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Widespread Occurrence of the Mycotoxin Fumonisin B_2 in Wine

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Fumonisins are important mycotoxins because they are suspected to cause human and animal toxicoses by the consumption of contaminated corn-based food and feeds. However, with the discovery of fumonisin production in grapes by *Aspergillus niger*, wine may also be a fumonisin-containing commodity. In the present study, we have developed a simple and quantitative cation-exchange-based purification method for the subsequent isotope dilution liquid chromatography−tandem mass spectrometry (LC−MS/MS) determination of fumonisins in wine. A comparative study of seven different solid-phase extraction (SPE) columns showed that polymeric mixed-mode reversed-phase (RP) cation-exchange columns were superior to classic silica-based cation and mixed-mode cation-exchange columns. A total of 77 wine samples from 13 countries were subsequently tested, and surprisingly, 18 (23%) were found to contain fumonisin B_2 in the range of 1−25 μg/L. These findings were further confirmed by immunoaffinity purification and re-analysis of the positive cation-exchanged extracts.

KEYWORDS: Fumonisin; wine; grapes; *Aspergillus niger*

INTRODUCTION

Black aspergilli are some of the most common fungi responsible for postharvest decay of fresh fruit (*1*). Of these are *Aspergillus carbonarius* and *Aspergillus niger*, very important opportunistic pathogens of grapes causing bunch rot or berryrot (*2*). Because *A. carbonarius* can produce large amounts of ochratoxin A, the main focus concerning grapes, wine, and black aspergilli has therefore been on this fungus and ochratoxin A (*3*−*6*). The recent discovery of fumonisins B_2 (*7*), B_4 (*8*), and B_6 (*9*) (**Figure 1**) from *A. niger* and the production of fumonisins by *A. niger* in coffee and especially grapes, raisins, and must (*8*, *10*, *11*) have raised concerns of the possible general presence of fumonisins in wine.

Fumonisins are important mycotoxins because they are suspected to cause human and animal toxicoses by the consumption of contaminated corn-based food and feeds (*12*). To avoid possible detrimental health effects, the United States Food and Drug Administration (U.S. FDA) recommends that maize should not be used for human consumption when contaminated with more than 2−4 mg/kg total fumonisins (*13*), whereas the European Union (EU) has a regulatory limit of 0.2−2 mg/kg (*14*).

Thus far, no detection method for fumonisins in wine has been described. Non-purified but highly concentrated extracts of must, grapes, and raisins have been analyzed using liquid chromatography−tandem mass spectrometry (LC−MS/MS) (*10*, *11*). However, for low concentrations in a high complexity of this product, wine (e.g., containing polyphenolics, flavonoids, tannins, and anthocyanins), this approach will clearly limit sensitivity and poses ion-suppression problems unless a much more sensitive instrument is used and, thus, less sample can be injected. We therefore decided to develop a fast solid-phase extraction method with subsequent LC−MS/MS analysis for the determination of fumonisins in wine. The method described here is to our knowledge the first quantitative method for fumonisins using cation exchange.

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MATERIALS AND METHODS

Unless otherwise stated, all solvents were high-performance liquid chromatography (HPLC)-grade and other chemicals were analytical-grade and from Sigma-Aldrich (St. Louis, MO). Water was purified on a Milli-Q system (Millipore, Bedford, MA). The fumonisin standard was a mixed certified standard containing both fumonisins B_1 and B_2, with concentrations of 50.2 and 51.0 mg/L (Biopure, Tulln, Austria). Relevant samples were added U-$^{13}C_{34}$-fumonisin B_2 (10.52 μg/mL, Biopure) to a total concentration of 10 μg/L.

Sample Preparation. Wine samples of 10 mL (spiked or non-spiked, pH∼3.5−4) were, unless otherwise stated, passed through a 30 mg/3 mL Strata X-C column (Phenomenex, Torrance, CA), which was previously conditioned with 1 mL of methanol and 1 mL of water. To further acidify the column, 1 mL of water with 2% formic acid was added. Afterward, the column was washed with 1 mL of methanol and the fumonisins were eluted with methanol/water (1:4) with 2% NH_4OH water into a 2 mL HPLC vial and used directly for analysis.

The following other cation-exchange columns were tested with the above method as well as small variations of this: Oasis MCX, 30 mg (Waters, Milford, MA) and the silica based ones, HCX, 130 mg (C_8, SCX, Biotage, Uppsala, Sweden); HCX-3, 130 mg (C_{18}, SCX, Biotage); HCX-5, 130 mg (C_4, SCX, Biotage); and SCX, 100 mg (Phenomenex).

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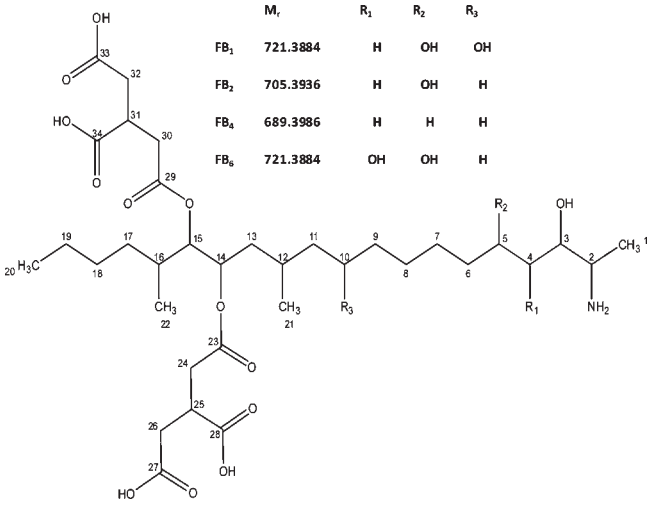

Figure 1. Structure of fumonisin B_2 and a comparison to fumonisins B_1, B_4, and B_6.

SAX purification was also tested on 100 mg columns (Phenomenex). The following procedure was used: The pH of the wine was adjusted to 7.0 ± 0.2 with 1M NaOH, and the columns were conditioned with 1 mL of methanol and 1 mL of water, followed by the addition of wine. The cartridge was washed with 1 mL of methanol/water (7:3) and 1 mL of methanol. The samples were eluted with methanol with 2% formic acid and used directly for analysis. Each sample was spiked with an internal standard of U-$^{13}C_{34}$-fumonisin B_2 to a total concentration of 10 μg/L.

Validation. The fumonisin B_2 extraction was validated by spiking portions of 10 mL of wine to the following concentrations of fumonisin B_2: 0 (blank), 2, 5, 15, 50, 150, and 450 μg/L. The experiments were performed on different days, with all levels in triplicates each day. The apparent recovery was calculated from methanol/water (20:80) diluted samples with the following concentrations of fumonisin B_2: 2, 5, 15, 50, 150, and 450 μg/L. An internal standard of U-$^{13}C_{34}$-fumonisin B_2 was added to each sample to a total volume of 10 μg/L. To determine the apparent recovery, the slope of spiked wine samples and MeOH standards was compared for similar concentrations.

Wine Samples. Wine samples for screening were either bought in a local supermarket or kindly provided by employees at the Department of Systems Biology, Technical University of Denmark. In total, 77 wines from 13 countries were tested, including red (n = 56), white (n = 13), port (n = 6), champagne (n = 1), rosé (n = 1), and madeira (n = 1) wines. The wines were from the years 1991−2009 and from Argentina (n = 1), Australia (n = 6), Chile (n = 3), China (n = 1), France (n = 22), Germany (n = 1), Italy (n = 23), New Zealand (n = 1), Portugal (n = 7), Romania (n = 1), Spain (n = 9), South Africa (n = 2), and the U.S. (California) (n = 1). All information on the wines was taken from the label of the bottle.

LC−MS/MS Conditions. LC−MS/MS analysis was performed as previously described (*11*) but with minor changes. Briefly described, the LC−MS/MS analysis was performed on a Quattro Ultima triple mass spectrometer (Micromass, Manchester, U.K.) with a z-spray electrospray ionization (ESI) source. Separations was performed on a 50 × 2 mm inner diameter, 3 μm Gemini C_6-phenyl column (Phenomenex, Torrance, CA). Using a linear gradient starting from 20% acetonitrile in water (both supplemented with 20 mM formic acid) to 55% acetonitrile for 6 min at a flow rate of 0.3 mL/min, which was then increased to 100% acetonitrile in 30 s at a flow of 0.5 mL/min, keeping this for 3.5 min before returning to the start conditions in 6 min. MS/MS was performed in ESI^+, and the MS operated in multi reaction monitoring (MRM) mode at the following transitions: fumonisin B_2, quantifier m/z 706 → 336, cone, 50 V; collision, 40 V; dwell time, 50 ms; qualifier m/z 706 → 512, cone, 50 V; collision, 25 V; dwell time, 100 ms; fumonisin B_4, quantifier m/z 690 → 320, cone, 50 V; collision, 35 V; dwell time, 50 ms; qualifier m/z 690 → 514, cone, 50 V; collision, 30 V; dwell time, 100 ms; fumonisins B_1 and B_6, quantifier m/z 722 → 334, cone, 50 V; collision, 40 V; dwell time, 50 ms; qualifier m/z 722 → 528, cone, 50 V; collision, 25 V; dwell time, 100 ms; and U-$^{13}C_{34}$-fumonisin B_2 m/z 740 → 358, cone, 50 V; collision, 50 V; dwell time, 50 ms.

Verification of Positive Samples. Because a significant number of the positive samples were close to the limit of quantitation (LOQ), it was decided to verify the findings by purification on FumoniTest immunoaffinity columns (Vicam, Watertown, MA). Half of the purified extract (500 μL) from the LC−MS/MS screening was diluted with phosphate-buffered saline (PBS) buffer to a final volume of 10 mL. The pH was adjusted to 7 ± 0.3 with 1 M HCl before it was loaded onto the immunoaffinity column (1−2 drops/s). The column was washed with 10 mL of PBS buffer; air was pushed through to remove buffer; and possible fumonisins were eluted with 1.5 mL of methanol (1 drop/s). The eluate was evaporated to dryness under a stream of nitrogen at approximately 45 °C, and the dried residue was redissolved in 100 μL of methanol/water (1:4) and re-analyzed by LC−MS/MS (5 μL injected).

RESULTS AND DISCUSSION

Sample Purification. Fumonisins have mainly been purified by anion-exchange or immunoaffinity columns (*15*, *16*). Our first approach was the anion-exchange approach using SAX columns, which could be used to target not only fumonisins but also ochratoxin A. However, SAX purification was irreproducible and suffered from poor recovery (≪50%), as also observed in a previous study on the analysis of fumonisins from grapes, presumably because of the competition from organic acids from the grapes (*11*). Subsequently, it was decided to use cation exchange, because fumonisins have an amine group, thereby avoiding the anionic compounds. In our first attempt, we compared Strata SCX (silica-based) to polymeric Strata X-C, with the latter being superior, because of stable flow and better recovery. To find an optimal column, we further compared five different mixed-mode reversed-phase (RP) cation-exchange SPE columns for their abilities to extract fumonisins from wine. The worst tested was clearly the anion-exchange column (SAX) with only 11% extracted fumonisin compared to the best column (MCX). Concerning the C_4, C_8, and C_{18} mixed-mode SCX columns, there was a recovery of 71, 50, and 12%, respectively, indicating that an increase in the hydrophobicity of the column resulted in a more inefficient recovery of fumonisins. Two of the six tested columns, Oasis MCX and Strata X-C, clearly gave recoveries of 86 and 83%, respectively, with the latter being slightly more reproducible.

To determine the loading capacity of fumonisins on the Strata X-C, seven concentrations within the range of 100−1500 μg/L fumonisin spiked wine were loaded onto columns and, after LC−MS/MS analysis, the eluted fumonisin showed a linear relationship with no signs of reaching a maximal capacity, indicating a maximal capacity above 1500 μg/L.

Validation. The three calibration curves, one from each day, from spiked samples, had the following R^2 values: 0.9928, 0.9957, and 0.9978, with a relative standard deviation (RSD) of the slope being 10%. The RSD of the spiked samples (N = 9) varied within (0.6−35%), with an average RSD of 15%. The lowest concentration (2 μg/L) gave a s/n of 20:1, indicating that the limit of detection (LOD) was approximately 4 times lower (assuming a s/n of 5:1). Recovery was found to be 60−93%, with an average of 83% and an average RSD of 12%.

Screening of Wine Samples. Analysis of the 77 wine samples demonstrated the occurrence of fumonisin B_2 in 23% of the samples (18 of 77). This method gives four identification points via retention time and two MRMs and, thus, exceeding the requirements for identification (three points) (*17*). Because this is the first report describing fumonisin B_2 in wine and that a high number of samples were close to the LOQ, it was decided to further confirm the results by immunoaffinity purification. This was not formally validated because the use of the ^{13}C standard validates each purification. By comparison of the peak areas of

Table 1. Overview of the Tested Wine Samples and Their Fumonisin Content[a]

sample number	country	grape sort	year	wine type	fumonisin (μg/L)
1	Australia	Chardonnay	2008	white	nd[b]
2	Spain	Monastrell		red	2.1
3	Australia	Shiraz, Cabernet Sauvignon	2006	red	nd
4	Italy			red	nd
5	South Africa	Cabernet Sauvignon	2007	red	2.5
6	Australia	Shiraz		rose	nd
7	Romania	Pinot Noir	2006	red	20
8	Spain	Tempranillo Garnacha	2007	red	nd
9	Italy	Merlot, Corvina	2007	red	7.0
10	Australia	Shiraz	2008	red	1.0
14	Italy	Corvina, Rondinella, Molinarea, Negrana	2007	red	6.7
17	France	Sauvignon Blanc, Semillion Muscadelle	2003	white	nd
18	Italy	Corvina, Rondinella	2007	red	nd
19	France	Chardonnay	2008	white	nd
20	France		2007	champagne	nd
21	Chile	Chardonnay	2008	white	nd
24	France	Carignan, Grenache, Syrah	2003	red	5.1
25	Italy		2005	red	nd
26	France	Chardonnay	2008	white	nd
27	Spain	Tempranillo, Gaunacha	2005	red	1.9
29	Italy	Shiraz	2007	red	nd
30	Italy		2003	red	nd
31	Italy		1998	red	nd
33	California, U.S.A.	Zinfandel	1998	red	25
34	Portugal		2007	port	nd
36	Italy	Grecanico, Chardonnay	2008	white	nd
37	France	Grenache, Syrah	2003	red	1.3
38	Portugal		2002	madeira	nd
39	Italy	Montepulciano, Aglianico	2005	red	2.1
40	Italy	Merlot, Corbina	2007	red	3.9
42	Chile	Chardonnay	2008	white	nd
43	Australia		2008	white	nd
44	South Africa		2008	white	nd
45	France	Grenache, Syrah	2007	red	nd
46	Italy	Malvasia, Trebbiano	2008	white	nd
47	Italy	Montalcino, Sangiovese Grosso, Brunello	2000	red	nd
49	Italy	Nebbiolo	1996	red	nd
50	Australia	Shiraz	2007	red	nd
51	China	Cabernet Sauvignon	2007	red	nd
52	Germany	Pinot Noir	2004	red	nd
53	France	Cabernet Sauvignon, Cabernet Franc, Merlot, Petit Verdot	1999	red	nd
54	France		1998	red	nd
57	New Zealand	Sauvignon Blanc	2008	white	nd
58	Italy	Negroamaro, Primitivo	2006	red	1.6
59	France		2003	red	nd
63	Italy	Primitivo di Manduria	2007	red	1.2
64	Italy		2005	red	nd
65	France		2007	red	nd
66	Italy	Corvina, Rondinello, Molinara	2004	red	3.2
67	Spain	Monastrell	2008	red	nd
68	France	Grenache, Syrah, Mourvedre	2008	red	6.5
71	France	Merlot, Cabernet Sauvignon, Cabernet Franc	2003	red	nd
72	France		2002	red	nd
73	Portugal			port	nd
74	France			white	nd
78	Italy	Cabernet Sauvignon, Cabernet Franc	2001	red	nd
79	France	Riesling	2008	white	nd
80	Spain		2004	red	nd
84	France	Cabernet Sauvignon	1996	red	nd
89	Italy		2008	white	1.0
90	France		2007	red	nd
91	Italy	Sangiovese, Rondinella, Corvina	2006	red	nd
92	Spain	Tempranillo	2005	red	nd
93	France		1991	red	nd
94	France		1996	red	nd
95	Italy			red	nd
96	Chile	Carmenere	2008	red	nd

Table 1. Continued

sample number	country	grape sort	year	wine type	fumonisin (μg/L)
99	Argentina	Cabernet, Merlot	2008	red	nd
100	Italy			rose	nd
101	Spain		1996	red	nd
102	France		1998	red	nd
103	Portugal			port	nd
104	Portugal			port	2.8
105	Portugal			port	nd
106	Spain		2003	red	nd
107	Portugal		1991	red	nd
108	France	Cabernet Sauvignon, Merlot Noir, Cabernet Franc	1994	red	nd

[a] The description of the wines was read off the label of the bottle. Blank cells indicate that information was not available. [b] nd = not detected.

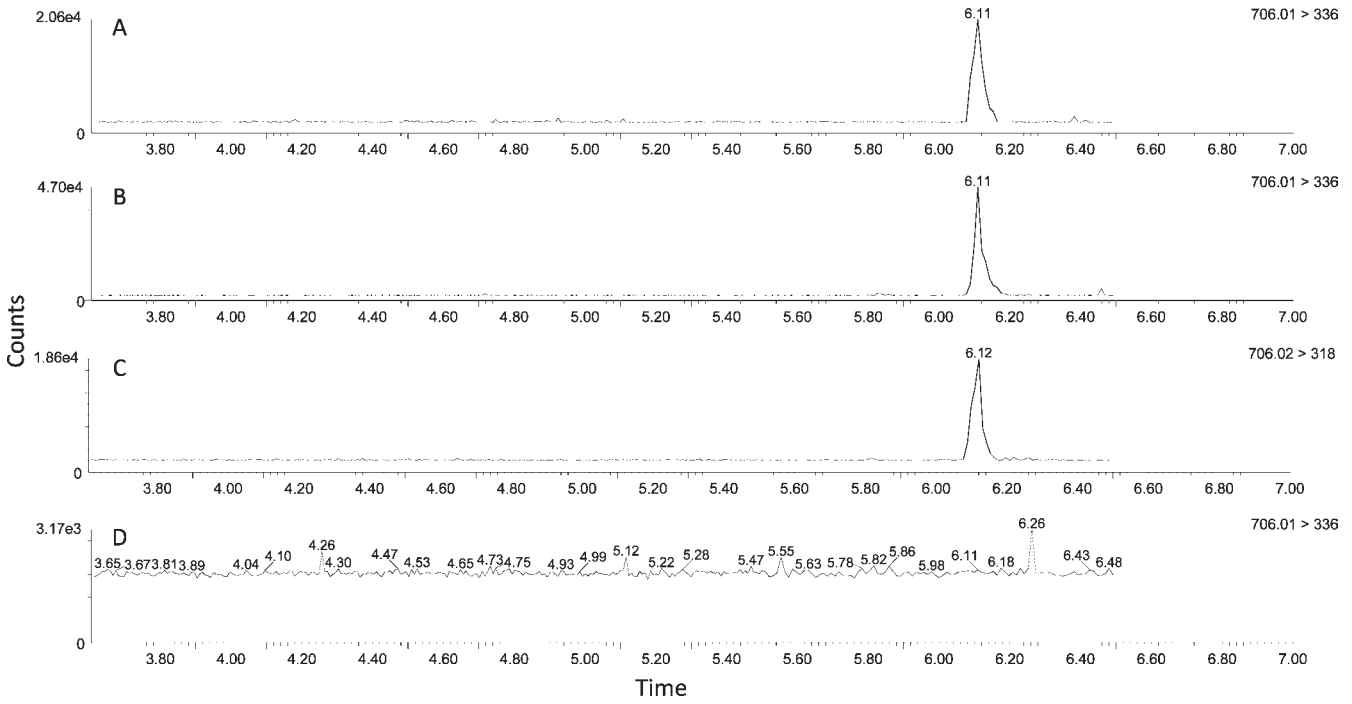

Figure 2. Results from the LC−MS/MS analysis. Transitions of fumonisin B_2 in spiked wine and a wine sample. (A) Quantifier of fumonisin B_2 from spiked wine (45 μg/L). (B) Quantifier of fumonisin B_2 from a wine sample (25 μg/L). (C) Qualifier of fumonisin B_2 from a wine sample (25 μg/L). (D) Quantifier of fumonisin B_2 from a blank sample (0 μg/L).

the ^{13}C fumonisin B_2, the recovery seemed to be better than 90%. However, clearly, it should not be necessary to use immunoaffinity purification in combination with LC−MS/MS because of the selectivity of the latter technique. The extra purification of 24 samples included 5 control samples (1 blank and 4 spiked with 2−45 μg/L). Re-analysis of the 18 initial positive samples did not change the number of positive samples, further confirming that the findings were true positive results. Of the 18 positive samples, 16 were red wine, 1 was white wine, and 1 was port wine (**Table 1**). The positive samples contained 1−25 μg/L fumonisin B_2 (average, 5.2 μg/L; median, 2.6 μg/L). Selected tested samples are shown in **Figure 2**. On the basis of the relative low amounts of samples, it is indicated that the fumonisin contamination could pose a larger problem in red wine (28% positive) compared to white wine (7% positive). This is similar to the ochratoxin A contamination, which is found to be at a higher concentration and coincidence in red wine compared to white wine (*18*, *19*). The findings of fumonisin B_2 with such a high frequency show that *A. niger* is apparently commonly growing on grapes in the fields. However, the frequency of heavily infected berries was low because the detected levels are 100−400 lower than those found in highly infected berries (2−8 mg/L) (*10*, *11*). Levels of ochratoxin A in wine have been found to be at 0.002−7.63 μg/L (*19*, *20*), which are lower than the found fumonisin concentrations. There were no statistically significant differences between the fumonisin concentration and countries and years, because of the relatively few samples investigated here.

The maximum permitted level of ochratoxin A in wine is set at 2.0 μg/L by the European Community (EC); this is ca. 2−3 times lower than the average maximum permitted level of ochratoxin A in food (*21*, *22*). Thus, a crude comparison to fumonisin, one could expect a 2−3-fold lower level than the 1−2 mg/kg set by the EC (*14*) and U.S. FDA (*13*), respectively. Thus, the maximum level detected (25 μg/L) was 40−80 times lower than an anticipated regulatory limit set by the EC and U.S. FDA.

However, even though the detected fumonisin concentrations are below such a limit, it is necessary to perform a larger survey to establish if wines with even higher concentrations are produced, because climatic and production conditions vary. Exposure assessment needs to be performed, especially concerning groups

with a high intake of wine alone or combined with other fumonisin-contaminated products.

In conclusion, this is the first report on the detection of fumonisin B_2 in wine, showing that almost a quarter of the samples were positive. This clearly points toward a much larger survey of how widespread fumonisins are in wine, as well as a determination of the fate of fumonisins in the winemaking process.

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