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Running head A. Pietri et al. Transfer of AFB<sub>1</sub> and FB<sub>1</sub> from raw materials to beer

**Transfer of aflatoxin B<sub>1</sub> and fumonisin B<sub>1</sub> from naturally contaminated raw materials to beer during an industrial brewing process.**

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**Abstract**

The aim of this research was to determine the fate of aflatoxins (AFs) and fumonisins (FBs) naturally occurring in raw materials (maize grit and malted barley) during four industrial brewing processes. The aflatoxin B<sub>1</sub> (AFB<sub>1</sub>) level in raw materials varied from 0.31 to 14.85 µg kg<sup>-1</sup>, while the fumonisin B<sub>1</sub> (FB<sub>1</sub>) level (only in maize grit) varied from 1146 to 3194 µg kg<sup>-1</sup>. The concentration in finished beer ranged from 0.0015 to 0.022 µg l<sup>-1</sup> for AFB<sub>1</sub> and from 37 to 89 µg l<sup>-1</sup> for FB<sub>1</sub>; the other aflatoxins and fumonisin B<sub>2</sub> were not found in beer samples. The average percentage of toxins recovered in finished beer, referring to the amounts contained in raw materials, were 1.5±0.8% for AFB<sub>1</sub> and 50.7±4.7% for FB<sub>1</sub>. These results were mainly due to the different solubility of the two mycotoxins during the mashing process. If raw materials comply with the limits fixed by the EC regulation, the contribution of a moderate daily consumption of beer to AFB<sub>1</sub> and FB<sub>1</sub> intake does not contribute significantly to the exposure of the consumer.

**Keywords:** aflatoxin B<sub>1</sub>, fumonisin B<sub>1</sub>, brewing, beer, maize grit, malted barley.

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## Introduction

In Italy it is common practice to brew beer from a mixture of malted barley and maize grit; according to the Italian Regulation (Gazzetta Ufficiale della Repubblica Italiana 1962) and its subsequent modifications, adjuncts (usually maize-derived products), can replace malted barley up to a maximum of 40%. The natural occurrence in maize of mycotoxins such as aflatoxins, fumonisins, trichothecenes and zearalenone, has been reported in various studies. Nearly 91% of the Italian production of maize is grown in northern Italy (Istat 2006). In this area, which ranges from Piedmont to Friuli Venezia Giulia, the mycotoxins that deserve special attention are aflatoxins, for their extreme acute and chronic toxicity, and fumonisins for their high and widespread contamination, that cause several disease states in animals and could affect human health (Pietri et al. 2004; Battilani et al. 2005).

Aflatoxins B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub> and G<sub>2</sub> are mainly produced by *Aspergillus flavus* and *A. parasiticus*, fungal species that grow in warm conditions in tropical and sub-tropical regions of the world. Aflatoxin B<sub>1</sub> (AFB<sub>1</sub>), the prevalent and most toxic aflatoxin, is a potent genotoxic carcinogen in laboratory animals and there is strong evidence that it is a liver carcinogen in humans. The International Agency for Research on Cancer (IARC) has classified AFB<sub>1</sub> as a Class 1 (human carcinogen) (IARC 1993). Food and health bodies have not set a tolerable daily intake (TDI) as AFB<sub>1</sub> is a genotoxic carcinogen; therefore, contamination in food should be reduced to the lowest possible level.

Fumonisin B<sub>1</sub>, B<sub>2</sub> and B<sub>3</sub> are a group of closely related compounds mainly produced by *Fusarium verticillioides* and *F. proliferatum*, fungi that commonly contaminate maize in many growing regions of the world. Fumonisin B<sub>1</sub> (FB<sub>1</sub>), the prevalent and most toxic fumonisin, has been associated with the high incidence of human oesophageal cancer in some regions of South Africa and China (Sydenham et al. 1990; Sun et al. 2007). Recent findings suggest that FB<sub>1</sub>

1  
2  
3 1 might increase the risk of neural tube defects in populations consuming large amounts of fumonisin-  
4  
5 2 contaminated maize (Missmer et al. 2006). FB<sub>1</sub> has been categorised by the IARC as class 2B  
6  
7  
8 3 (possible human carcinogen) (IARC 2002).  
9

10 4 As a consequence of the use of maize adjuncts, normally maize grit, in the brewing process,  
11  
12 5 aflatoxins and fumonisins may potentially occur in beer; moreover, aflatoxins can be occasionally  
13  
14 6 found also in malted barley (Fukal et al. 1990). Surveys on different types of beer reported that AFB<sub>1</sub>  
15  
16 7 was detected in 5 of 8 samples imported from Mexico and in one from Brazil (0.0028-0.049 µg l<sup>-1</sup>)  
17  
18 8 (Scott and Lawrence 1997), in 2 of 22 (0.0005 and 0.0008 µg l<sup>-1</sup>) and in 11 of 94 (0.0005-0.0831  
19  
20 9 µg l<sup>-1</sup>) Japanese and imported beer samples (Nakajima et al. 1999). Successively, in a survey on 304  
21  
22 10 samples of Canadian and imported beer, the toxin was found in 4 of 5 samples from India (0.096-  
23  
24 11 0.230 µg l<sup>-1</sup>), 2 of 6 from Spain, 1 of 5 from Portugal and in 5 of 49 from Mexico (0.0044-0.018 µg l<sup>-1</sup>)  
25  
26 12 (Mably et al. 2005). Regarding FB<sub>1</sub>, several surveys reported concentrations generally lower than 100  
27  
28 13 µg l<sup>-1</sup> (Scott and Lawrence 1995; Kawashima et al. 2007; Torres et al. 1998; Hlywka and Bullerman  
29  
30 14 1999; Dasko et al. 2005). Generally, with the exception of a survey on 75 bottled Kenyan lager beers  
31  
32 15 (Mbugua and Gathumbi 2004), much higher levels of AFB<sub>1</sub> and FB<sub>1</sub> were found in some types of  
33  
34 16 traditional African beer (aflatoxins: 200-400 µg l<sup>-1</sup>; FB<sub>1</sub>: 38-1066 µg l<sup>-1</sup>) (Scott 1996; Odhav and  
35  
36 17 Naicker 2002; Shephard et al. 2005).  
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43 18 Since aflatoxins (AFs) and fumonisins (FBs) can be present in beer, the aim of this study was to  
44  
45 19 investigate, during full scale industrial brewing process, the carry-over of AFs and FBs from naturally  
46  
47 20 contaminated raw materials to the different intermediate and final products.  
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50  
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52

## 53 22 **Materials and methods**

### 54 23 *Brewing process*

55 24 The study was carried out in an industrial brewery, located in northern Italy, that produces bottom  
56  
57 25 fermented pilsner type beer from a mixture of malted barley and de-germed maize grit (Figure 1) with  
58  
59  
60

1 a particle size > 500 µm. First, finely ground malted barley and maize grit were mashed in order to  
2 convert starch into fermentable sugars and dextrins and to provide mashing liquors with nitrogenous  
3 fractions which can be assimilated by yeast. The mashing process was performed according to a single  
4 decoction process, which requires two different kettles to be completed. In the adjunct kettles, a  
5 fraction of the finely ground malted barley and maize grit were boiled with water (*cooked mash*); in  
6 the second tun, finely ground malted barley and water were mixed and heated at two different  
7 saccharification rest temperatures according to a specific protocol (*malt mash*). In this process the  
8 content of the cooked mash was added back to the main malt mash to increase the main malt mash  
9 temperature up to the first saccharification rest (decoction process). The following are the essentials of  
10 the mashing operations. Malted barley ( $\cong 800$  kg), maize grit ( $\cong 5000$  kg) and water ( $\cong 15000$  l) were  
11 mixed in the adjunct kettle and held at 65 °C for 17 min. Then, the temperature was increased to 99.5  
12 °C in 40 min and the mash was boiled for 20 min. In the main mash tun, finely ground malted barley  
13 ( $\cong 7000$  kg) and water ( $\cong 20000$  l) were mixed at 37 °C for 15 min and the mixture was allowed to stand  
14 at 37 °C for 15 min. Then, the cooked mash was added and the temperature was raised to 63 °C. A  
15 first stand for 45 min was programmed. Successively, the temperature was increased to 69 °C and a  
16 second shorter stand (20 min) programmed. Finally, heating up to 78 °C (mashing off) in 20 min  
17 preceded the separation of the sweet wort. During the process, the pH of the malt mash was adjusted to  
18  $5.6 \pm 0.1$  by means of phosphoric acid. No addition of exogenous enzymes occurred during mashing  
19 nor acidification by means of lactic acid bacteria.

20 The mashing liquors were separated from spent grains by means of a Meura filter, which is a thin  
21 bed membrane-assisted mash filter (*lautering*). Lautering occurred in two stages: 1) the running off of  
22 the first wort, that is the aqueous solution of the extract recovered from mashing; 2) the sparging of the  
23 spent grains at 78 °C to wash out the extract retained by the spent grains. Sparging was performed  
24 until the last run off wort concentration was about 1.6 °P. At the end of the filtration process, spent  
25 grains were discharged. Sweet wort and spargings were gathered in a buffer tank which served the

1  
2  
3 1 wort boiling kettle. Successively, sweet wort was boiled for 1 h and hopped (*boiling and hopping*)  
4  
5 2 using a combination of CO<sub>2</sub> hop extract (53.4%  $\alpha$ -acids per 500 g of product) and type 90 pellets (15%  
6  
7  
8 3  $\alpha$ -acids per 1 kg of product) in a fixed proportion, in order to obtain a cast wort of 35 European  
9  
10 4 Brewery Convention (EBC) bitter units. During boiling, a small amount of water evaporated  
11  
12 5 (maximum 4%). Then, the cast wort was treated in a whirlpool at 90 °C, for 20 min in order to  
13  
14 6 separate protein-polyphenol aggregates formed during wort boiling (hot trub). After removal of the hot  
15  
16 7 trub (about 4000 l), the wort was cooled at 15 °C, aerated to avoid adverse yeast behaviors during cell  
17  
18 8 division and pitched (pitching rate 0,007 kg l<sup>-1</sup>) with a lager strain of *Saccharomyces cerevisiae*,  
19  
20 9 originating from a former Hürlimann brewery collection (*fermentation*).  
21  
22  
23

24  
25 10 The fermentation was performed in 140000 l horizontal stainless steel vessels at 15 °C for an  
26  
27 11 average period of 10 days; normally a small amount of water was added (about 2500 l). Fermentation  
28  
29 12 time may vary according to the reduction of the diacetyl level in green beer below 50  $\mu$ g kg<sup>-1</sup>. Then,  
30  
31 13 the green beer was cooled at 7 °C for 2 days. At the end of fermentation, beer was centrifuged. Then,  
32  
33 14 beer was clarified and stabilized by using diatomaceous earth and polyvinyl polypyrrolidone (PVPP)  
34  
35 15 as the main filter aids, respectively. A sheet type filter was used for clarification, supplied with a fully  
36  
37 16 automatic kieselguhr dosing unit. Stabilization with PVPP was performed in a recycling process. The  
38  
39 17 stabilizing solution was made dissolving 350 kg of PVPP in 5000 l of water at 80 °C; PVPP dosing  
40  
41 18 into beer was set at 0.35 g l<sup>-1</sup> (*stabilization*). Successively, dilution to the final beer alcohol content  
42  
43 19 was performed by means of de-aerated water. A trap filter, installed downstream, helped retain  
44  
45 20 kieselguhr particles that bleed through the sheet filter and PVPP fines. Finally, the finished beer was  
46  
47 21 recovered in 100000 l horizontal bright beer tanks; after bottling, the beer was tunnel pasteurized; the  
48  
49 22 bottled beer was kept at 64 °C for 20 min and then cooled to room temperature.  
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## 58 24 *Samples*

59  
60 25 In accordance with the brewing program, four brews (A, B, C and D) were brewed on a 65000 l

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3 1 scale. Wort A and B were fermented in two different horizontal fermentors with a capacity of 140000  
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5 2 l. Wort C and D were jointly fermented using a horizontal fermentor of equal full capacity. The  
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8 3 utilization of fermentors of such size, usually used for the production of extremely limited volumes of  
9  
10 4 specialty beer in substitution of classical 450000 l cylindroconical units, helped us optimize the  
11  
12  
13 5 sampling plan and avoided there being an overwhelming number of samples to be monitored for each  
14  
15 6 brew at the fermentation stage. Sampling of malted barley and maize grit was performed according to  
16  
17 7 the EC Regulation 401/2006 (Commission of the European Communities 2006a). Primary samples of  
18  
19 8 about 200 g of malted barley and maize grit were separately collected in continuum at a fixed and  
20  
21 9 constant flow rate by means of a sampling flux deviator applied to the raw material conveyors, which  
22  
23 10 connected the weighing machines to the silos. Using this flux deviator, 40 incremental samples were  
24  
25 11 collected in a time period as long as the time required for the off-take of a single brew; two aggregate  
26  
27 12 samples of about 8 kg of malted barley and maize grit were obtained. Samples of intermediate  
28  
29 13 products and finished beer were taken at the end of the corresponding process (numbered in figure 1).  
30  
31 14 For these products, two samples of 1.5 kg or liter were collected and put together. Solid samples  
32  
33 15 (malted barley and maize grit) were ground with a hammer mill (1 mm sieve) and homogenized for 10  
34  
35 16 min using a horizontal mixer. From the industrial plant were collected: malted barley (1), maize grit  
36  
37 17 (2), malt mash (3), sweet wort (4), boiled wort (5), wort after whirlpool process (6), hot trub (7), green  
38  
39 18 beer (8), beer after kieselguhr filtration (9), beer after PVPP stabilization (10), beer after water dilution  
40  
41 19 and trap filtration (11), bottled beer (12). All the samples were stored at -20 °C until the time of  
42  
43 20 analysis. For each brewing, the amounts of malted barley and maize grit and the volumes of water,  
44  
45 21 wort and beer are shown in table 1. The volumes were measured in l with an approximation of  $\pm 200$  l.  
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47 22 These data derived from measurements carried out by plant-technicians at the time of brewing.  
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24 *Final product*



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3 1 The bottled beer was a standard product, whose main parameters are shown in table 2. The  
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5 2 parameters were measured by means of an AlcoLyzer Plus (Anton Paar GmbH, Graz, Austria) coupled  
6  
7  
8 3 with a DMA 4500 density meter and with a SP-1m automatic sample changer. Bitterness and diacetyl  
9  
10 4 were measured in compliance with Analytica EBC methods 9.8 and 9.24.1, respectively (European  
11  
12 5 Brewery Convention, 1998).  
13  
14  
15 6

#### 17 *Analytical Standards*

18  
19 8 Aflatoxin and fumonisin standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). For  
20  
21 9 each aflatoxin, a stock solution of 5-8  $\mu\text{g ml}^{-1}$  was prepared in benzene:acetonitrile (98:2, v/v, 2 ml)  
22  
23 10 and stored at  $-20^{\circ}\text{C}$ . The solution was calibrated spectrophotometrically at 350 nm (AOAC  
24  
25 11 International, 2005a). The working standard solution was prepared after evaporation under nitrogen of  
26  
27 12 an aliquot (50  $\mu\text{l}$ ) of the stock solution and re-dissolution in chloroform (10 ml) by ultrasonication. An  
28  
29 13 aliquot (50 or 100  $\mu\text{l}$ ) of this solution was evaporated under nitrogen and re-dissolved in the HPLC  
30  
31 14 mobile phase (1-25 ml), to obtain calibrant solutions at individual concentrations of AFB<sub>1</sub>, AFB<sub>2</sub>,  
32  
33 15 AFG<sub>1</sub>, AFG<sub>2</sub> between 0.1 and 6  $\mu\text{g l}^{-1}$ . FB<sub>1</sub> and FB<sub>2</sub> (10 mg) were separately dissolved in 50 ml  
34  
35 16 acetonitrile:water (1:1, v/v). These solutions were diluted to obtain HPLC calibrant solutions in  
36  
37 17 acetonitrile:water (30:70, v/v) at individual concentrations of FB<sub>1</sub> and FB<sub>2</sub> between 2.5 and 50  $\mu\text{g l}^{-1}$ .  
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#### 46 *Analysis for aflatoxins*

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48 20 Regarding solid samples, aflatoxins were extracted from 25 g of sample with 250 ml acetone:water  
49  
50 21 (85:15 v/v) according to the AOAC method 2003.02 (AOAC International 2005b). For liquid samples  
51  
52 22 with solid materials (cooked and malt mash), 120 ml of acetone was added to a homogenized aliquot  
53  
54 23 of 50 g. The extraction was performed using a rotary-shaking stirrer for 45 min. After filtration of the  
55  
56 24 extracts through a folded filter-paper, an aliquot of the filtrate (5 ml) was diluted with distilled water  
57  
58  
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1 (45 ml) and the solution was purified through an immunoaffinity column (R-Biopharm Rhône LTD,  
2 Glasgow, Scotland, UK).

3 For liquid samples (wort and beer), 20 ml was diluted with 20 ml of phosphate buffered saline  
4 (PBS, pH 7.4) and the solution was shaken for about 1 min. The pH was controlled and adjusted, if  
5 necessary, between 6.5 and 7.5 using NaOH 0.1 N; then the solution was purified through the  
6 immunoaffinity column.

7 After washing of the column with 5 ml of distilled water, aflatoxins were eluted into a graduated  
8 glass vial with methanol (2.5 ml). The eluate, evaporated under a gentle stream of nitrogen, was  
9 redissolved in 1 ml of acetonitrile:water (25:75, v/v), and vortex-mixed for a few seconds; then, the  
10 extract was filtered (Millipore Corporation, Bedford, Massachusetts, USA, HV 0.45  $\mu\text{m}$ ) and injected  
11 (30 or 100  $\mu\text{l}$ ).

12 Analysis was performed using an HPLC instrument, consisting of two PU-1580 chromatographic  
13 pumps, an AS 1555 sampling system, an FP 1520 fluorescence detector and a post-column  
14 derivatization system (Jasco Corporation, Tokyo, Japan); the instrument was controlled by Borwin 1.5  
15 software (Jasco). A Superspher RP-18 column (4  $\mu\text{m}$  particle size, 125x4 mm i.d., Merck) was used at  
16 ambient temperature, with a mobile phase of water:methanol:acetonitrile (64:23:13, v/v/v), at 1.0 ml  
17  $\text{min}^{-1}$ . A solution of pyridinium bromide perbromide (25 mg in 500 ml of HPLC-grade water) was  
18 used as a derivatizing agent. The flow of the post-column derivatizing solution was set at 0.1 ml  $\text{min}^{-1}$   
19 and the reaction tubing was 500  $\mu\text{l}$ . The detector was set at  $\lambda_{\text{ex}}=365$  nm and  $\lambda_{\text{em}}=440$  nm.

#### 21 *Analysis for fumonisins*

22 Regarding solid samples (malted barley, maize grit, spent grains), fumonisins were analysed  
23 according to the method proposed by Visconti et al. (2001). Fumonisins were extracted from 20 g of  
24 sample in a plastic centrifuge bottle with 100 ml of acetonitrile:methanol:water (25:25:50, v/v/v).  
25 After extraction for 45 min using a rotary-shaking stirrer and centrifugation at 4500 g for 6 min, the

1  
2  
3 1 supernatant was poured into a flask. Another 100 ml of the same solution was added to the residue in  
4  
5 2 the centrifuge bottle, and a second extraction performed for 30 min; then, the extracts were combined.  
6  
7  
8 3 For liquid samples with solid materials (cooked and malt mash), 40 ml of acetonitrile:methanol (50:50  
9  
10 4 v/v) were added to a homogenized aliquot of 50 g; the extraction was performed using a rotary-  
11  
12 5 shaking stirrer for 45 min. After filtration of the extracts through a folded filter-paper, an aliquot of the  
13  
14 6 filtrate (2 ml) was diluted with 20 ml of PBS and purified through an immunoaffinity column (R-  
15  
16 7 Biopharm Rhône LTD, Glasgow, Scotland, UK).

17  
18  
19  
20 8 For liquid samples, 20 ml was diluted with 20 ml of PBS and the solution was shaken for about 1  
21  
22 9 min. The pH was controlled and adjusted, if necessary, between 6.5 and 7.5 using NaOH 0.1 N; then  
23  
24 10 the solution was purified through the immunoaffinity column.

25  
26  
27 11 After washing the column with PBS (2 ml), fumonisins were slowly eluted ( $0.5 \text{ ml min}^{-1}$ ) with  
28  
29 12 methanol (6 ml) into a graduated glass vial; subsequently, the eluate was concentrated to 2 ml under a  
30  
31 13 gentle stream of nitrogen.

32  
33  
34 14 Analysis was carried out using a LC-MS/MS system, consisting of a LC 1.4 Surveyor pump  
35  
36 15 (Thermo-Fisher Scientific, San Jose, CA, USA), a PAL 1.3.1 sampling system (CTC Analytcs AG,  
37  
38 16 Zwingen, Switzerland) and a Quantum Discovery Max triple-quadrupole mass spectrometer; the  
39  
40 17 system was controlled by Excalibur 1.4 software (Thermo-Fisher). After dilution of the extract (0.1 ml  
41  
42 18 brought to 1 ml) with acetonitrile:water (30:70 v/v), fumonisins were separated on a Betasil RP-18  
43  
44 19 column ( $5 \mu\text{m}$  particle size,  $150 \times 2.1 \text{ mm}$ , Thermo-Fisher) with mobile-phase gradient acetonitrile-  
45  
46 20 water (both acidified with 0.4% acetic acid) from 25:75 to 55:45 in 9 min, then isocratic for 3 min; the  
47  
48 21 flow rate was  $0.2 \text{ ml min}^{-1}$ . Ionisation was carried out with an ESI interface (Thermo-Fisher) in  
49  
50 22 positive mode as follows: spray capillary voltage 4.0 kV, sheath and auxiliary gas 35 and 14 psi,  
51  
52 23 respectively, temperature of the heated capillary  $270 \text{ }^\circ\text{C}$ . The mass spectrometric analysis was  
53  
54 24 operated in selected reaction monitoring (SRM). For fragmentation of  $[\text{M}+\text{H}]^+$  ions (722 and 706  $m/z$   
55  
56 25 for  $\text{FB}_1$  and  $\text{FB}_2$ , respectively), the argon collision pressure was set to 1.5 mTorr and the collision

1 energy to 36 V. The selected fragment ions were 704, 352 and 334  $m/z$  for FB<sub>1</sub> and 688, 336 and 318  
2  $m/z$  for FB<sub>2</sub>. Quantitative determination was performed using LC-Quan 2.0 software (Thermo-Fisher).

3 Both for aflatoxins and fumonisins, three replicates were analysed for each sample.

#### 4 5 *Other mycotoxins*

6 Malted barley and maize grit were also analyzed for deoxynivalenol (DON), zearalenone (ZEA) and  
7 ochratoxin A (OTA) by methods routinely used in our laboratory (Rossi et al. 2006; Pietri et al. 2004);  
8 since very low levels were found in all samples (DON < 200  $\mu\text{g kg}^{-1}$ , ZEA < 20  $\mu\text{g kg}^{-1}$  and OTA <  
9 0.2  $\mu\text{g kg}^{-1}$ ), the carry-over of these mycotoxins was not investigated.

## 10 11 **Results and discussion**

### 12 *Performance of the methods*

13 Percentage recoveries were determined by spiking known blank maize grit samples at levels of 2 and  
14 1000  $\mu\text{g kg}^{-1}$  and blank beer samples at levels of 0.1 and 50  $\mu\text{g kg}^{-1}$  for aflatoxins and fumonisins,  
15 respectively. Average recovery values were: 97.8  $\pm$ 1.6% and 98.3 $\pm$ 1.5% for AFB<sub>1</sub>, 94.9 $\pm$ 2.3% and  
16 97.6 $\pm$ 1.8% for AFB<sub>2</sub>, 95.5 $\pm$ 1.9% and 97.6 $\pm$ 1.7% for FB<sub>1</sub>, 93.7 $\pm$ 3.0% and 95.5 $\pm$ 1.9% for FB<sub>2</sub>, in maize  
17 grit and beer, respectively. Recovery values higher than 90% were also obtained for malt mash and  
18 boiled wort; for the other liquid intermediate products, being very similar to boiled wort and beer,  
19 recovery experiments were not carried out.

20 Certified reference materials (ground corn, R-Biopharm Rhône LTD), with AFB<sub>1</sub> and fumonisin  
21 contamination of 4.1 $\pm$ 1.0  $\mu\text{g kg}^{-1}$  and 2500 $\pm$ 600  $\mu\text{g kg}^{-1}$  (FB<sub>1</sub>=1600, FB<sub>2</sub>=700, FB<sub>3</sub>=200  $\mu\text{g kg}^{-1}$ ),  
22 respectively, were also analysed; our results were 4.2 $\pm$ 0.2  $\mu\text{g kg}^{-1}$  for AFB<sub>1</sub>, 1553 $\pm$ 39 and 721 $\pm$ 21  $\mu\text{g}$   
23  $\text{kg}^{-1}$  for FB<sub>1</sub> and FB<sub>2</sub>, respectively. The results of the analyses were not corrected for recovery; the  
24 relative standard deviations were always <13% for AFB<sub>1</sub> and <11% for FB<sub>1</sub>. The limits of detection  
25 (LOD, signal-to-noise ratio of 3:1) and of quantification (LOQ, signal-to-noise ratio of 10:1) were

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2  
3 1 respectively: 0.005 and 0.01  $\mu\text{g kg}^{-1}$  for AFB<sub>1</sub>, 0.01 and 0.02  $\mu\text{g kg}^{-1}$  for AFB<sub>2</sub>, 30 and 50  $\mu\text{g kg}^{-1}$  for  
4  
5  
6 2 FB<sub>1</sub> and FB<sub>2</sub>, in maize grit; 0.001 and 0.002  $\mu\text{g l}^{-1}$  for AFB<sub>1</sub>, 0.002 and 0.004  $\mu\text{g l}^{-1}$  for AFB<sub>2</sub>, 1 and  
7  
8 3 2.5  $\mu\text{g l}^{-1}$  for FB<sub>1</sub> and FB<sub>2</sub>, in beer.  
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13 5 *Aflatoxin B<sub>1</sub>*  
14

15 6 AFB<sub>1</sub> ranged in maize grit from 0.31 to 14.85  $\mu\text{g kg}^{-1}$ ; it was also found in malted barley, at levels  
16  
17 7 ranging from 0.20 to 4.07  $\mu\text{g kg}^{-1}$  (table 3). Malted barley and maize grit used for brewing A and  
18  
19 8 maize grit for brewing B showed concentrations higher than 2  $\mu\text{g kg}^{-1}$ , the maximum level fixed by EC  
20  
21 9 Regulation for all cereals and all products derived from cereals, including processed cereal products  
22  
23 10 (Commission of the European Communities 2006b). As regards the other aflatoxins, only AFB<sub>2</sub> was  
24  
25 11 detected in maize grits used for brew A and B production (1.2 and 0.7  $\mu\text{g kg}^{-1}$ , respectively); however,  
26  
27 12 it resulted <LOD in the bottled beer and therefore the very low levels found in some intermediate  
28  
29 13 products are not reported. For each stage of the brewing, the AFB<sub>1</sub> percentage in the product, referring  
30  
31 14 to the total amount in the raw materials (TARM), was calculated ( $(\text{conc}_{\text{product}} * \text{kg}(\text{or l})_{\text{product}}) / (\text{conc}_{\text{malted}}$   
32  
33 15  $\text{barley} * \text{kg}_{\text{malted barley}} + \text{conc}_{\text{maize grit}} * \text{kg}_{\text{maize grit}}) * 100$ ). A relatively small amount of the toxin was transferred  
34  
35 16 to the liquid phase during mashing; in fact, averaging the 4 brewing results, only 14.3±2.8% of the  
36  
37 17 AFB<sub>1</sub> TARM was found in the liquor after mashing: this is probably due to the low solubility of the  
38  
39 18 toxin in water. In the sweet wort the AFB<sub>1</sub> level was reduced both because of the dilution of the  
40  
41 19 product, and mainly because of the filtration step: in effect, lautering caused a consistent reduction (-  
42  
43 20 45.4±14.3%) of the AFB<sub>1</sub> present in the liquor and only an average of 7.9±3.0% of the AFB<sub>1</sub> TARM  
44  
45 21 was found in the sweet wort. In the following steps, the reductions were more moderate (boiling  
46  
47 22 -28.5±10.5%, whirlpooling -2.6±14.9%, fermentation -14.3±9.5%), while a further strong decrease  
48  
49 23 (-59.6±17.3%) was observed passing from green to bottled beer. The percentages of AFB<sub>1</sub> TARM  
50  
51 24 found in the bottled beer varied from 0.6 to 2.2% (mean 1.5±0.8%) and the concentrations, because of  
52  
53 25 the high dilution, were in the low part-per-trillion level. These results are in agreement with a previous  
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1 study (Steiner and Länzlinger 1978), where about 5% of AFB<sub>1</sub> and AFB<sub>2</sub> were recovered in the final  
2 beer brewed from fortified malt. Differently, Chu et al. (1975), in a micro-brewing process, after  
3 addition of AFB<sub>1</sub> (dissolved in chloroform) at 1 and 10 µg g<sup>-1</sup>, at the beginning of mashing, found 18  
4 and 27% of the toxin, respectively, in bottled beer; Okoye (1986) found a carry-over of 41% from red  
5 guinea corn into a Nigerian native beer. These higher recoveries were likely due to the modality of the  
6 trials and to the very high level of spiking.

#### 7 8 *Fumonisin B<sub>1</sub>*

9 The FB<sub>1</sub> levels in maize grit varied from 1146 to 3194 µg kg<sup>-1</sup> (table 4); FB<sub>2</sub> occurred as well, at  
10 levels from 347 to 908 µg kg<sup>-1</sup>. Since FB<sub>2</sub> was not detected in the bottled beer, the very low levels  
11 found in some intermediate products are not reported. Only in the case of maize grit used for brewing  
12 B (FB<sub>1</sub>+FB<sub>2</sub>=1493 µg kg<sup>-1</sup>), was the concentration of fumonisins close to the level fixed by the EC  
13 Regulation 1126/2007 (Commission of the European Communities 2007) for milling fractions of  
14 maize with particle size > 500 µm not intended for direct human consumption (1400 µg kg<sup>-1</sup>); on the  
15 contrary, the fumonisin level exceeded the limit in the case of the grit used in the other brewing  
16 processes. Fumonisin were not detected in malted barley. For each stage of the brewing, the FB<sub>1</sub>  
17 percentage in the product, referring to the TARM, was calculated  $((\text{conc}_{\text{product}} * \text{kg}(\text{or l})_{\text{product}}) / (\text{conc}_{\text{maize}} * \text{kg}_{\text{maize}} * \text{kg}_{\text{grit}}) * 100)$ . Unlike AFB<sub>1</sub>, most of the FB<sub>1</sub> entering the brewing was extracted into the  
18 mashing liquors (average 84.3±10.3%). During the following steps, the reduction was quite low:  
19 -7.3±2.5% after lautering, -8.3±3.6% at the end of the boiling process, -10.3±2.5% after whirlpooling.  
20 Likewise, the fermentation process did not appreciably decrease the amount of FB<sub>1</sub> (-4.2±2.6%); this  
21 was in agreement with the study of Scott et al. (1995), in which a loss of FB<sub>1</sub> in the range 3-28% was  
22 estimated. Finally, a further low reduction (on average -16.4±10.0%) was observed from green beer to  
23 bottled beer. The clarification and stabilization processes, which employed kieselguhr and PVPP,  
24 respectively, did not appreciably reduce the amount of FB<sub>1</sub>. These materials showed a nearly full

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3 1 retentive indifference towards FB<sub>1</sub>; this held true also for celite used for the brewing process, as  
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5 2 reported by Belajova et al. (2007). In bottled beer 50.7±4.7% of the FB<sub>1</sub> TARM was recovered; the  
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7 3 concentrations varied from 37 to 89 µg l<sup>-1</sup>. These results indicate that FB<sub>1</sub> can occur in beer when  
8  
9 4 maize-based solid adjuncts are used. FB<sub>1</sub> occurrence in finished beer is mainly due to its high  
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11 5 solubility in water and to the relative stability to heat treatment.  
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## 18 7 **Conclusions**

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20 8 The four industrial brewing processes gave quite similar results; as expected, AFB<sub>1</sub> and FB<sub>1</sub>  
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22 9 showed different behaviors during brewing, mainly due to their different solubility in water; however,  
23  
24 10 also the process determined a different transfer rate of the two solubilised toxins into the bottled beer  
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26 11 (10.5 vs. 60.1% for AFB<sub>1</sub> and FB<sub>1</sub>, respectively). Consequently, the concentration of AFB<sub>1</sub> retrieved in  
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28 12 bottled beer was very low. In Italy the consumption of beer is about 30 l per person year<sup>-1</sup> (Assobirra  
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30 13 2007), corresponding to 0.082 l per person day<sup>-1</sup>; if a consumer drinks beer with contamination like  
31  
32 14 that obtained from brewing A, the AFB<sub>1</sub> intake would be 1.8 ng or 0.03 ng kg<sup>-1</sup> b.w., that is 3.6-0.5%  
33  
34 15 of the estimated range of daily exposure to total aflatoxins in Italy (0.838-1.934 ng kg<sup>-1</sup> b.w.; EFSA  
35  
36 16 2007). When the EU limits for AFB<sub>1</sub> in raw materials are respected, like in brewing C+D, the  
37  
38 17 corresponding figures for a consumer are 0.12 ng of AFB<sub>1</sub> or 0.002 ng kg<sup>-1</sup> b.w., that is 0.2-0.1% of the  
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40 18 estimated range of daily exposure in Italy. In the latter case, the contribution of beer to exposure is  
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42 19 negligible.  
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48 20 Analogous precautions should be taken for the fumonisin contamination in maize adjuncts. If the  
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50 21 fumonisin contamination clearly exceeds the EU limit, as in maize adjuncts used in brewing C+D, a  
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52 22 daily exposure of 0.12 µg kg<sup>-1</sup> b.w. can be calculated for a consumer, that represents about 6% of the  
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54 23 TDI of 2 µg kg<sup>-1</sup> b.w., fixed by the Scientific Committee on Food (2000). If the EU limit is respected,  
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56 24 as in maize adjuncts used in brewing B, the corresponding figure for a consumer is 0.05 µg kg<sup>-1</sup> b.w.  
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58 25 per day, that represents only about 2% of the TDI.  
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3 1 However, it must be considered that in Italy about 2% of males and about 3% of young males (25-  
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5 2 44 years) consume more than 0.5 l of beer; for this fraction of population, the calculated mean  
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8 3 exposure must be multiplied by six or more.

10 4 It is evident that when the raw materials used in the brewing process exceed the EU limits for  
11  
12 5 AFB<sub>1</sub> and FB<sub>1</sub>, beer can become a more significant source of exposure to the two toxins.

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Table 1. Amounts (kg) of malted barley and maize grit and volumes (l) of wort and beer.

|                     | brewing A | brewing B | brewing C | brewing D |
|---------------------|-----------|-----------|-----------|-----------|
| Malted barley       | 7633      | 7885      | 7853      | 7857      |
| Maize grit          | 4999      | 5036      | 5000      | 5000      |
| Water added in mash | 37500     | 35000     | 34600     | 35500     |
| Sweet wort          | 69700     | 68000     | 70800     | 70700     |
| Boiled wort         | 69500     | 66900     | 69000     | 69000     |
| Wort                | 65700     | 62900     | 65000     | 65000     |
| Hot trub            | 3800      | 4000      | 4000      | 4000      |
| Green beer          | 67700     | 66000     |           | 133000    |
| Filtered beer       | 67500     | 65800     |           | 128000    |
| Stabilized beer     | 67500     | 65800     |           | 128000    |
| Diluted beer        | 81500     | 79400     |           | 156500    |
| Bottled beer        | 81500     | 79400     |           | 156500    |

Table. 2. Main parameters of bottled beer.

| Parameters                          | Range    |
|-------------------------------------|----------|
| Original Extract (%°P)              | 13-14    |
| ABV (alcohol by volume %)           | 4.2-4.7  |
| RDF (real degree of fermentation %) | 63-70    |
| pH                                  | 3.7-4.1  |
| Colour (EBC)                        | 7.5-11.5 |
| Bitterness (BU)                     | 15-25    |
| Diacetyl ( $\mu\text{g kg}^{-1}$ )  | < 50     |

Table 3. Transfer of aflatoxin B<sub>1</sub> (mean of three replicates) from raw materials to the different intermediate and final products during the brewing processes.

|                 | <i>brewing A</i>                                 |                                  | <i>brewing B</i>                                 |                                  | <i>brewing C</i>                                 |                                  | <i>brewing D</i>                                 |                                  |
|-----------------|--|----------------------------------|--|----------------------------------|--|----------------------------------|--|----------------------------------|
|                 | $\mu\text{g kg}^{-1}$<br>or $\mu\text{g l}^{-1}$ | Percentage<br>(%) <sup>(1)</sup> | $\mu\text{g kg}^{-1}$<br>or $\mu\text{g l}^{-1}$ | Percentage<br>(%) <sup>(1)</sup> | $\mu\text{g kg}^{-1}$<br>or $\mu\text{g l}^{-1}$ | Percentage<br>(%) <sup>(1)</sup> | $\mu\text{g kg}^{-1}$<br>or $\mu\text{g l}^{-1}$ | Percentage<br>(%) <sup>(1)</sup> |
| Malted barley   | 4.07   | 100                              | 1.36   | 100                              | 0.38   | 100                              | 0.20   | 100                              |
| Maize grit      | 14.85  |                                  | 10.93  |                                  | 0.92   |                                  | 0.31   |                                  |
| Malt mash       | 0.437  | 15.6                             | 0.202  | 10.7                             | 0.038  | 17.3                             | 0.012  | 13.6                             |
| Sweet wort      | 0.178  | 11.8                             | 0.046  | 4.8                              | 0.009  | 8.4                              | 0.003  | 6.8                              |
| Boiled wort     | 0.114  | 7.5                              | 0.033  | 3.4                              | 0.008  | 7.3                              | 0.002  | 4.2                              |
| Wort            | 0.089  | 5.5                              | 0.029  | 2.8                              | 0.005  | 4.2                              | 0.002  | 4.2                              |
| Hot trub        | 0.141  | 0.5                              | 0.024  | 0.2                              | 0.008  | 0.4                              | 0.003  | 0.4                              |
|                 |  |                                  |  |                                  | <i>brewing C+D</i>                               |                                  |  |                                  |
|                 |  |                                  |  |                                  | $\mu\text{g l}^{-1}$                             | Percentage (%)                   |  |                                  |
| Green beer      | 0.082  | 5.3                              | 0.021  | 2.1                              | 0.003  | 3.6                              |  |                                  |
| Filtered beer   | 0.060  | 3.8                              | 0.019  | 1.9                              | 0.003  | 3.6                              |  |                                  |
| Stabilized beer | 0.058  | 3.7                              | 0.019  | 1.9                              | 0.003  | 3.6                              |  |                                  |
| Diluted beer    | 0.046  | 3.6                              | 0.013  | 1.6                              | 0.002  | 2.9                              |  |                                  |
| Bottled beer    | 0.022  | 1.7                              | 0.005  | 0.6                              | 0.0015   | 2.2                              |  |                                  |

<sup>(1)</sup>  $(\text{conc}_{\text{product}} * \text{kg}(\text{or l})_{\text{product}}) / (\text{conc}_{\text{malted barley}} * \text{kg}_{\text{malted barley}} + \text{conc}_{\text{maize grit}} * \text{kg}_{\text{maize grit}}) * 100$ .

Table 4. Transfer of fumonisin B<sub>1</sub> (mean of three replicates) from maize grit to the different intermediate and final products during the brewing processes.

|                 | <i>brewing A</i>                                 |                                  | <i>brewing B</i>                                 |                                  | <i>brewing C</i>                                 |                                  | <i>brewing D</i>                                 |                                  |
|-----------------|--|----------------------------------|--|----------------------------------|--|----------------------------------|--|----------------------------------|
|                 | $\mu\text{g kg}^{-1}$<br>or $\mu\text{g l}^{-1}$ | Percentage<br>(%) <sup>(1)</sup> | $\mu\text{g kg}^{-1}$<br>or $\mu\text{g l}^{-1}$ | Percentage<br>(%) <sup>(1)</sup> | $\mu\text{g kg}^{-1}$ or<br>$\mu\text{g l}^{-1}$ | Percentage<br>(%) <sup>(1)</sup> | $\mu\text{g kg}^{-1}$<br>or $\mu\text{g l}^{-1}$ | Percentage<br>(%) <sup>(1)</sup> |
| Malted barley   | < 30   | 100                              | < 30   | 100                              | < 30   | 100                              | < 30   | 100                              |
| Maize grit      | 1741   |                                  | 1146   |                                  | 2880   |                                  | 3194   |                                  |
| Malt mash       | 207  | 89.2                             | 116  | 70.3                             | 347  | 83.4                             | 424  | 94.2                             |
| Sweet wort      | 102  | 81.7                             | 56   | 66.0                             | 162  | 79.6                             | 191  | 84.6                             |
| Boiled wort     | 97   | 77.5                             | 52   | 60.3                             | 156  | 74.7                             | 170  | 73.4                             |
| Wort            | 95   | 71.7                             | 50   | 54.5                             | 145  | 65.4                             | 159  | 64.7                             |
| Hot trub        | 134  | 5.8                              | 66   | 4.6                              | 227  | 6.3                              | 211  | 5.3                              |
|                 |  |                                  |  |                                  | <i>brewing C+D</i>                               |                                  |  |                                  |
|                 |  |                                  |  |                                  | $\mu\text{g l}^{-1}$                             | Percentage (%)                   |  |                                  |
| Green beer      | 88   | 68.4                             | 47   | 53.7                             | 142  | 60.8                             |  |                                  |
| Filtered beer   | 83   | 64.4                             | 46   | 52.4                             | 137  | 57.7                             |  |                                  |
| Stabilized beer | 81   | 62.8                             | 45   | 51.3                             | 132  | 55.6                             |  |                                  |
| Diluted beer    | 62   | 58.1                             | 38   | 52.3                             | 98   | 50.5                             |  |                                  |
| Bottled beer    | 59   | 55.2                             | 37   | 50.9                             | 89   | 45.9                             |  |                                  |

<sup>(1)</sup>  $(\text{conc}_{\text{product}} * \text{kg}(\text{or l})_{\text{product}}) / (\text{conc}_{\text{maize grit}} * \text{kg}_{\text{maize grit}}) * 100$ .



Figure 1: layout of the industrial plant showing sampling points (numbered).

