Aflatoxin monitoring in Italian hazelnut products by LC-MS

Monitoring of aflatoxins (B1, B2, G1, G2) in hazelnut products commercialized in Italy with liquid chromatography tandem mass spectrometry

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Abstract

Hazelnut samples of different origin were collected in different stores of Northern Italy and analysed for aflatoxin (AF) contamination by a sensitive chromatographic method based on a tandem mass spectrometer with electrospray ionization. The effects of two extracting solvent mixtures (methanol/water and acetonitrile/water) and different extraction times were tested and compared in terms of recovery. The analysis showed that 35 out of 93 samples (37.6%) were contaminated by AFs. The incidence of positive samples was higher in the Turkish (66.7%) than in the Italian samples (35.9%). The mean AF contamination was higher (p=0.045, Kruskal-Wallis test) in the Turkish samples (0.33 µg kg^{-1}), compared to the Italian (0.14 µg kg^{-1}) ones. Generally, a low level of AFs contamination was found in the positive samples (0.64 µg kg^{-1}), far below the maximum threshold admitted by the European legislation. AFG_1 was the most diffused aflatoxin, found in 19 samples.

Keywords: Aspergillus; electrospray ionization; Northern Italy; contamination.

Word count: 4,275

Introduction

Aflatoxins (AFs) are highly toxin secondary metabolites produced by fungi belonging to the genus *Aspergillus* (Kimiko et al. 1999). *A. flavus* produces aflatoxin B_1 (AFB_1) and aflatoxin B_2 (AFB_2), whereas *A. parasiticus* generates aflatoxins B, as well as aflatoxin G1 (AFG_1) and aflatoxin G2 (AFG_2). These fungi can grow on a wide variety of foods and feeds under favourable temperature and humidity. Generally, tropical conditions, such as high temperature and high relative humidity, unseasonal rains during harvest and flash floods lead to fungal proliferation and AFs production. Moreover, poor agronomic practices, improper storage and suboptimal conditions during transport, increase the risk of contamination by AFs, that can take place at any point along the food chain, from the field, harvest, handling, transport to the storage (Bhat and Vasanthi 2003, Giray et al. 2007).

These mycotoxins have been clearly identified as highly toxic, mutagenic, teratogen and carcinogenic compounds and have been implicated as causal agents of human hepatic and extra hepatic carcinogenesis (Castells et al. 2008). Thus, AFs contamination is a worldwide problem with regard to food and feed safety, and many countries established AFs restrictions in food. The European Union (EU) fixed a maximum admitted level of 8 µg kg^{-1} for aflatoxin B_1, and 15 µg kg^{-1} for total AFs (B_1, B_2, G_1 and G_2) on dried fruit intended for further processing, and a threshold of 5 µg kg^{-1} for aflatoxin B_1, and 10 µg kg^{-1} for total AFs in ready to eat groundnuts, nuts, dried fruits and cereals (EC 2010).

Because hazelnuts prefer regions with mild, moist winters and cool summers, *Aspergillus* spp. contamination can be found on their fruits with subsequent production of AFs. Moreover, hazelnuts can be contaminated during various steps from harvesting to the final product, especially after dehulling.

Hazelnuts are commercially produced in a relatively few countries, such as Turkey (73% of the market), Italy (15%), the United States (4%) and Spain (3%) (HPG, 2005). Since hazelnuts are components of many commercial products, such as baked products, ice cream, and chocolate bars, it is necessary a sensitive and simple method to determine the presence and the content of AFs in various commercial products to evaluate the risks associated with their human consumption.

Several analytical methods have been used for AFs monitoring, such as thin-layer chromatography (TLC) (Stroka et al. 2000; Trucksess et al. 1984), high-performance liquid chromatography (HPLC) coupled to fluorescence or mass spectrometry detectors (Ghali et al. 2009, Huang et al. 2010) and immunological methods (Reddy et al. 2001, Saha et al. 2007). TLC and immunological techniques provide rapid methods of screening for aflatoxins and play an important role in developing countries for surveillance purposes and control of regulatory limits (Gilbert and Anklam 2002). HPLC methods coupled with fluorescence detection are sensitive and the most widely adopted, but often require pre- or post-column derivatisation because of the weak native fluorescence of the analytes. On the other hand, liquid chromatography coupled with mass spectrometry (LC-MS) or liquid chromatography coupled with tandem mass spectrometry (LC-MS-MS) methods are specific and sensitive, and their use is becoming increasingly widespread (Di Mavungu et al. 2009).

Extraction of AFs from various food matrices generally involves aqueous mixtures of polar organic solvents such as methanol (Leong et al. 2010), acetone or acetonitrile (Khayoon et al. 2010), whereas extraction with chloroform (Alvito et al. 2010) has been abandoned in order to reduce the use of chlorinated solvents. Immunoaffinity columns (Kumagai et al. 2008) and solid-phase extraction (SPE) columns (Saha et al. 2007) have been used in clean up procedures.

Previous reports on the occurrence of aflatoxins in Italy took in consideration few samples coming from stores of Central/Southern Italy. In particular, Bacaloni et al. (2008) analysed 38 hazelnuts from Rome, and Campone et al. (2009) analysed an unspecified number of nuts (almond, hazelnuts and peanuts) from Salerno.

Due to scarce data on aflatoxin content in Italian hazelnuts, the aim of this study was to analyse a larger number of hazelnut samples collected in different stores of Northern Italy using an efficient, very sensitive and simple method to extract, purify and analyse simultaneously aflatoxins B_1, B_2, G_1 and G_2 by LC-MS-MS with electrospray ionization (ESI).

Materials and Methods

Chemical and reagents

Standards of AFB_1, AFB_2, AFG_1 and AFG_2 were purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved in methanol to prepare a working standard solution at the concentration of 10 µg ml^{-1}. Aflatoxin M_1 (AFM_1) was used as an internal standard at 20 ng ml^{-1}, because it is an hydroxylated metabolite of AFB_1 and it is not present in plant samples. AFM_1 was purchased from Supelco (Bellefonte, PA, USA) and diluted in acetonitrile to make a stock solution at the concentration of 0.5 µg ml^{-1}. These standard solutions were stored in the dark at 4°C. LC-MS grade methanol, acetonitrile, acetic acid, 2-propanol and water, used as mobile phases in the LC-MS and as extraction solutions were purchased from Sigma-Aldrich. NaCl, KCl, Na_2HPO_4, KH_2PO_4 and Tween 20 (Merck, Darmstadt, Germany) were dissolved in ultrapure water (Maina system, Turin, Italy) to prepare the phosphate buffer saline (PBS) solution. AflaTest WB immunoaffinity columns were obtained from Vicam (Watertown, MA, USA).

Samples

Whole hazelnuts (with shell, dehulled, raw, roasted and also hazelnut grain, or flour samples were purchased randomly in Northwestern Italy stores and analysed. Samples (0.5 kg) were stored in the dark before the analysis, at low relative humidity and 20°C. After analysis, all samples were sealed up in plastic bags and stored at 4°C. The hazelnuts with shell were dehulled prior to sample preparation. Whole samples, except the hazelnut flour, were pulverized using a Polymix System PX-MFC 90D mill, until homogeneous and the powder was sieved with a 0.2 mm mesh sieve. They were divided based on the agricultural practice adopted (conventional/organic), their geographical origin (Italian, Turkish and other countries), and their commercial typology (raw dehulled, roasted dehulled, with shell, in grain and floor).

Extraction and clean up

The extraction method described by Stroka et al. (2000) was used with some modifications. After sieving, 5 g were taken from 5 representative zones and mixed. The 25 g sample was extracted with 125 ml of different solvents in water mixtures for various contact times, by shaking at high speed. To evaluate the best extraction mixture, methanol and acetonitrile were tested as solvents. Five grams of sodium chloride were added to the sample prior to extraction. The extract was filtered through Whatman (Whatman GmbH, Dassel, Germany) No. 4 filter paper to eliminate the most solid part and then by using a Whatman PVDF 0.45 µm syringe filter. An aliquot of 10 ml of filtrate was diluted 1:1 in ultrapure water. To concentrate and clean up the samples immunoaffinity columns were used. After conditioning the immunoaffinity columns in their internal solution, 10 ml of diluted filtrate were loaded at a flow rate of 0.5 ml min^{-1}. The columns were washed with 10 ml of PBS solution and then

with 10 ml of water, before air drying. Aflatoxins were eluted with 3 ml methanol into an amber glass vial. The eluate was evaporated at 37°C under air flow and 1 ml of eluent (water:methanol, 90:10, with 0.1% of acetic acid and the internal standard AFM1 at 0.02 µg ml^{-1}) was added to the residue and vortexed for 1 minute.

LC-MS/MS conditions

Liquid chromatography was performed with a Varian Model 212-LC micro pumps (Hansen Way, CA, USA) coupled with a Varian autosampler Model 410 Prostar equipped with a 100 µl loop. The analytical column used for LC separation was a Pursuit XRs Ultra C18 (100 mm x 2.0 mm, 2.8 µm particle size, Varian). The chromatographic conditions were: column temperature: 30°C; mobile phase consisting of eluent A (water with 0.1% acetic acid) and eluent B (methanol with 0.1% acetic acid), using a flow rate of 0.2 ml min^{-1}. A gradient elution was applied as follows: 0-25 min: from 90% A to 15% A; 25-28 min: from 15% A to 90% A; 28-30 min: 90% A. The injection volume was 10 µl.

The LC system was coupled to a triple quadrupole mass spectrometer, Varian 310-MS, and was operated in the positive electrospray ionization mode (ESI^+). The ionization source conditions were: needle voltage of 6 kV, capillary voltage of 55-77 V, source temperature of 50 °C, desolvation temperature of 300°C, cone gas flow rate of 50 psi, desolvation gas flow rate of 20 psi with nitrogen. Multiple reaction monitoring (MRM) mode of operation was used. The $[M+H]^+$ ions of aflatoxins were used as parent ions. The most intense daughter ions, resulting from collision-induced dissociation with argon, were used to detect and quantify aflatoxin content. The argon pressure was set at 1.8 psi. The most intense daughter ions detected were: m/z 284.9 at 14 eV of collision energy (CE) for B1, m/z 286.9 at 18 eV CE for B2, m/z 242.9 at 18 eV CE for G1, m/z 245 at 24 eV CE for G2 and m/z 301 at 15 eV CE for M1.

Method validation

Validation of the method was carried out according to harmonized guidelines for methods of analysis in single-laboratory (Thompson et al. 2002). Precision was calculated in terms of intra-day repeatability (n = 10) and inter-day reproducibility (3 different days) at 1 µg kg^{-1} spiking level. The precision was calculated as relative standard deviation of replicate measurements. Linearity was done by injecting triplicate AF standard solutions at different concentrations. In this report, the limit of detection (LOD) for each aflatoxin was defined as three times the electronic baseline noise and the limit of quantification (LOQ) as six times the level of baseline noise. Recovery tests to evaluate the extraction efficacy were determined on roasted hazelnut samples of 1000 g spiked with four concentrations of each AF (0.5, 5, 10, and 25 µm kg^{-1}). Each test and the sample analysis were performed three times and experimental results were reported as mean ± standard deviation. When AFs concentration had to be confirmed in putatively positive samples, the standard addition approach was applied.

Statistical analysis

Normal distribution of toxin contents, means, standard errors and validation data were analyzed with SPSS software (SPSS Institute, Inc, 2000, Version 18.0). The calibration curves used for quantification was calculated by least-squares method. Samples with a concentration of AFs higher than the LOD were considered positives, whereas samples with concentrations lower than the LOD were considered negatives. Mean AFs concentrations were calculated only on the positive samples higher than the LOQ. Experimental results are reported as mean ± standard deviation. The Kruskal-Wallis test was used to compare the mean AF levels among the different typologies (with shell, raw dehulled, roasted dehulled, grain and flour), and geographical origins of the hazelnut samples, while the Mann-Whitney test

was used to compare the mean AF levels in conventional / organic hazelnut samples, using the null hypothesis that the levels were not different.

Results and Discussion

Optimization of extraction, clean up and LC-MS/MS analysis

The ESI positive ion mode resulted a common ionization procedure for aflatoxins determination (Bacaloni et al., 2008; Imperato et al., 2011), and it was chosen to evaluate and determinate the presence of AFs in the samples. The mobile phase was chosen based on the ionization and separation efficiencies: besides methanol and water, 0.1% acetic acid was added in the mobile phase, and AFs separation was obtained by varying the gradient conditions (Figure 1).

Different concentrations of AFs, ranging from 0.1 to 50 ng ml^{-1}, were analysed to test the linearity of the calibration curve. These curves were built by plotting the aflatoxins peak area against the internal standard peak area at a concentration of 20 ng ml^{-1}. Table 1 shows the analytical method performance: LOD, LOQ, recovery range, RSDr, RSDi and RSDI for each AF, calculated in accordance with the European standards (EC Directive 2002/27).

Instrumental precision was assessed by analysing standard solution at 1 µg kg^{-1} 10 times a day for 3 consecutive days; the intra-day repeatability (RSDi) and the inter-day reproducibility (RSDI) were lower than 4% for all AFs.

To evaluate the extraction efficiency of solvents (methanol and acetonitrile) on hazelnuts, AFs were spiked at 10 µg kg^{-1}. Preliminary tests with different combinations of solvents and water were investigated, by keeping them in contact with the samples for 120 minutes. As shown in Figure 2, the best extraction was obtained by using acetonitrile:water and methanol:water respectively at a ratio of 90:10 and 80:20. To determine the most efficient

extraction time, different extractions were tested by mixing the solution with solid sample for 30, 60 and 120 min. Methanol:water (80:20) at 120 min of mixing provided the best recovery (Table 2): at least 70% of each of the four AFs were extracted at the three levels of concentration tested. Moreover, Stroka et al. (1999) showed that aqueous acetonitrile extraction of AFs was unsuitable because it resulted in recoveries too high compared to aqueous methanol. The recovery percentages obtained at different spiking levels (Table 1) were higher for AFB_1 and AFB_2, and lower values for AFG_1 and AFG_2, as in previous studies (Campone et al. 2009; Ozay et al. 2007).

Sample analysis

Considering other monitoring of AFs in hazelnut products, the present study analysed the highest number of samples in Italy. Three other Italian studies, Bacaloni et al. (2008), Blesa et al. (2004) and Imperato et al. (2011) analysed respectively 38, 5 and 41 hazelnut samples. A study with the largest number of hazelnut samples (3,188) was recently carried out in Turkey (Baltaci et al., 2012).

AFB_1, AFB_2, AFG_1 and AFG_2 were simultaneously determined for 93 hazelnut samples commercialized in Italy (Table 3). Thirty-five samples (37.6%) resulted positive to one or more of the four aflatoxins considered. Out of the positive samples, the contamination level was lower than the LOQ in 19 samples, and it could be quantified in 16 samples. Generally, a low level of contamination was found in the positive samples (0.64 µg kg^{-1}). In two samples the level of AFs was higher than 1 µg kg^{-1}. The sample with the highest AF contamination (1.22 µg kg^{-1} of AFG_1) was an Italian one of dehulled raw hazelnuts. Not any sample contained AFs above the maximum admitted threshold established by the European legislation (Regulation 165/2010) in 10 µg kg^{-1}. The Regulation establishes also a maximum threshold specific for AFB_1 in 5 µg kg^{-1} and, among the samples analysed, the highest AFB_1

level was 0.31 µg kg^{-1}, 16 times lower than the threshold. In general, AFG_1 was the most widespread aflatoxin found in 19 samples, followed by AFB_1 (in 14 samples), AFG_2 (in 11 samples), and AFB_2 present in just 5 samples.

Bircan et al. (2008) found AFs in 2 (2.5%) out of 80 Turkish samples with total aflatoxins in the range 5.46 - 6.55 µg kg^{-1}; Ayacicek et al. (2005) in 43 (84.3%) out of 51 dehulled Turkish hazelnuts, ranging from <1 to 10 µg kg^{-1}; Bacaloni et al. (2008) in 6 (15.8%) out of 38 Italian samples; and Blesa et al. (2004) in 1 out of 5 Spanish hazelnut samples with 0.92 µg kg^{-1}.

The incidence and level of aflatoxins were related to the typology of food, the geographical origin and the cultivation system adopted (Table 3). The samples analysed were coming from the main countries cultivating hazelnuts, 78 from Italy, 9 from Turkey, 4 from the U.S.A. and 2 from Spain. The incidence of positive samples was higher in the Turkish samples (66.7%) compared to the Italian ones (35.9%) or the samples coming from the other countries (16.7%). The χ^2-test showed that the frequencies of AFs occurrence in the different countries were not comparable ($p=8x10^{-8}$). Also the mean AF contamination was statistically higher ($p=0.045$, Kruskal-Wallis test) in the Turkish samples (mean of all the samples: 0.33 µg kg^{-1}), compared to the Italian (0.14 µg kg^{-1}) or other samples (0.19 µg kg^{-1}). Hazelnuts produced in Turkey are more prone to be contaminated by aflatoxins (Baltaci et al., 2012), due to the climatic conditions of the country, that favours the occurrence and development of aflatoxigenic strains of *Aspergillus* (Kabak and Dobson 2006).

According to the typology of product, 42 samples were composed by dehulled and roasted whole hazelnuts, 32 by dehulled and raw whole hazelnuts, 8 by hazelnuts with shell, and 11 by hazelnut grain and flour. The highest incidence of positive samples to aflatoxins was found in grain and flour (45.5%), while the lowest incidence was found in raw dehulled hazelnuts. Anyway, the lowest level of AFs was found in the positive samples of hazelnut grain and flour (0.53 µg kg^{-1}). The magnitude between the means of AF levels in the typologies

considered was not statistically significant (p=0.399, Kruskal-Wallis test). Among the positive samples, the highest level of contamination was found in the hazelnuts with shell, due to the presence of one United States sample with 1.16 µg kg^{-1} of total AF (mainly AFG_1). Considering the agricultural system used to cultivate the hazelnuts, most samples were conventional (89) and just 4 samples were organic. The incidence of positive samples is higher in the organic samples (2 /4) compared to the conventional samples (37.1%), but the difference is not significant, due to the low number of organic samples considered in the current study.

In conclusion, in this paper, we developed and described a sensitive method to monitor the occurrence of AFs in the hazelnut samples and to get information even at very low levels of contamination. A large number of hazelnuts, purchased in Italy, has been analysed and the average level of AFs contamination founded was far lower than the level imposed by European regulation. However, AF contamination in hazelnuts could occur in ready-to-eat commercial products, so a continuous monitoring to ensure food safety is necessary.

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Table 1. Analytical method performance.

Analyte	Matrix	LOD (µg kg^{-1})	LOQ (µg kg^{-1})	Recovery range %	RSDr% (n=4)	RSDi (%)	RSDI (%)	Accreditation
B1	roasted dehulled halzenut	0.17	0.3	84.57-108.01	0.8-8.7	3	3.18	no
B2	roasted dehulled halzenut	0.17	0.2	83.98-109.89	1.2-9.2	3.3	3.41	no
G1	roasted dehulled halzenut	0.06	0.28	52.46-101.51	1.5-7.1	2.79	3.14	no
G2	roasted dehulled halzenut	0.35	0.44	54.35-99.37	1.7-9.1	2.83	3.49	no

Table 2. Recovery of AFs spiked at 1 ng g^{-1} into hazelnuts with different extraction times with methanol water (80 :20) as extraction solution.

Time (min)	AFB1 (%)	AFB2 (%)	AFG1 (%)	AFG2 (%)
30	50.15	45.24	30.05	7.27
60	54.49	52.56	33.4	13.38
120	93.69	87.36	71.15	64.05
180	91.69	86.81	72.81	62.98

Table 3. AFs levels in hazelnut samples from Italy and other countries.

Matrix	*n*	positive (%)		Sample distribution			Mean ± SD* (µg kg^{-1})
				<LOQ	LOQ-1	>1	
With shell	8	3	37.5	2	0	1	1.16±0.00
Raw dehulled	32	10	31.3	4	5	1	0.63±0.35
Roasted dehulled	42	17	40.5	10	7	0	0.62±0.11
Grain and flour	11	5	45.5	3	2	0	0.53±0.33
Italian	78	28	35.9	17	10	1	0.63±0.28
Turkish	9	6	66.7	2	4	0	0.57±0.06
Other countries	6	1	16.7	0	0	1	1.16±0.00
Conventional	89	33	37.1	17	14	2	0.64±0.27
Organic	4	2	50.0	2	0	0	0.00±0.00
Total	**93**	**35**	**37.6**	**19**	**14**	**2**	**0.64±0.27**

* The mean ± SD is calculated only on the positive samples higher than the LOQ.

Figure captions

Figure 1. LC/ESI-MS/MS chromatogram in Multiple Reaction Monitoring of AFs at 25 µg/l.

Figure 2. Effect of extracting solvent mixtures: A, acetonitrile : water, B, methanol: water on the efficiency of AFs extraction on hazelnuts spiked at 20 ng g^{-1}.