Aflatoxin monitoring in Italian hazelnut products by LC-MS

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

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28 **Abstract**

29 Hazelnut samples of different origin were collected in different stores of Northern Italy and

30 analysed for aflatoxin (AF) contamination by a sensitive chromatographic method based on a

31 tandem mass spectrometer with electrospray ionization. The effects of two extracting solvent

32 mixtures (methanol/water and acetonitrile/water) and different extraction times were tested

33 and compared in terms of recovery. The analysis showed that 35 out of 93 samples (37.6%)

34 were contaminated by AFs. The incidence of positive samples was higher in the Turkish

35 (66.7%) than in the Italian samples (35.9%). The mean AF contamination was higher

36 (p=0.045, Kruskal-Wallis test) in the Turkish samples (0.33 µg kg^{-1}), compared to the Italian

37 (0.14 µg kg^{-1}) ones. Generally, a low level of AFs contamination was found in the positive

38 samples (0.64 µg kg^{-1}), far below the maximum threshold admitted by the European

39 legislation. AFG$_1$ was the most diffused aflatoxin, found in 19 samples.

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

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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.

70 Hazelnuts are commercially produced in a relatively few countries, such as Turkey (73% of

71 the market), Italy (15%), the United States (4%) and Spain (3%) (HPG, 2005). Since

72 hazelnuts are components of many commercial products, such as baked products, ice cream,

73 and chocolate bars, it is necessary a sensitive and simple method to determine the presence

74 and the content of AFs in various commercial products to evaluate the risks associated with

75 their human consumption.

76 Several analytical methods have been used for AFs monitoring, such as thin-layer

77 chromatography (TLC) (Stroka et al. 2000; Trucksess et al. 1984), high-performance liquid

78 chromatography (HPLC) coupled to fluorescence or mass spectrometry detectors (Ghali et al.

79 2009, Huang et al. 2010) and immunological methods (Reddy et al. 2001, Saha et al. 2007).

80 TLC and immunological techniques provide rapid methods of screening for aflatoxins and

81 play an important role in developing countries for surveillance purposes and control of

82 regulatory limits (Gilbert and Anklam 2002). HPLC methods coupled with fluorescence

83 detection are sensitive and the most widely adopted, but often require pre- or post-column

84 derivatisation because of the weak native fluorescence of the analytes. On the other hand,

85 liquid chromatography coupled with mass spectrometry (LC-MS) or liquid chromatography

86 coupled with tandem mass spectrometry (LC-MS-MS) methods are specific and sensitive, and

87 their use is becoming increasingly widespread (Di Mavungu et al. 2009).

88 Extraction of AFs from various food matrices generally involves aqueous mixtures of polar

89 organic solvents such as methanol (Leong et al. 2010), acetone or acetonitrile (Khayoon et al.

90 2010), whereas extraction with chloroform (Alvito et al. 2010) has been abandoned in order to

91 reduce the use of chlorinated solvents. Immunoaffinity columns (Kumagai et al. 2008) and

92 solid-phase extraction (SPE) columns (Saha et al. 2007) have been used in clean up

93 procedures.

94 Previous reports on the occurrence of aflatoxins in Italy took in consideration few samples

95 coming from stores of Central/Southern Italy. In particular, Bacaloni et al. (2008) analysed 38

96 hazelnuts from Rome, and Campone et al. (2009) analysed an unspecified number of nuts

97 (almond, hazelnuts and peanuts) from Salerno.

98 Due to scarce data on aflatoxin content in Italian hazelnuts, the aim of this study was to

99 analyse a larger number of hazelnut samples collected in different stores of Northern Italy

100 using an efficient, very sensitive and simple method to extract, purify and analyse

101 simultaneously aflatoxins B_1, B_2, G_1 and G_2 by LC-MS-MS with electrospray ionization

102 (ESI).

103

104 **Materials and Methods**

105

106 **Chemical and reagents**

107 Standards of AFB_1, AFB_2, AFG_1 and AFG_2 were purchased from Sigma-Aldrich (St Louis,

108 MO, USA) and dissolved in methanol to prepare a working standard solution at the

109 concentration of 10 μg ml^{-1}. Aflatoxin M_1 (AFM_1) was used as an internal standard at 20 ng

110 ml^{-1}, because it is an hydroxylated metabolite of AFB_1 and it is not present in plant samples.

111 AFM_1 was purchased from Supelco (Bellefonte, PA, USA) and diluted in acetonitrile to make

112 a stock solution at the concentration of 0.5 μg ml^{-1}. These standard solutions were stored in

113 the dark at 4°C. LC-MS grade methanol, acetonitrile, acetic acid, 2-propanol and water, used

114 as mobile phases in the LC-MS and as extraction solutions were purchased from Sigma-

115 Aldrich. NaCl, KCl, Na_2HPO_4, KH_2PO_4 and Tween 20 (Merck, Darmstadt, Germany) were

116 dissolved in ultrapure water (Maina system, Turin, Italy) to prepare the phosphate buffer

117 saline (PBS) solution. AflaTest WB immunoaffinity columns were obtained from Vicam

118 (Watertown, MA, USA).

119

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

144 with 10 ml of water, before air drying. Aflatoxins were eluted with 3 ml methanol into an

145 amber glass vial. The eluate was evaporated at 37°C under air flow and 1 ml of eluent

146 (water:methanol, 90:10, with 0.1% of acetic acid and the internal standard AFM1 at 0.02 µg

147 ml^{-1}) was added to the residue and vortexed for 1 minute.

148

149 **LC-MS/MS conditions**

150 Liquid chromatography was performed with a Varian Model 212-LC micro pumps (Hansen

151 Way, CA, USA) coupled with a Varian autosampler Model 410 Prostar equipped with a 100

152 µl loop. The analytical column used for LC separation was a Pursuit XRs Ultra C18 (100 mm

153 x 2.0 mm, 2.8 µm particle size, Varian). The chromatographic conditions were: column

154 temperature: 30°C; mobile phase consisting of eluent A (water with 0.1% acetic acid) and

155 eluent B (methanol with 0.1% acetic acid), using a flow rate of 0.2 ml min^{-1}. A gradient

156 elution was applied as follows: 0-25 min: from 90% A to 15% A; 25-28 min: from 15% A to

157 90% A; 28-30 min: 90% A. The injection volume was 10 µl.

158 The LC system was coupled to a triple quadrupole mass spectrometer, Varian 310-MS, and

159 was operated in the positive electrospray ionization mode (ESI$^+$). The ionization source

160 conditions were: needle voltage of 6 kV, capillary voltage of 55-77 V, source temperature of

161 50 °C, desolvation temperature of 300°C, cone gas flow rate of 50 psi, desolvation gas flow

162 rate of 20 psi with nitrogen. Multiple reaction monitoring (MRM) mode of operation was

163 used. The [M+H]$^+$ ions of aflatoxins were used as parent ions. The most intense daughter

164 ions, resulting from collision-induced dissociation with argon, were used to detect and

165 quantify aflatoxin content. The argon pressure was set at 1.8 psi. The most intense daughter

166 ions detected were: m/z 284.9 at 14 eV of collision energy (CE) for B1, m/z 286.9 at 18 eV

167 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

168 CE for M1.

169

Method validation

171 Validation of the method was carried out according to harmonized guidelines for methods of

172 analysis in single-laboratory (Thompson et al. 2002). Precision was calculated in terms of intra-

173 day repeatability ($n = 10$) and inter-day reproducibility (3 different days) at 1 µg kg^{-1} spiking

174 level. The precision was calculated as relative standard deviation of replicate measurements.

175 Linearity was done by injecting triplicate AF standard solutions at different concentrations. In

176 this report, the limit of detection (LOD) for each aflatoxin was defined as three times the

177 electronic baseline noise and the limit of quantification (LOQ) as six times the level of

178 baseline noise. Recovery tests to evaluate the extraction efficacy were determined on roasted

179 hazelnut samples of 1000 g spiked with four concentrations of each AF (0.5, 5, 10, and 25 µm

180 kg^{-1}). Each test and the sample analysis were performed three times and experimental results

181 were reported as mean ± standard deviation. When AFs concentration had to be confirmed in

182 putatively positive samples, the standard addition approach was applied.

183

Statistical analysis

185 Normal distribution of toxin contents, means, standard errors and validation data were

186 analyzed with SPSS software (SPSS Institute, Inc, 2000, Version 18.0). The calibration

187 curves used for quantification was calculated by least-squares method. Samples with a

188 concentration of AFs higher than the LOD were considered positives, whereas samples with

189 concentrations lower than the LOD were considered negatives. Mean AFs concentrations

190 were calculated only on the positive samples higher than the LOQ. Experimental results are

191 reported as mean ± standard deviation. The Kruskal-Wallis test was used to compare the mean

192 AF levels among the different typologies (with shell, raw dehulled, roasted dehulled, grain

193 and flour), and geographical origins of the hazelnut samples, while the Mann-Whitney test

194 was used to compare the mean AF levels in conventional / organic hazelnut samples, using

195 the null hypothesis that the levels were not different.

196

197 **Results and Discussion**

198

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

200 The ESI positive ion mode resulted a common ionization procedure for aflatoxins

201 determination (Bacaloni et al., 2008; Imperato et al., 2011), and it was chosen to evaluate and

202 determinate the presence of AFs in the samples. The mobile phase was chosen based on the

203 ionization and separation efficiencies: besides methanol and water, 0.1% acetic acid was

204 added in the mobile phase, and AFs separation was obtained by varying the gradient

205 conditions (Figure 1).

206 Different concentrations of AFs, ranging from 0.1 to 50 ng ml^{-1}, were analysed to test the

207 linearity of the calibration curve. These curves were built by plotting the aflatoxins peak area

208 against the internal standard peak area at a concentration of 20 ng ml^{-1}. Table 1 shows the

209 analytical method performance: LOD, LOQ, recovery range, RSDr, RSDi and RSDI for each

210 AF, calculated in accordance with the European standards (EC Directive 2002/27).

211 Instrumental precision was assessed by analysing standard solution at 1 µg kg^{-1} 10 times a day

212 for 3 consecutive days; the intra-day repeatability (RSDi) and the inter-day reproducibility

213 (RSDI) were lower than 4% for all AFs.

214 To evaluate the extraction efficiency of solvents (methanol and acetonitrile) on hazelnuts,

215 AFs were spiked at 10 µg kg^{-1}. Preliminary tests with different combinations of solvents and

216 water were investigated, by keeping them in contact with the samples for 120 minutes. As

217 shown in Figure 2, the best extraction was obtained by using acetonitrile:water and

218 methanol:water respectively at a ratio of 90:10 and 80:20. To determine the most efficient

219 extraction time, different extractions were tested by mixing the solution with solid sample for

220 30, 60 and 120 min. Methanol:water (80:20) at 120 min of mixing provided the best recovery

221 (Table 2): at least 70% of each of the four AFs were extracted at the three levels of

222 concentration tested. Moreover, Stroka et al. (1999) showed that aqueous acetonitrile

223 extraction of AFs was unsuitable because it resulted in recoveries too high compared to

224 aqueous methanol. The recovery percentages obtained at different spiking levels (Table 1)

225 were higher for AFB_1 and AFB_2, and lower values for AFG_1 and AFG_2, as in previous studies

226 (Campone et al. 2009; Ozay et al. 2007).

227

228 **Sample analysis**

229 Considering other monitoring of AFs in hazelnut products, the present study analysed the

230 highest number of samples in Italy. Three other Italian studies, Bacaloni et al. (2008), Blesa et

231 al. (2004) and Imperato et al. (2011) analysed respectively 38, 5 and 41 hazelnut samples. A

232 study with the largest number of hazelnut samples (3,188) was recently carried out in Turkey

233 (Baltaci et al., 2012).

234 AFB_1, AFB_2, AFG_1 and AFG_2 were simultaneously determined for 93 hazelnut samples

235 commercialized in Italy (Table 3). Thirty-five samples (37.6%) resulted positive to one or

236 more of the four aflatoxins considered. Out of the positive samples, the contamination level

237 was lower than the LOQ in 19 samples, and it could be quantified in 16 samples. Generally, a

238 low level of contamination was found in the positive samples (0.64 µg kg^{-1}). In two samples

239 the level of AFs was higher than 1 µg kg^{-1}. The sample with the highest AF contamination

240 (1.22 µg kg^{-1} of AFG_1) was an Italian one of dehulled raw hazelnuts. Not any sample

241 contained AFs above the maximum admitted threshold established by the European

242 legislation (Regulation 165/2010) in 10 µg kg^{-1}. The Regulation establishes also a maximum

243 threshold specific for AFB_1 in 5 µg kg^{-1} and, among the samples analysed, the highest AFB_1

244   level was 0.31 µg kg^{-1}, 16 times lower than the threshold. In general, AFG$_1$ was the most

245   widespread aflatoxin found in 19 samples, followed by AFB$_1$ (in 14 samples), AFG$_2$ (in 11

246   samples), and AFB$_2$ present in just 5 samples.

247   Bircan et al. (2008) found AFs in 2 (2.5%) out of 80 Turkish samples with total aflatoxins in

248   the range 5.46 - 6.55 µg kg^{-1}; Ayacicek et al. (2005) in 43 (84.3%) out of 51 dehulled Turkish

249   hazelnuts, ranging from <1 to 10 µg kg^{-1}; Bacaloni et al. (2008) in 6 (15.8%) out of 38 Italian

250   samples; and Blesa et al. (2004) in 1 out of 5 Spanish hazelnut samples with 0.92 µg kg^{-1}.

251   The incidence and level of aflatoxins were related to the typology of food, the geographical

252   origin and the cultivation system adopted (Table 3). The samples analysed were coming from

253   the main countries cultivating hazelnuts, 78 from Italy, 9 from Turkey, 4 from the U.S.A. and

254   2 from Spain. The incidence of positive samples was higher in the Turkish samples (66.7%)

255   compared to the Italian ones (35.9%) or the samples coming from the other countries (16.7%).

256   The χ^2-test showed that the frequencies of AFs occurrence in the different countries were not

257   comparable (p=8x10^{-8}). Also the mean AF contamination was statistically higher (p=0.045,

258   Kruskal-Wallis test) in the Turkish samples (mean of all the samples: 0.33 µg kg^{-1}), compared

259   to the Italian (0.14 µg kg^{-1}) or other samples (0.19 µg kg^{-1}). Hazelnuts produced in Turkey are

260   more prone to be contaminated by aflatoxins (Baltaci et al., 2012), due to the climatic

261   conditions of the country, that favours the occurrence and development of aflatoxigenic

262   strains of *Aspergillus* (Kabak and Dobson 2006).

263   According to the typology of product, 42 samples were composed by dehulled and roasted

264   whole hazelnuts, 32 by dehulled and raw whole hazelnuts, 8 by hazelnuts with shell, and 11

265   by hazelnut grain and flour. The highest incidence of positive samples to aflatoxins was found

266   in grain and flour (45.5%), while the lowest incidence was found in raw dehulled hazelnuts.

267   Anyway, the lowest level of AFs was found in the positive samples of hazelnut grain and

268   flour (0.53 µg kg^{-1}). The magnitude between the means of AF levels in the typologies

269 considered was not statistically significant (p=0.399, Kruskal-Wallis test). Among the

270 positive samples, the highest level of contamination was found in the hazelnuts with shell, due

271 to the presence of one United States sample with 1.16 µg kg^{-1} of total AF (mainly AFG$_1$).

272 Considering the agricultural system used to cultivate the hazelnuts, most samples were

273 conventional (89) and just 4 samples were organic. The incidence of positive samples is

274 higher in the organic samples (2/4) compared to the conventional samples (37.1%), but the

275 difference is not significant, due to the low number of organic samples considered in the

276 current study.

277 In conclusion, in this paper, we developed and described a sensitive method to monitor the

278 occurrence of AFs in the hazelnut samples and to get information even at very low levels of

279 contamination. A large number of hazelnuts, purchased in Italy, has been analysed and the

280 average level of AFs contamination founded was far lower than the level imposed by

281 European regulation. However, AF contamination in hazelnuts could occur in ready-to-eat

282 commercial products, so a continuous monitoring to ensure food safety is necessary.

283

288

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370

371   **Table 1.** Analytical method performance.

Analyte	Matrix	LOD ($\mu g\ kg^{-1}$)	LOQ ($\mu g\ kg^{-1}$)	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

372

373

374 **Table 2.** Recovery of AFs spiked at 1 ng g^{-1} into hazelnuts with different extraction times

375 with methanol water (80 :20) as extraction solution.

376

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

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378

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

380

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**

381

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

383

384 **Figure captions**

385

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

387

388 **Figure 2.** Effect of extracting solvent mixtures: A, acetonitrile : water, B, methanol: water on

389 the efficiency of AFs extraction on hazelnuts spiked at 20 ng g^{-1}.