinoxyfen 250 SC |
| Campania 2 | **2** Sulfur 80 Pb | **6** Fenarimol 12 SC |
| Campania 3 | **2** Sulfur 80 Pb | **6** Azoxystrobin 250 SC |
| Campania 4 | **2** Sulfur 50 PS | **6** Sulfur 50 PS |
| Campania 5 | **2** Dinocap350EC | **6** Penconazole 100 EC |
| Campania 6 | **2** Sulfur 80 Pb | **6** Dinocap 350 EC |
| Campania 7 | **2** Water | **6** Water |
| Campania 8 | **2** Sulfur 80 Pb | **6** Sulfur 80 Pb |

Table 3
Pesticide treatments on "Sangiovese" (Tuscany) variety of grapes

| Wine samples | No. of pesticide and treatments during grape ripening | |
|---|---|---|
| Tuscan 1 | **2** Sulfur 80 Pb | **9** Quinoxyfen 250 SC |
| Tuscan 2 | **2** Sulfur 80 Pb | **9** Fenarimol 12 SC |
| Tuscan 3 | **2** Sulfur 80 Pb | **9** Azoxystrobin 250 SC |
| Tuscan 4 | **2** Dinocap 350 EC | **9** Penconazole 100 EC |
| Tuscan 5 | **2** Sulfur Powder | **9** Sulfur Polvere |
| Tuscan 6 | **2** Sulfur 80 PBWG | **9** Sulfur 80 PBWG |
| Tuscan 7 | **2** Dinocap 350 EC | **6**+**3** Sulfur + Quinoxyfen 250 SC |

## 2. Materials and methods

### 2.1. Chemicals and reagents

Standards of OTA, sodium chloride, polyethylene glycol (Peg 8000), sodium hydrogencarbonate, glacial acetic acid and toluene were obtained from Fluka (Milano-Italy). Immunoaffinity columns were purchased from Vicam (Waterton, MA, USA).

Acetonitrile, methanol and water (HPLC grade) were supplied from Carlo Erba Reagents (Milan-Italy).

### 2.2. Wine samples

A total of 23 wine samples (16 white 7 red samples), produced in the year 2000 by three experimental viticultures situated in three areas, "Avellino"—Campania, "Catania"—Sicily and "Grosseto"—Tuscany, were analysed.

### 2.3. Wine characteristics and pesticide treatments

White wines came from Sicily and Campania in the crop year 2000. Sicilian wines were produced from 15 to 20 year-old plants, grown on Etna (300 masl, S. Venerina, Catania, Italy), in volcanic soil. Vines were grafted with Inzolia and Carricante varieties in a 1:1 ratio.

Wines from Campania were produced from 25 year-old plants grown on the Montefredane hills (700 masl, Avellino, Italy), in a clayey soil. Vines were grafted with the Fiano d'Avellino variety. Red wines from Tuscany were produced from 25 year-old plants, cultivated in Maremma Toscana coast (100 masl, Grosseto, Italy), on a calcareous soil. Vines were grafted with the Sangiovese variety, Morellino clone.

The vinification process was run within 24 h after grape harvesting and it was run using the following scheme:

### 2.4. White vinification

Newly cropped *Vitis vinifera* grapes from Sicily and Campania were crushed, destemmed and then soft-pressed by a pneumatic press. Must was treated with $SO_2$ (30 g/hl), pectolytic enzymes (1.5 g/hl) and vitamin C (5 g/hl) to favour clarification before fermentation; temperature was maintained at 8 °C for 24 h. Clear must was spiked with 20 g/hl of thiamine and ammonium phosphate as fermentation coadjuvants and 30 g/hl of selected yeasts and fermentation was run at 15 °C. In order to remove leaves, after fermentation, wine was decanted into a tank and spiked with $SO_2$ (5 g/hl). Ten days later wine was decanted again and treated with $SO_2$ (2–3 g/hl), bottled in dark bottles and maintained at 4 °C for the duration of the experimentation. Each grape sample was separately vinificated, following the earlier mentioned protocol.

### 2.5. Red vinification

Newly cropped *Vitis vinifera* grapes from Tuscany were crushed and destemmed, then spiked with $SO_2$ (5 g/hl), treated with selected yeasts (30 g/hl) and allowed to ferment for 10 days at 28 °C, making three fillings per day. Wine was drawn from the vat and the vinasses pressed by a hydraulic press. After 4 weeks, lees were removed and the wine spiked with $SO_2$ (2–3 g/hl). Wine was spiked again with $SO_2$ (2–3 g/hl) and bottled in dark bottles and maintained at 4 °C for the duration of the experimentation.

Each grape sample was separately vinificated following the earlier mentioned protocol. All wines were stored in the dark at 4 °C, and each sample was opened immediately prior to analysis.

### 2.6. Pesticide treatments

Pesticides were used at the doses recommended by the manufacturer and were sprayed with a manual sprayer onto the three Italian vines. Each of the Sicilian samples but four, was subjected to a sulfur treatment, followed by organic pesticide treatments, applied during grape ripening and repeated six times every 15 days. Two samples were treated only with sulfur (dry and wettable powder), one sample was treated only with organic pesticides (Dinocap–Penconazole) and one only with water, in order to give a comparison with the treated samples (Table 1). Each sample from Campania but four, was subjected twice to sulfur treatments every 10 days, followed by organic pesticide treatments applied during the vine maturation phase and repeated siz times every 15 days. Two samples were treated only with sulfur (dry and wettable powder), one sample was treated only with organic pesticides (Dinocap–Penconazole) and one sample only with water (Table 2). Each Tuscan sample but four, was subjected twice to sulfur treatments, applied during the vine maturation phase and repeated nine times every 12 days. Two samples were treated only with sulfur (dry and wettable powder), one sample was treated only with organic pesticides (Dinocap–Penconazole) and one sample only with water (Table 3).

### 2.7. Apparatus

The analytical method proposed by Visconti et al. (1999, 2000) for OTA determination in wine was utilized. Commercial immunoaffinity columns and HPLC equipped with a RF detector were used for Ochratoxin A determination.

The chromatographic analysis was done with a Shimadzu HPLC system equipped with a System Controller SCL-10 A, an RF-1AXL detector ($\lambda_{ex} = 330$ nm, $\lambda_{ex} = 460$ nm), a LC 10A pump, a Rheodyne injector with a loop of 20 μl and a reversed-phase Supelco column $C_{18}$ (15 cm × 4.6 mm, 5 μm particles) equipped with a guard filter (0.5 μm). Analyses were run at room temperature under isocratic conditions with a mobile phase composed of water/acetonitrile/acetic acid 99:99:2 v/v/v at a flow rate of 1 ml/min. OTA quantification was done by measuring peak areas at OA retention time and by comparing them with a calibration curve. A wine sample with added OTA served as an OTA identification standard. An HPLC chromatogram of a standard solution of Ocratoxin A is shown in Fig. 1. The sensibility of the analytical method was of 0.01 ng/ml.

## 3. Results and discussion

Values of OTA found in wines are shown in Table 4. OTA was not found in white wines produced in Campania while, of eight samples from Sicily, only six were found to be contaminated; the highest value found was 0.03 ng/ml in the sample treated with dinocap. The sample treated with quinoxifen and that treated with water were found not to be contaminated with OTA. Because OTA concentration in this wine is very low, it is not possible to correlate its presence with the pesticide treatments.

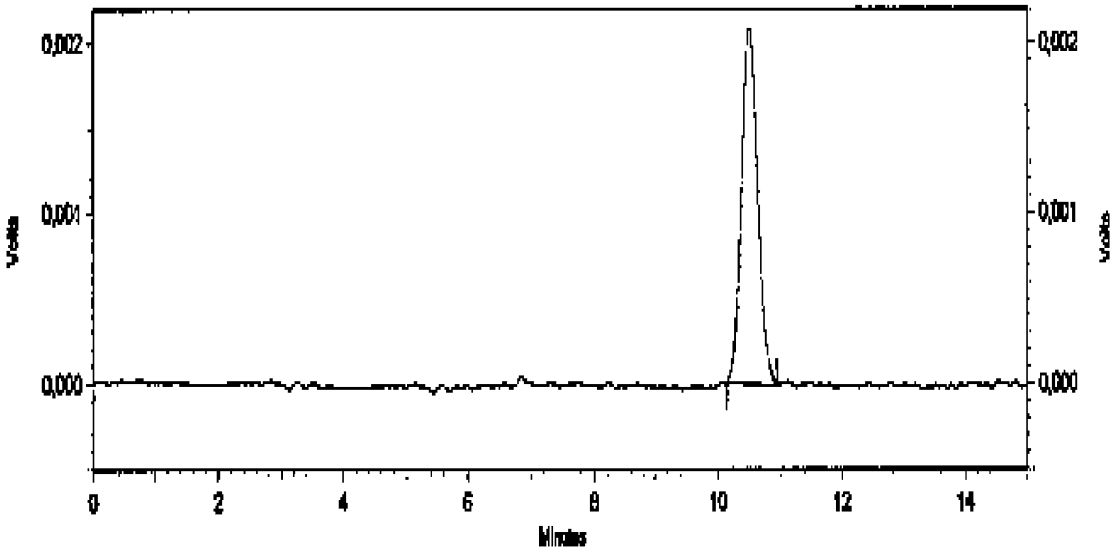


Fig. 1. HPLC chromatogram of standard OTA.

Values of OTA found in red wines show that all samples are contaminated. The sample produced with grapes treated only with sulfur 80 PBWG, showed an Ochratoxin A content (2.00 ng/ml) higher than the other samples, followed by the sample obtained from grapes treated with powdered sulfur (0.71 ng/ml). The concentration of OTA in the other samples ranged from 0.07 ng/mg in the sample treated with "azoxystrobin" to 0.24 ng/mg in the sample treated with "dinocap and penconazole". The values of OTA found, are comparable with those reported in the literature for mycotoxins in red wines.

The experimental viticulture located in Campania is not subject to pollution from OTA-producing moulds. The experimental Sicilian cultivar seems to be more easily contaminated with OTA since, of eight wine samples, six were found contaminated.

Table 4
Concentrations of OTA in wine samples

| Wine samples | Ochratoxin A (μg/l) |
|---|---|
| Sicily 1 | n.d. |
| Sicily 3 | 0.02 |
| Sicily 4 | 0.01 |
| Sicily 5 | 0.02 |
| Sicily 8 | 0.02 |
| Sicily 9 | 0.03 |
| Sicily 10 | 0.01 |
| Sicily 11 | Traces |
| Tuscan 1 | 0.22 |
| Tuscan 2 | 0.11 |
| Tuscan 3 | 0.07 |
| Tuscan 4 | 0.24 |
| Tuscan 5 | 0.71 |
| Tuscan 6 | 2.00 |
| Tuscan 7 | 0.10 |

n.d., not deteceted.

Values of OTA found in red wines show that the experimental viticulture located in Tuscany is strongly subject to infection by OTA-producing fungi. Synthetic pesticides can reduce OTA concentration from 96.5% in the sample treated with azoxystrobin, to 88% in the sample treated with dinocap and penconazole. Since OTA is strictly related to the growth of some toxigenic fungi on grapes, the different Ochratoxin A contents in wine samples can be considered to be an efficiency test of the pesticides used.

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