Occurrence of ochratoxin A before bottling in DOC and DOCG wines produced in Piedmont (northern Italy)

Occurrence of ochratoxin A before bottling in DOC and DOCG wines produced in Piedmont (northern Italy)

D. Spadaro[12]*, A. Lorè[2], A. Garibaldi[2], M.L. Gullino[2]

[1] DiVaPRA – Plant Pathology, University of Turin, Via L. da Vinci 44, I-10095, Grugliasco (TO), Italy

[2] AGROINNOVA – Centre of Competence for the Innovation in the Agro-environmental Sector, University of Turin, Via L. da Vinci 44, I-10095, Grugliasco (TO), Italy

*Corresponding author.

E-mail address: davide.spadaro@unito.it (D. Spadaro).

Abstract

Italy is one of the countries where ochratoxin A (OTA) contamination in wine poses more risks. Previous surveys on the occurrence of OTA have poorly considered north Italian wines. In this study, 1,206 red and white DOC and DOCG wines produced in Piedmont (Northern Italy) from 2000 to 2007 have been analyzed for OTA level (0.116 µg l^{-1}) and incidence (68.0%). The monitoring - the widest per number of Italian wine samples considered - analyzed the OTA contamination of wines in tanks just before bottling. OTA level and incidence were significantly higher in red (0.121 µg l^{-1}, 69.4%) than in white (0.086 µg l^{-1}, 61.3%) wines. Among the white wines, the incidence was significantly lower in the Moscato wines (7.3%), due to the different wine processing. The differences in the mean OTA level in the three main grapevine varieties of red wines could be related to the harvest period. Among the Nebbiolo appellations, a reduction of the OTA level was noticed with increasing the wood aging period. A significant effect of the vintage year was also registered.

Keywords: *Aspergillus carbonarius*, HPLC, mycotoxins, ochratoxin A, survey, wine.

1. Introduction

Ochratoxin A (OTA) was first detected in wine by Zimmerli and Dick (1996). On grapes, OTA is produced by *Aspergillus* belonging to the section *Nigri* (black aspergilli), and in particular by *Aspergillus carbonarius* (Battilani & Pietri, 2002). OTA constitutes a serious threat for the human and animal health, as it is a potent nephrotoxin, which also exhibits immunosuppressive, teratogenic and carcinogenic properties. Currently, the European

Union has a specific regulation for the OTA thresholds in different food, 2 µg/kg being the maximum level of the mycotoxin allowed for wine, grape must and grapes (European Commission, 2006).

There is a correlation between the mycotoxin contamination and the colour class of the wine: both the incidence of contamination and the concentration of the toxin are higher in red than in rosé or white wines (Otteneder & Majerus, 2000), due to the maceration of the must with grape skins, which might favour OTA extraction from the skin (Blesa, Soriano, Moltó, & Mañes, 2006). In Europe, a concentration gradient was also observed from the north to the south of Europe, especially for red wines (Pietri, Bertuzzi, Pallaroni, & Piva, 2001). This could be attributed to the hotter and more humid climatic conditions in southern countries that can better favour the growth of *A. carbonarius* and the consequent production of OTA. A geographical gradient has also been observed from north to south of Italy (Brera et al., 2008).

Piedmont is a northern Italian region vocated to the production of high quality red and white wines, including worldwide known denominations, such as Asti, Barbaresco, Barbera, Barolo, Dolcetto, Nebbiolo and Roero. Over 70% of the regional wine production is represented by Appellation of Controlled and Guaranteed Origin (DOCG) and Appellation of Controlled Origin (DOC) wines.

Generally, all the previous reports considering Italian wines, analyzed few samples from the northern regions and summarily concluded that the northern wines have very low levels or not detectable OTA contamination. In particular, Visconti, Pascale, and Centonze (1999), analyzed 56 samples of red (38), rosé (8), white (9) and dessert (1) wine, finding 87% of contamination, ranging from < 0.01 to 7.6 µg/kg, but the authors affirmed that most of the samples where coming from southern Italy. Pietri, Bertuzzi, Pallaroni, and Piva

(2001) analyzed 111 Italian wines, 23 of them produced in north-western Italy (9 in Piedmont): the mean was 0.011 µg/l and the incidence was 65%. An extensive study on the OTA contamination on Italian wines was carried out by Brera et al. (2008), that analyzed 1166 samples: none of the 34 wines produced in Piedmont trespassed the limit of detection (0.01 µg/l). All the above-mentioned papers analyzed wines in bottles purchased in local food stores. Anyway, Grazioli, Fumi, and Silva (2006) reported a significant reduction of the OTA content in wines after 12 months of bottle-aging. In our study, all the samples were withdrawn from wines still in the tanks of the wineries just before bottling.

The aim of this study was to assess the incidence and level of OTA in Piedmontese red and white wines DOC (Appellation of Controlled Origin) and DOCG (Appellation of Controlled and Guaranteed Origin), over a wide period (from 2000 to 2007). A second aim was to evaluate if the colour, the grapevine variety, the vintage year, and the wood aging period had an effect on the OTA content. In particular, the wines produced from Nebbiolo grapes can receive different appellations based on the wood and bottle aging period (Nebbiolo, Roero, Barbaresco, and Barolo).

2. Materials and methods

2.1. Samples

A total of 1,206 samples of wines produced in Piedmont were analyzed, all of them collected between 2004 and 2008 from 132 wineries distributed all around the Piedmont region, at the end of the winemaking process just before bottling. The wines were produced from grapes harvested in the period 2000-2007. A lower number of samples for the years 2000-2002 is related to the fact that just aged wines were collected for this period. The

106 wine samples - all of them DOC (Appellation of Controlled Origin) or DOCG (Appellation
107 of Controlled and Guaranteed Origin) - included 1,002 red wines and 204 white wines.
108
109 *2.2. Sample preparation and analysis*
110 The method developed by Visconti et al. (1999) with some modifications was used for
111 OTA determination. A 10 ml aliquot of each sample was diluted with 10 ml water solution
112 containing polyethyleneglycol (1%) and $NaHCO_3$ (5%), mixed and filtered through
113 Whatman® GF/A glass microfiber filter (GE Healthcare®, Piscataway, NJ, USA). Ten ml of
114 diluted extract were cleaned up through an OchraTest® (Vicam®, Milford, MA, USA)
115 immunoaffinity column. OTA was eluted by adding three times 0.75 ml methanol and
116 collected in silanized clean vial. The elute was dried using a microplate evaporator with air
117 flow at 40°C and recovered with 0.5 ml of HPLC mobile phase. Samples were analyzed in
118 a HPLC apparatus Agilent® 1100 series equipped with G1379 degasser, G1313A
119 autosampler, G1316A column thermostat set at 27°C, G1321A FLD - Fluorescence
120 Detector set at excitation and emission wavelengths of 333 and 460 nm, respectively,
121 G1311 quaternary pump and Agilent Chemstation G2170AA Windows XP operating
122 system (Agilent®, Waldbronn, Germany). An analytical column RP-18 (XTerra® Waters®,
123 Milford, MA, USA; 150 mm x 4.6 mm i.d., 5 µm) with a pre-column was used. The mobile
124 phase, eluting at 1 ml min^{-1}, consisted of an isocratic mixture of acetonitril:water:acetic
125 acid (99:99:2) for 18 min. 100 µl of sample were injected into the HPLC column and the
126 retention time of OTA was ca. 6.23 min.
127 The amount of OTA in the final solution was determined by using a calibration graph of
128 concentration versus peak area and expressed as ng/ml, achieved by injection into the
129 HPLC column of 100 µl of standard solutions of OTA (Sigma Chemical Co.®, St Louis,

MO, USA). The recovery (Table 1) was determined on a blank wine spiked at three concentrations of ochratoxin A (0.1, 2 and 10 µg l^{-1}). Each test was performed six times and the median recovery value were respectively 90.6%, 91.8% and 92.4%. The repeatability was, respectively, 2.64%, 2.71% and 2.82% (Table 1). The limit of detection (LOD) and the limit of quantification (LOQ), based on the IUPAC definition (Thompson, Ellison, & Wood, 2002) were respectively 0.0072 and 0.0093 µg l^{-1}. The high value of regression coefficient ($R^2 \geq 0.99$) obtained indicated a good linearity of the analytical response.

2.3. Statistical analysis

Samples with a concentration of OTA higher than the LOD were considered positive, whereas samples with concentrations lower than LOD were considered negative. Mean OTA concentrations were calculated by using 0 for negative samples. Experimental results are reported as mean ± standard deviation and maximum. The Kruskal-Wallis test was used to compare the mean OTA levels among the different vintage years, grapevine varieties of red or white wines, and appellations of Nebbiolo wines, while the Mann-Whitney test was used to compare the mean OTA levels in red / white wines, using the null hypothesis that the levels were not different. The χ^2 test was used to compare the OTA contamination frequencies of different categories of wines. Statistical analyses were performed by using the programme SPSS Release 12.01.

3. Results and discussion

Most of the studies carried out on the OTA level and incidence analyzed wines in bottle bought in the supermarkets or wine shops. A particularity of this study was to analyze the

154 OTA contamination of wines in tanks just before bottling and commercialization. Sample
155 population of the present study was composed of 1,206 units and 4 variables as follows:
156 colour of wine (red or white), grapevine variety, or appellation (Table 2), and year of
157 vintage (Table 3). The present study represents not only the widest monitoring on
158 Piedmontese wines for number of samples, but also on Italian wines and, excluding the
159 interlaboratory study realized for the European Commission in 2002 (Miraglia & Brera,
160 2002), it is the single monitoring with the highest number of sample analyzed. Previous
161 studies on Italian wines, carried out by Visconti et al. (1999), Pietri et al. (2001), and Brera
162 et al. (2008) analyzed respectively 56, 111, and 1166 Italian wine samples.
163 Comparing the results obtained in this study with similar monitoring, a generally low level
164 of contamination emerges in the wines produced in Piedmont, but the incidence of the
165 contamination is relatively high (68.0%). It means a high percentage of wines contaminated
166 with a low level of OTA. Brera et al. 2008 found an incidence of 64.3% on all the Italian
167 wine samples, but considering the north Italy wines, the incidence was only 7.3%. The
168 incidence resulted relatively high at European level (59.0%; Miraglia & Brera, 2002).
169 Studies about the relationship between presence of black aspergilla in vineyard and final
170 level of OTA contamination in the wines produced, showed that even low levels of
171 mycotoxigenic fungi can be related to significant ochratoxin A contamination (Battilani,
172 Giorni, Bertuzzi, Formenti, & Pietri, 2006). The presence of *Aspergillus* section *nigri* in
173 Piedmontese vineyards was very low, either at veraison or at harvest (Spadaro, Lorè,
174 Ciavorella, Garibaldi, & Gullino, 2008).
175 As far as wine colour is concerned, a higher incidence of positive samples was observed in
176 the red (69.4%) compared to the white wines (61.3%). The χ^2-test showed that the
177 frequencies of OTA occurrence in red and white wines were not comparable (p=7x10^{-137}).

Also the mean OTA concentration was statistically higher (p=0.001, Mann-Whitney test) in the red (0.121 µg l^{-1}) than in the white wines (0.086 µg l^{-1}). The percentage of red and white wines with over 0.1 µg l^{-1} OTA were 24.6% and 13.7% respectively. The different level of contamination in the red and white wines is related to the wine-making process – red wines have longer maceration that favour the OTA extraction from the pomace to the must (Grazioli et al., 2006) – and confirms previous studies (Otteneder & Majerus, 2000). The maximum level of OTA detected in a white wine – a Roero Arneis produced in 2007 – was 1.36 µg l^{-1}, and the maximum level in a red wine – a Nebbiolo produced in 2005 – was 2.63 µg l^{-1}. Two red wines out of 1206 samples (0.17%) trespassed the maximum admitted level of 2.0 µg l^{-1}, established by the European legislation (EC Regulation 1881/2006).

Considering the white wine varieties, the statistical analysis (p=0.001; Kruskal-Wallis test) showed a significant effect of the grape variety. In particular, the incidence (6.7%) of OTA contamination in the Moscato wines was significantly lower than in all the other white wines. The difference among the white wines could be explained by the different winemaking processes. In particular, the Moscato grapevines are used for the production of sparkling wines, such as Asti Spumante or Moscato d'Asti, where the grapes are immediately and softly pressed, and the juice is immediately cooled and fermented at controlled temperature.

Also among the red wines, a significant effect was shown by the grapevine variety tested (p=0.046; Kruskal-Wallis test). In particular, the magnitude between the mean OTA contamination levels of Barbera wines (0.087 µg l^{-1}) and Nebbiolo wines (0.129 µg l^{-1}) was statistically significant (p=0.011; Mann-Whitney test). The contamination level of Nebbiolo wines was also statistically different (p=0.049; Mann-Whitney test) from the level of the Dolcetto wines (0.117 µg l^{-1}). Moreover, the incidence of contaminated Nebbiolo wines

(72.0%) was higher than the incidence of Barbera (67.0%) or Dolcetto (66.3%) ones. Such difference could be explained with the different variety characteristics or harvesting period. The bunch structure of the Barbera grapes is very compact, while the Nebbiolo grapes are less compact, and the Dolcetto grapes are intermediate. This difference is accompanied by a higher susceptibility to *Botrytis cinerea* of the Barbera grapes, and a lower susceptibility of the Nebbiolo variety. Anyway, in our study such characteristics were not related to the ochratoxin level found in the wines produced, as in previous studies (Battilani et al. 2004). Dolcetto grapes are generally harvested after September 15, followed by the harvest of Barbera grapes at the beginning of October, and finally by the vintage of Nebbiolo grapes, in the second half of October. The late harvest of Nebbiolo grapes could favour a longer contamination by *A. carbonarius* and a higher production of OTA. Generally the wines produced from grapes left for longer times on the vines have higher OTA contamination (Valero, Marín, Ramos, & Sanchis, 2008).

Considering the wines produced from Nebbiolo grapes, four are the appellations, depending also on the aging period before commercialization. In particular, the Nebbiolo appellation does not include a compulsory aging, while the Roero, Barbaresco, and Barolo appellations foresee an aging period (20, 24, and 36 months, respectively), including a period in wood barrels (at least 6, 12, and 24 months, respectively). Such wood aging period in oak barrels could contribute to reduce the mean OTA contamination observed in the Barolo wines (0.098 µg l^{-1}), compared to the Barbaresco (0.133 µg l^{-1}), Roero (0.175 µg l^{-1}), or Nebbiolo wines (0.179 µg l^{-1}). Previously, Savino, Limosani, and Garcia-Moruno (2007) found that the OTA contamination in red wines can be reduced by oak wood fragments. Other studies did not find any change in the OTA concentration in wine after 1-year aging (Visconti, Perrone, Cozzi, & Solfrizzo, 2008).

Table 3 reports the incidence and level of contamination in the wines based on the year of vintage. A relatively low level of OTA contamination was found in all the years taken in consideration, ranging from 0.048 µg l^{-1} in 2007 to 0.096 µg l^{-1} in 2006. The magnitude between the means of OTA levels in the years considered was statistically significant (p=0.002, Kruskal-Wallis test). The incidence of OTA contamination was relatively high for every year considered (ranging from 51.5% to 77.8%). Anyway it should be noticed that the LOQ of the method, originally published by Visconti et. al (1999) and recommended by the European Standard rEn 14133, was relatively low (0.0093 µg l^{-1}), permitting to find a high number of positive samples. The years with a higher number of positive samples were 2000, 2003, 2006, and 2007. An explanation of this trend could be attributed to the climate conditions that occurred in the harvest season of those years characterized by a September – October period with intense rain and relatively high temperatures. Clouvel, Bonvarlet, Martinez, Lagouarde, Dieng, and Martin (2008) found critical levels of OTA concentration in French wines with humid and warm conditions during the 20 days before harvest.

Although Northern Italy is not at the moment a geographical area where OTA contamination represents a significant risk in the vineyards, and the wines produced present generally low levels of the mycotoxin, future scenarios of climatic changes, characterized by a general increase of temperature and decrease of precipitation in this region (Salinari et al., 2006) could favour *A. carbonarius* contamination. To prevent severe OTA contaminations, a constant monitoring of the black aspergilli in vineyard and of the OTA level on the wines should performed.

Acknowledgements

Work carried out with grants from the Piedmont Region “Mycotoxin risk evaluation, prevention and management in the Piedmontese wine chain” and the Cassa di Risparmio di Torino Foundation “Presence of mycotoxins in the grapes and wine chain”. The authors acknowledge Dr. Fabrizio Stecca (Enocontrol S.c.a.r.l., Alba, Italy) for providing the wine samples.

References

Battilani, P., & Pietri, A. (2002). Ochratoxin A in grapes and wine. *European Journal of Plant Pathology, 108*, 639-643.

Battilani, P., Logrieco, A., Giorni, P., Cozzi, G., Bertuzzi, T., & Pietri, A. (2004). Ochratoxin A production by *Aspergillus carbonarius* on some grape varieties grown in Italy. *Journal of the Science of Food and Agriculture, 84*, 1736-1740.

Battilani, P., Giorni, P., Bertuzzi, T., Formenti, S., & Pietri, A. (2006). Black aspergilli and ochratoxin A in grapes in Italy. *International Journal of Food Microbiology, 111*, S53-S60.

Blesa, J., Soriano, J.M., Moltó, J. C., & Mañes, J. (2006). Factors affecting the presence of ochratoxin A in wines. *Critical Reviews in Food Science and Nutrition, 46*, 473–478.

Brera, C., Debegnach, F., Minardi, V., Prantera, E., Pannunzi, E., Faleo, S., De Santis, B., & Miraglia, M. (2008). Ochratoxin A contamination in Italian wine samples and evaluation of the exposure in the Italian population. *Journal of Agricultural and Food Chemistry, 56*, 10611–10618.

Clouvel, P., Bonvarlet, L., Martinez, A., Lagouarde, P., Dieng, I., & Martin, P. (2008). Wine contamination by ochratoxin A in relation to vine environment. *International Journal of Food Microbiology, 123*, 74-80.

European Commission (2006). Commission Regulation No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union*, L365/5 – L365-24.

Grazioli, B., Fumi, M.D., & Silva, A. (2006).The role of processing on ochratoxin A content in Italian must and wine: A study on naturally contaminated grapes. *International Journal of Food Microbiology, 11*, S93-S96.

Miraglia, M., & Brera, C. (2002). Reports on tasks for scientific cooperation, Task 3.2.7. Assessment of dietary intake of ochratoxin A by the population of EU. Member States. *SCOOP Report*. Brussels, Belgium: European Commission. Available: http://ec.europa.eu/food/fs/scoop/3.2.7_en.pdf. Accessed 1 July 2009.

Otteneder, H., & Majerus, P. (2000). Occurrence of ochratoxin A (OTA) in wines: influence of the type of wine and its geographical origin. *Food Additives and Contaminants 17*, 793–798.

Pietri, A., Bertuzzi, T., Pallaroni, L., & Piva, G. (2001). Occurrence of ochratoxin A in Italian wines. *Food Additives and Contaminants 18*, 647–654.

Salinari, F., Giosuè, S., Tubiello, F.N., Rettori, A., Rossi, V., Spanna, F., Rosenzweig, C., & Gullino, M.L. (2006). Downy mildew (*Plasmopara viticola*) epidemics on grapevine under climate change. *Global Change Biology, 12,* 1299-1307.

Savino, M., Limosani, P., & Garcia-Moruno, E. (2007). Reduction of ochratoxin A contamination in red wines by oak wood fragments. *American Journal of Enology and Viticulture, 58,* 97-101.

Spadaro, D., Lorè, A., Ciavorella, A., Garibaldi, A., & Gullino, M.L. (2008). Wines produced in Piedmont: presence of *Aspergillus* spp. in vineyard and of ochratoxin A in wines. *Journal of Plant Pathology, 90*, S2, 326-327.

297 Thompson, M., Ellison, S.L.R., & Wood, R., (2002). Harmonized guidelines for single-
298 laboratory validation of methods of analysis (IUPAC Technical Report). *Pure and Applied*
299 *Chemistry, 74,* 835-855.
300 Turrini, A., Saba, A., Perrone, D., Ciafala, E., & D'Amicis, A. (2001). Food consumption
301 pattern in Italy: The INN-CA study 1994-1996. *European Journal of Clinical Nutrition, 55,*
302 571-588.
303 Valero, A., Marín, S., Ramos, A.J., & Sanchis, V. (2008). Survey: Ochratoxin A in
304 European special wines. *Food Chemistry, 108,* 593-599.
305 Visconti, A., Pascale, M., & Centonze, G. (1999). Determination of ochratoxin A in wine
306 by means of immunoaffinity column clean-up and HPLC. *Journal of Chromatography A,*
307 *864,* 89-101.
308 Visconti, A., Perrone, G., Cozzi, G., & Solfrizzo, M. (2008). Managing ochratoxin A risk
309 in the grape-wine food chain. *Food Additives and Contaminants, 25,* 193-202.
310 Zimmerli, B., & Dick, R. (1996). Ochratoxin A in table wine and grape juice: occurrence
311 and risk assessment. *Food Additives and Contaminants, 13,* 655-668.