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Short title: *Fumonisin B₂ in wine*

Natural occurrence of fumonisin B₂ in red wine from Italy

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Abstract

The potential risk of exposure to fumonisin B₂ (FB₂) in the grape-wine chain has been recently pointed out after reporting *Aspergillus niger* in grapes and its ability to produce FB₂ and FB₄. The occurrence of these two fumonisins in wine was investigated by LC/MS/MS in fifty-one market samples (45 red, 5 white and one rosè wine) produced in various Italian regions. Nine samples of red wine were found contaminated by fumonisin B₂ at levels ranging from 0.4 to 2.4 ng/mL, while FB₄ was not detected in any of the tested samples. This is the first report on the natural occurrence of FB₂ in wine indicating that, although at low levels, there is a potential risk of FB₂ exposure for the wine-consumer.

Keywords: Fumonisins; *Aspergillus* species; wine; mycotoxins; food safety

Introduction

Grapes and derived products such as grape juices and wines have been reported as potentially contaminated with ochratoxin A (Zimmerli and Dick 1996; Visconti et al. 1999). “Black aspergilli”, mainly *Aspergillus carbonarius* followed by *A. niger* and *A. tubingensis*, are the main source of contamination by ochratoxin A of grapes and wines (Abarca et al. 2001; Battilani et al. 2003; Belli et al. 2004; Perrone et al. 2006). Ochratoxin A has been shown to be nephrotoxic, immunotoxic and teratogenic to several species of animals and to cause kidney and liver tumours in mice and rats (WHO, 2001).

Recently, some industrially important strains of *A. niger* were reported to produce, in addition to ochratoxin A, the mycotoxin fumonisin B₂ (FB₂) (Frisvad et al. 2007). Then, it has been shown that strains of *A. niger* from coffee beans and raisins produced together with FB₂ also FB₄ (Noonim et al. 2009; Mogensen et al. 2009). Logrieco et al. (2009) reported some strains of *A. niger* from grape producing FB₂ and the occurrence of FB₂ in two must samples, and pointed out the potential mycotoxicological risk of grape and derived products colonized by this species. Fumonisin have been extensively studied as mycotoxins with cancer-promoting activity and are associated with a number of animal and human diseases (Marasas 2001). Mogensen et al. (2009) has recently shown that *A. niger* strains isolated from raisins are able to produce both FB₂ and FB₄ when cultured on either grapes or raisins.

The objective of this study was to investigate the presence of FB₂ and FB₄ in market wines in order to evaluate the potential risk for wine consumers.

Materials and methods

Wine samples. Fifty-one bottled samples of red (45), white (5) and rosè (1) wine were randomly purchased from the market in Apulia (Table 1). These wines originated from most Italian wine producing areas including Veneto (10), Apulia (6), Piedmont (6), Trentino Alto Adige (5), Campania (4), Tuscany (4), Lombardy (3) Emilia Romagna (3), Sardinia (2), Sicily (2), Friuli Venezia Giulia (1), Lazio (1), Umbria (1). The year of production ranged from 2004 to 2008 with most in 2007.

Fumonisin analysis

Fumonisin B₂ standard was purchased from Sigma-Aldrich (Milan, Italy), while fumonisin B₄ was kindly offered by Mark Busman (National Center for Agricultural Utilization Research, Peoria, Illinois, USA). Standards were dissolved in methanol at 1 mg mL⁻¹ concentration, working solution were prepared using a solution CH₃OH/H₂O (70:30, v/v). In Figure 1 are reported the chemical structures of FB₂ and FB₄. Fumonisin B₁ (Sigma-Aldrich, Milan, Italy) was also analyzed in the tested wines.

FB₂ and FB₄ were analysed using 7.5 mL of wine sample mixed with 2.5 mL of methanol and stirred for 2 min at room temperature. The sample was acidified to pH 3 using 1 M hydrochloric acid and then loaded on a reversed phase Strata C18-E 500 mg cartridge (Phenomenex, Torrance, CA, USA), previously conditioned with methanol (5 mL) and water (5 mL). The column was washed with water (3 mL), methanol/water (1:3, 5 mL) and then fumonisins mixture was eluted with 10 mL methanol. The eluate was dried

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1 using a centrifuge evaporator (Savant Instruments Inc., Farmingdale, NY, USA), dissolved
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7 2 in 750 μ L of methanol/water (70:30), ultracentrifuged at 13,400 g for 3 min and then the
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10 3 residue was used for LC/MS/MS analyses. Recoveries for the toxins were measured by
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12 4 spiking 7.5 mL of wine with FB₂ and FB₄ at three different concentrations (1 ng/mL, 5
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14 5 ng/mL and 10 ng/mL); each experiment was repeated three times on two non consecutive
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16 6 days by different operators. FB₂ and FB₄ recoveries in spiked samples averaged 83.2 % and
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18 7 79.4 %, respectively. The calibration curve showed good linearity in the range 1-1000
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21 8 ng/mL. All chromatographic points of calibration curve were run in triplicate and the mean
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24 9 values are reported. The standard deviation was less than 5%.

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26 10 The detection limit (LOD with a signal to noise ratio of 3) for FB₂ was 0.25 ng/mL while
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28 11 the quantification limit (LOQ with a signal to noise ratio of 9) was 0.5 ng/mL. The detection
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30 12 limit (LOD with a signal to noise ratio of 3) for FB₄ was 0.7 ng/mL while the quantification
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33 13 limit (LOQ with a signal to noise ratio of 9) was 1.5 ng/mL.

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35 14 Chromatographic separation of extracts was performed using an HPLC apparatus
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38 15 equipped with two micropumps Series 200 (Perkin Elmer, Canada Shelton, USA) and a
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40 16 Gemini 5u C₁₈ 110 Å column (150 x 2 mm) (Phenomenex, Torrance, CA, USA). The
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42 17 chromatographic solvents were water 0.1% formic acid (eluent A) and CH₃CN/MeOH
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44 18 (80:20 v/v), 0.1% formic acid (eluent B). The gradient program was as follow: 30-100 % B
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46 19 (8 min), 100 % B (3 min), 100-30% B (3 min), at a constant flow of 0.2 mL/min. The
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48 20 injection volume was 20 μ L. MS/MS analyses of FB₂ and FB₄ were performed on an API
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50 21 3000 triple quadrupole mass spectrometer (Applied Biosystems, Canada) equipped with a
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53 22 TurboIonspray source. The following setting were used: Capillary Voltage (IS) 5,000 V,
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Declustering Potential (DP) 70 V, focus potential 340 V. Collision energy (CE) 51 V and 42 V generated daughter ions 336.2 and 512.2 m/z , for FB₂; whereas for FB₄ collision energy (CE) 48V and 37V generated daughter ions 338.5 and 514.3 m/z . These mass parameters were optimised by preliminary experiments with a direct infusion of pure compounds (10 µg/mL) at a constant flow rate of 6 µL/min using a model 11 syringe pump (Harvard Apparatus, Holliston, MA, USA) into the mass spectrometer source. Drying gas (air) was heated to 350°C. Analyses were performed in the positive ion mode in MRM (multiple reaction monitoring), ion mode, FB₂ showed an [M+H]⁺ ion at m/z 706.4, FB₄ showed an [M+H]⁺ ion at m/z 690.4.

In Figure 2 is reported the Total Ion Chromatogram (TIC) of a sample of wine artificially contaminated with 1 µg/L of FB₂ and FB₄ been using to evaluate the recovery performances of fumonisins from contaminated natural samples and to identify eventual molecules interfering in the fumonisin analysis.

Results and discussion

This is the first report relevant to FB₂ and FB₄ surveillance in wine samples. Shephard et al. (2005) reported fumonisins determination in maize beer in South Africa by reversed-phase HPLC, after *o*-phthaldialdehyde (OPA) post-run derivatization. The analytical method reported in this paper represents a real advance in the field of mycotoxin surveillance in foods by using tandem mass spectrometry techniques. In particular, these minimal changes made to the method of Shephard et al. (2005) allow to reduce the time of the analysis and

the handling of samples of alcoholic beverages at risk of contamination by fumonisins with benefits both on the values of recovery of the analytes and cost analysis.

Frisvad et al. (2007) reported a semiquantitative method for fumonisins analysis on agar using selective ion monitoring electrospray LC-MS with a time-of-flight mass spectrometer. Our work was a quantitative analysis of FB₂ and FB₄ on wine by liquid chromatography coupled to electrospray ionization tandem mass spectrometry (LC/MS/MS). Acquisition was in positive ion mode, FB₁ showed an [M+H]⁺ ion at *m/z* 722.4, FB₂ showed an [M+H]⁺ ion at *m/z* 706.4, FB₄ showed an [M+H]⁺ ion at *m/z* 690.4. CAD (Collision Activated Dissociation) of these protonated molecules produced product ions and the two main daughter peaks were selected to be monitored in the MRM mode. Previous studies on fumonisins analysis by LC/MS/MS (Faber et al. 2005; Bartók et al. 2006) showed that the most abundant products ion were *m/z* 352.0 and *m/z* 336.0, respectively for FB₁ and FB₂, corresponding to the loss of water and two tricarboxylic acids (TCA) from the alkyl backbone [MH-2TCA-H₂O]⁺. In addition to these ions, our MS/MS spectra showed the product ions at *m/z* 528.1 and *m/z* 512.2 corresponding to the loss of water and a tricarboxylic acid [MH-TCA-H₂O]⁺ (Table 1). For FB₄ analysis the product ions *m/z* 514.3 and 338.5 were monitored, corresponding to [MH-TCA]⁺ and [MH-2TCA]⁺ (Bartók et al. 2006).

Using chromatographic conditions described in this work, fumonisins showed retention times of 3.7, 4.4 and 5.1 min for FB₁, FB₂ and FB₄, respectively.

The occurrence of FB₂ and FB₄ was investigated in a total of 51 wine samples collected in Apulia markets. The chemical identification of FB₂ and FB₄ was performed using the

molecular ion peaks, peak fragments and chromatographic retention time. The distribution of FB₂ in wine is resumed in Table 1. FB₂ was detected in 9 samples representing 17.6% of the whole samples and FB₂ concentrations in the positive samples ranged from 0.4 to 2.4 ng/mL. TIC of wine sample #43 positive for FB₂ is reported in Figure 3. This study shows that there is a real risk of contamination in wine with FB₂. No FB₄ was detected in the wine samples. Likewise no FB₁ was detected, as expected due to the fact that this toxin is not produced by *A. niger*. Fumonisin, including FB₂, have been found in various plants and plant products including corn and corn based products (Marasas 2001), beer (Shephard et al. 2005), black tea leaves (Martins et al. 2001) and asparagus (Logrieco et al. 1998). The regulatory limits in corn and corn based products in USA and Europe are of 2-4 and 0.2-4 µg/g total fumonisins, respectively (FDA 2001; European Commission 2007). *Aspergillus niger* represents the major fumonisin producing species among black *Aspergilli* occurring on grapes (Logrieco et al. 2009) with producing strains accounting for about 75 % of all *A. niger* strains (Nielsen et al. 2009). This risk is real also considering the consistent presence of *A. niger* on grapes during the growing season, generally higher than that of other black *Aspergilli* (Leong et al. 2004; Chulze et al. 2006).

Wine is also exposed to other mycotoxicological risk due to the natural occurrence of ochratoxin A mainly due to the presence of *A. carbonarius* in field (Abarca et al. 2001; Battilani et al. 2003). An European report estimated that wine is the second major source of OTA intake in European populations (European Commission, 2002) and a maximum limit for OTA in wine of 2 µg/kg was regulated in the EU (European Commission, 2006).

By assuming a daily consumption of 200 mL wine for a 70 kg person, i.e. one or two glasses of wine as recommended by the World Health Organization for an healthy response

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1 to wine exposure, and assuming a contamination level (approximated per excess) equal to
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1 to wine exposure, and assuming a contamination level (approximated per excess) equal to
2 the average of the positive samples of this study, i.e. about 1 ng/mL FB₂, the average
3 exposure to fumonisins through wine consumption would be of about 0.003 µg/kg of body
4 weight (b.w.) per day. Such level of exposure would be far below the guideline provided for
5 risk characterization by the WHO-FAO joint expert committee for food and additives
6 (JECFA) that established the provisory maximum tolerable daily intake (PMTDI) of 2
7 µg/kg of body weight to FB₁, FB₂ and FB₃ alone or in combination. The contribution of
8 wine to mean daily intake of fumonisins can be considered negligible in the case of people
9 drinking wine manufactured in Italy. Wine alone does not supply the diet with an amount of
10 fumonisins corresponding to PMTDI, but combined with possible fumonisins exposure
11 from the maize based staple diet, it could become a cause for concern when climatic
12 conditions are particularly favourable to the development of toxigenic *A. niger* strains on
13 grape-vines and the consequent transfer of FB₂ to wine. Nevertheless, the number of wine
14 samples included in this study was limited and relevant to Italy only. Additional wine
15 samples from other wine producing countries (e.g. Spain, France, Australia, South Africa,
16 etc) should be investigated for their contamination by fumonisins as well as ochratoxin A in
17 order to have a general view of mycotoxicological risk for the grape-wine chain.

18 Further studies are needed to better define the environmental conditions and other
19 factors influencing the toxin production, as well as the widespread of fumonisins producing
20 *Aspergillus*, and the fate of fumonisins during the winemaking process. Geographic
21 differences have been shown in *Aspergillus* and ochratoxin A contamination of grapes and
22 wines, indicating higher incidence and contamination levels in wines and grapes from the

southern regions of Europe and the lower latitude (Visconti et al. 2008). *Aspergillus niger*, which includes FB₂ producing strains, has been frequently isolated from grapes in Europe (Logrieco et al. 2003), Australia (Leong et al. 2004) and South America (Chulze et al. 2006) and show significant differences in the physiology and ecology with respect to *A. carbonarius*, the main responsible for wine contamination by ochratoxin A.

In conclusion, this report provides a new knowledge about the FB₂ occurrence in wine that, added to previous data relevant to its occurrence in grapes and musts, provides a clear evidence that FB₂ contamination can occur in the grape-wine chain and, under conditions particularly favourable to its formation, could result in a potential risk for human and animal health.

Acknowledgments

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Figure 1: Chemical structures of Fumonisin B₂ (FB₂) and B₄ (FB₄)

Figure 2: Total Ion Chromatogram (TIC) of a blank sample spiked with Fumonisin B₂ and Fumonisin B₄ standards at 1 µg/L

Figure 3: Total Ion Chromatogram (TIC) of wine sample #43 positive for FB₂

Table 1. Distribution of wine samples, with reference to production region and year, and relevant occurrence of Fumonisin B₂

N°	Origin	Wine	Year	FB ₂ (ng/mL)
1	Apulia	Red	2005	n.d.*
2	Apulia	Red	2007	n.d.
3	Apulia	Red	2007	0.6
4	Apulia	Red	2007	0.8
5	Apulia	Red	2006	n.d.
6	Apulia	Red	2005	n.d.
7	Veneto	Red	2007	n.d.
8	Veneto	Red	2007	n.d.
9	Veneto	Red	2007	n.d.
10	Veneto	Red	2006	n.d.
11	Veneto	Red	2007	n.d.
12	Veneto	Red	2007	1.2
13	Veneto	Red	2006	n.d.
14	Veneto	Red	2005	n.d.
15	Veneto	Red	2007	n.d.
16	Veneto	Red	2006	n.d.
17	Piemonte	Red	2006	1.1
18	Piedmont	Red	2007	n.d.
19	Piedmont	Red	2007	n.d.
20	Piedmont	Red	2007	n.d.
21	Piedmont	Red	2007	n.d.
22	Piedmont	Red	2008	0.5
23	Trentino Alto Adige	Red	2007	n.d.
24	Trentino Alto Adige	Red	2007	n.d.
25	Trentino Alto Adige	Red	2007	n.d.
26	Trentino Alto Adige	Red	2007	n.d.
27	Trentino Alto Adige	White	2006	n.d.
28	Tuscany	Red	2004	0.5
29	Tuscany	Red	2004	0.7
30	Tuscany	Red	2006	n.d.
31	Tuscany	Red	2007	n.d.
32	Lombardy	Red	2006	n.d.
33	Lombardy	Red	2007	n.d.
34	Lombardy	Red	2007	n.d.
35	Basilicata	Red	2007	n.d.

36	Emilia Romagna	Red	2007	n.d.
37	Emilia Romagna	Red	2007	n.d.
38	Emilia Romagna	Red	2007	0.6
39	Friuli Venezia Giulia	Red	2007	n.d.
40	Abruzzi	Red	2007	n.d.
41	Marche	Red	2007	n.d.
42	Sardinia	Red	2007	n.d.
43	Sardinia	Red	2006	2.4
44	Campania	Red	2006	n.d.
45	Campania	Red	2007	n.d.
46	Campania	Rosè	2006	n.d.
47	Campania	White	2007	n.d.
48	Sicily	Red	2007	n.d.
49	Sicily	White	2007	n.d.
50	Lazio	White	2007	n.d.
51	Umbria	White	2007	n.d.

* n.d. = not detected (< 0.3 ng/mL)