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Research Article

Analysis of aflatoxins in nonalcoholic beer using liquid–liquid extraction and ultraperformance LC-MS/MS

Aflatoxins AFB1, AFB2, AFG1, and AFG2 are toxic secondary metabolites produced by *Aspergillus flavus* and *Aspergillus parasiticus* and posses a potential threat to food safety. In the present work, liquid–liquid extraction and ultraperformance LC-MS/MS method has been applied for the determination of four naturally occurring aflatoxins AFB1, AFB2, AFG1, and AFG2 in nonalcoholic beer. Aflatoxins extraction from nonalcoholic beer was carried out using liquid–liquid extraction procedure. The effects of solvent-types were studied to obtain maximum recovery of the target analytes with minimum contamination. Among different solvents, the aflatoxins extraction was best achieved using ethyl acetate. The obtained recoveries were ranged from 85 to 96% with good quality parameters: LOD values between 0.001 and 0.003 ng/mL, linearity of the calibration curve ($r^2 > 0.999$), and repeatability (run-to-run) and reproducibility (day-to-day) precisions with RSDs lower than 5% ($n = 5$) achieved at 0.50 ng/mL concentration. The optimized liquid–liquid extraction in combination with ultraperformance LC-MS/MS was applied successfully to the analysis of AFB1, AFB2, AFG1, and AFG2 aflatoxins in 11 nonalcoholic beers and were detected up to 15.31 ng/L in some of the samples.

1 Introduction

Diet is the major way through which humans as well as animals are exposed to natural carcinogens. Aflatoxins AFB1, AFB2, AFG1, and AFG2 are one of the most potent toxic carcinogens that occur naturally. These are a group of closely related mycotoxins produced by species of Aspergilli, especially *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nomius* [1, 2]. These mycotoxins occur commonly in cereals, cereal products, nuts and spices, beer, dry fruits, and baby food [3–8]. The International Agency for Research on Cancer (IARC) has defined AFB1 as carcinogen, included in the Group 1, while other aflatoxins have been classified as possible carcinogens (Group 2B) [9]. In order to protect public health, recently the European Union food safety legislation on mycotoxins levels is becoming ever stricter with the latest regulations setting lower limits in certain foods [10]. A reliable risk assessment of mycotoxin contamination for humans and animals relies basically on their unambiguous identification

and accurate quantification in food and feedstuffs [11]. The occurrence of mycotoxins is unavoidable and its incidence in beer has been reported in various literature [5, 12–15].

At the present time, in order to accomplish highest concentrations set up by the European Commission many fast sensitive and reliable analytical methodologies are available. ELISA-based techniques have been used for rapid mycotoxins screening [16]. Recently, LC-MS/MS has turn into the most widespread approach for mycotoxin analysis in single run [12, 17–19]. Furthermore, the introduction of ultraperformance LC (UPLC) hyphenated with MS/MS, high-resolution MS (orbitrap MS) or TOF-MS technology offers fast analysis, improved peak resolution, low LODs and high sample throughput. A number of applications of UPLC hyphenated with MS/MS, orbitrap MS, or TOFMS techniques on mycotoxin analysis have been recently reported [20–23].

The analysis of mycotoxins is quite challenging for food researchers because they are present at very low concentrations in foodstuffs. Hence, an intensive clean-up or enrichment procedure is usually required to adequately determine mycotoxins at the low European Union established limit. At present, several multimycotoxin methods based on SPE procedure have been developed in different food commodities. The target analytes were extracted using ACN alone or a mixture of ACN and methanol [24, 25]. In addition, the different sorbents for mycotoxins clean up were studied to obtaining the best results with multifunctional cartridges using ACN aqueous solution as an extraction solvent [26]. Other authors

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Abbreviations: IAC, immunoaffinity columns; **UPLC**, ultraperformance LC

have also put forward the use of immunoaffinity columns (IAC) as they present high specificity and selectivity for selected analytes, removing matrix interferences [27–29]. IAC are generally designed for only one type of toxin, reducing the method multiresiduality. However, being an interesting substitute that decreases sample throughput and permits the simultaneous analysis of aflatoxins, this disadvantage could be resolved with the introduction of columns with particular antibodies for aflatoxins [30, 31]. In many reported works, authors have also evaded clean-up steps, injecting the crude extract, in order to enhance the multianalyte capacity of the technique [18, 32, 33].

The aim of this study was to analyze aflatoxins AFB1, AFB2, AFG1, and AFG2 in nonalcoholic beers using liquid–liquid extraction and UPLC-MS/MS techniques. To the best of our knowledge, this is the first report on the application of liquid–liquid extraction and UPLC–MS/MS method, which can be used to improve the quality of results in the determination of aflatoxins in beer samples.

2 Experimental

2.1 Chemicals and materials

All solvents used were of HPLC grade and were purchased from BDH Chemicals (Poole, England). Formic acid for mobile phase preparation was obtained from Panreac (Barcelona, Spain). Water was purified through a Milli-Q water purification system (Millipore, Bedford, MA, USA). Aflatoxins were purchased from FERMENTEK Chemical (Jerusalem, Israel). The chemical structure of the aflatoxins studied is shown in Fig. 1.

Individual stock standard solutions of the AFB1, AFB2, AFG1, and AFG2 at 1000 ng/mL were prepared in ACN and used for further dilutions. Standard mixtures of aflatoxins at different concentration levels (0.01–100 ng/mL) were prepared by weight for calibration curves. Standards and samples were filtered through a 0.22-μm PVDF syringe filter
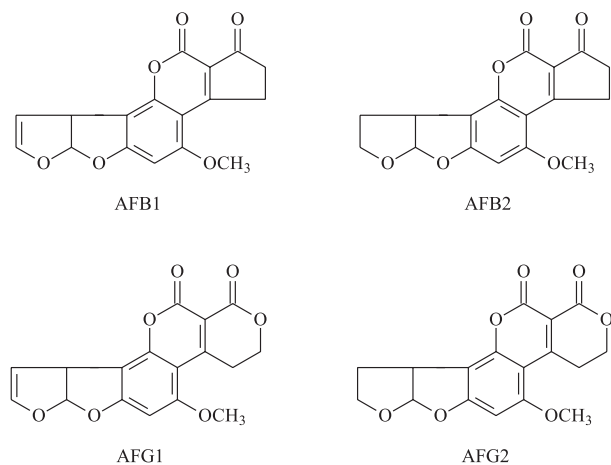
(Membrane Solutions, TX, USA) before being injected into the UPLC-MS system.

2.2 Instrumentation and MS conditions

The chromatographic separation of aflatoxins was carried out on a Waters acquity UPLC system, equipped with a quaternary pump system (Milford, MA, USA), using an acquity BEH C_{18} column (Waters, Milford) of dimension 100 mm $\times$ 2.1 mm id, and 1.7-μm particle size. The optimum separation was achieved with a binary mobile phase at flow rate of 0.3 mL/min. Solvent A: methanol; solvent B: water with formic acid (0.1%, v/v). The linear gradient elution program was as follows: 0–0.5 min 30% A, 0.5–6 min 30–50% A. The sample volume injected was 5 μL.

The UPLC system was coupled to a Quattro Premier triple quadrupole MS (Micromass, Milford, MA, USA) using the ESI source Z spray. The MS instrument was operated in the positive mode and the data were acquired in multiple reaction monitoring form using the protonated molecular ion of each compound as a precursor ion. The optimal ionization source parameters for monitoring aflatoxins were as follows: cone voltage, 55 V; capillary voltage, 3 kV; source temperature, 150°C; desolvation temperature, 300°C; cone gas flow rate, 70 L/h; desolvation gas flow rate, 700 L/h. Nitrogen (99.99% purity, Peak Scientific, model NM30LA nitrogen generator, Inchinann, UK) and high-purity argon (99.99%, Specialty Gas Centre, Jeddah, Saudi Arabia) were used as cone and collision gases, respectively. An Oerlikon rotary pump, model SOGEVAC SV40 BI (France) provided the primary vacuum to the MS. The multiple reaction monitoring transitions as well as the individual collision energy voltages applied for the analysis are summarized in Table 1. The most abundant product ion was monitored to quantify the aflatoxins, and the second most abundant product ion was monitored to confirm aflatoxin identification. Data acquisition and processing were carried out by MassLynx V4.1 software [34].

2.3 Extraction of aflatoxins from nonalcoholic beer samples

Eleven nonalcoholic beers with different flavors of different brands (Moussi classic, Moussi apple, Moussi strawberry, Moussi green tea, Barbican lemon, Barbican apple, Holsten classic, Holsten mango, Holsten lemon, Bario classic, and Rokers classic) were purchased from local market (Riyadh) for analysis of AFB1, AFB2, AFG1, and AFG2 contents. All samples were stored under the recommended refrigerated conditions until use. The aliquot of 5 mL beer sample was taken in a glass tube and degassed in ultrasonic bath for 10 min. The degassed beer sample was transferred to separating funnel by adding ethyl acetate (15 mL). The contents were vigorously shaken for approximately 10 min. The 5-mL aliquot of the supernatant was evaporated to dryness under nitrogen stream and the residue was reconstituted in

Figure 1. Chemical structures of aflatoxins used in this study.

Table 1. Data acquisition parameters of multiple reaction monitoring transitions for each aflatoxin used in triple quadrupole (QqQ) instrument[a)]

Aflatoxins	Retention time time (min)	Precursor ion $[M + H]^+$ (*m/z*)	Quantification transition		Confirmation transition	
			Product (*m/z*)	Collision energy (eV)	Product ion (*m/z*)	Collision energy (eV)
AFB1	3.67	313	285	42	241	50
AFB2	3.49	315	259	42	243	50
AFG1	3.31	329	243	36	311	46
AFG2	3.08	331	245	38	313	28

a) Dwell time was 0.1 s in all cases.

0.5 mL ACN. To avoid obstruction of the UPLC, microfiltration with 0.22-μm PVDF syringe filter was performed prior to injection.

2.4 Identification and quantification of aflatoxins

Quantification and recoveries estimation were carried out by the standard addition method, which comprised two non-spiked and four spiked samples at different levels; level I (50%, 5 ng/L), level II (100%, 10 ng/L), level III (200%, 20 ng/L), and level IV (400%, 40 ng/L). Recoveries were calculated from the slope of the linear regression obtained between the added analyte concentration and the measured analyte concentration. LOD and LOQ were determined by spiked samples based on S/N ratio of 3:1 for LOD and 10:1 for LOQ. To check the linearity of the method, calibration curves based on the peak area were constructed in the range of 0.01–100 ng/mL. To evaluate run-to-run precision, five replicate extractions were carried at same day. To assess day-to-day precision, 15 replicate extractions were performed over three consecutive days (five replicates each day).

3 Results and discussion

3.1 Optimization of liquid–liquid extraction solvent

As there are no previous investigations into the extraction of aflatoxins from nonalcoholic beer samples using liquid–liquid extraction and UPLC-MS/MS. To find the best possible liquid–liquid extraction solvent for aflatoxins extraction from beer samples, different organic solvents such as dichloromethane, ethyl acetate, methanol, ACN, and acetone were tested. The preliminary studies were carried out on standard solution of aflatoxins to find the optimum extraction solvent. Preliminary experiments showed that dichloromethane, methanol, ACN, acetone did not extract aflatoxins efficiently except ethyl acetate that yields recoveries up to 99% in some cases. Two solvent mixtures of different polarities such as dichloromethane/ethyl acetate (50:50, v/v) and dichloromethane/acetone (50:50, v/v)
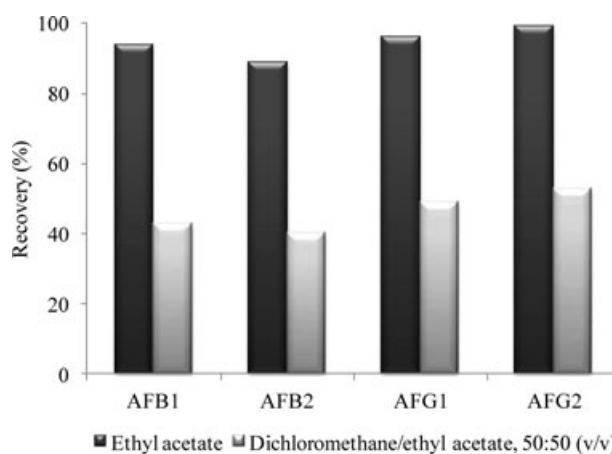

Figure 2. Effect of solvent on aflatoxins recovery rate.

were also investigated. The obtained results showed that the dichloromethane/acetone (50:50, v/v) did not extract aflatoxins, whereas lower recoveries up to 53% were achieved with dichloromethane/ethyl acetate (50:50, v/v). From all the solvent studied, the ethyl acetate alone yielded highest recoveries. Therefore, ethyl acetate was selected as the optimal extraction solvent. As can be seen in Fig. 2, ethyl acetate produced relatively high recoveries for all aflatoxins, ranging from 89 to 99%, whereas, lower recoveries were achieved with dichloromethane/ethyl acetate (50:50, v/v) between 40 and 53%.

The main benefits of the proposed liquid—liquid extraction method are the speediness, the application facility, and the low cost, since it needs a small quantity of organic solvent. This selected extraction method offers advantages in eliminating the extensive clean-up process of extracts prior to analysis, reducing the sample manipulation and total extraction time compared to the conventional techniques such as SPE or IAC.

3.2 Performance of the analytical method

In order to evaluate the performance of the analytical method, the quality parameters such as LOD, LOQ, linearity,

Table 2. Linear regression data, LOD, LOQ, and precision of aflatoxins by liquid–liquid and UPLC-MS/MS

Aflatoxins	Range (ng/mL)	Regression line		Correlation coefficients (r^2)	LOD[a] (ng/mL)	LOQ[b] (ng/mL)	Precision[c] (RSD %)	
		Slope	Intercept				run-to-run	day-to-day
AFB1	0.01–100	1.699	−7.02	0.999	0.003	0.010	2.5	3.7
AFB2	0.01–100	1.427	−6.10	0.999	0.003	0.010	3.2	4.9
AFG1	0.01–100	1.771	−11.84	1.000	0.001	0.004	1.9	2.5
AFG2	0.01–100	1.379	−15.60	0.999	0.002	0.007	2.3	3.4

a) S/N = 3.
b) S/N = 10.
c) *n* = 5.

repeatability (run-to-run precision), and reproducibility (day-to-day precision) were studied. The values of abovementioned quality parameters are presented in Table 2. For liquid–liquid extraction and UPLC-MS/MS, LOD ranged between 0.001 and 0.003 ng/mL and LOQ ranged between 0.004 and 0.010 ng/mL. These results are closer than those previously obtained using the IAC techniques [5, 13, 14].

Calibration curves based on the peak area were constructed. They were linear across the concentration range studied (0.01–100 ng/mL) and the correlation coefficients were higher than 0.999 for all the analytes. For run-to-run precision, five replicates of the extraction experiment were carried out at level 0.5 ng/mL aflatoxin mixture at same day. To assess day-to-day precision, 15 replicate extractions were performed with the same solutions over three consecutive days (five replicates each day). High repeatability and reproducibility was achieved for all analytes with RSD lower than 5% in all cases. This confirms that the method is successful in providing acceptable values of repeatability and reproducibility required for an accurate aflatoxins analysis. From these results, it can be concluded that liquid–liquid extraction in combination with UPLC-MS/MS can be used in the routine analysis of AFB1, AFB2, AFG1, and AFG2 aflatoxins in beer samples.

3.3 Application

3.3.1 Analysis of nonalcoholic beer samples

In order to evaluate the applicability of the liquid–liquid extraction method for the determination of aflatoxins in nonalcoholic beer samples, 11 nonalcoholic beer samples with different flavors of above-mentioned brands were analyzed. The samples were analyzed in triplicate using the standard addition quantification method two nonspiked and four spiked samples at different level (50, 100, 200 and 400%). Table 3 shows the aflatoxins amount and estimated recovery rates. The level of aflatoxins was relatively low in most products except classics ones. In classics samples, AFB1, AFB2, AFG1, and AFG2 were found at a high concentration between 4.62 and 15.31 ng/L (Moussi classic), 2.89 and 9.63 ng/L (Holsten classic), 2.91 and 4.25 ng/L (Bario classic), and 2.65 and 8.02

ng/L (Rokers classic), respectively. Figure 3 displays, as an example, the chromatograms obtained by the proposed liquid–liquid extraction and UPLC-MS/MS method for Moussi beer sample. The chromatograms did not show any interference, as no detectable matrix peak was eluted in the retention time of the AFB1, AFB2, AFG1, and AFG2 aflatoxins. The recoveries of studied compounds that were calculated from the regression slope of the added quantity versus the measured quantity were in the range of 85–96%, depending on the types of sample. These results are closer than those previously reported using the IAC techniques [5, 13, 14].

4 Concluding remarks

Aflatoxins AFB1, AFB2, AFG1, and AFG2 were determined at nanogram per liter levels in nonalcoholic beer by liquid–liquid extraction and UPLC-MS/MS. Different solvents have been tested out in order to get maximum recovery and minimize interferences with the aim of reaching the required selectivity and sensitivity in this type of analysis. Using ethyl acetate as an extracting solvent, the estimated recovery rates of analyzed aflatoxins in beer samples were ranging from 85 to 96%. The LOD was 0.003 ng/mL for aflatoxins AFB1 and AFB2 and 0.001–0.002 ng/mL for aflatoxins AFG1 and AFG2. Analysis of 11 imported beer samples were performed, the majority showed contamination of aflatoxins and were detected up to 15.31 ng/L. In addition, the proposed extraction technique in combination with UPLC-MS/MS offers advantages in eliminating the extensive SPE clean-up process of extracts prior to analysis. The results of the quality parameters achieved with this system, as well as the results obtained in the analysis of nonalcoholic beer samples, confirm that they are sufficient to propose liquid–liquid extraction coupled with UPLC-MS/MS as a new methodology for the fast, inexpensive, and reliable analysis of aflatoxins in beer samples.

The authors have declared no conflict of interest.

Table 3. Aflatoxins level in nonalcoholic beer samples ± SD (ng/L) and estimated recovery rates (%)

Sample	AFB1	Recovery	AFB2	Recovery	AFG1	Recovery	AFG2	Recovery
Moussi classic	4.62 ± 1.23	89	7.21 ± 1.92	91	12.23 ± 2.30	93	15.31 ± 2.01	96
Moussi apple	ND	92	ND	88	1.83 ± 0.50	91	0.89 ± 0.12	89
Moussi strawberry	ND	86	ND	87	<0.01[a)]	90	ND	90
Moussi green tea	ND	86	0.39 ± 0.08	85	0.86 ± 0.10	92	0.55 ± 0.09	89
Barbican lemon	0.25 ± 0.10	91	ND	93	0.10 ± 0.02	88	ND	90
Barbican apple	ND	91	1.38 ± 0.33	91	2.80 ± 0.73	91	1.53 ± 0.42	92
Holsten classic	3.64 ± 1.04	88	2.89 ± 0.79	92	7.94 ± 2.03	93	9.63 ± 2.02	89
Holsten mango	ND	85	0.20 ± 0.01	89	1.65 ± 0.50	90	2.01 ± 0.91	93
Holsten lemon	ND	90	1.02 ± 0.05	87	1.83 ± 0.63	94	0.90 ± 0.06	89
Bario classic	3.72 ± 1.03	87	3.98 ± 1.00	86	4.25 ± 1.11	95	2.91 ± 0.90	92
Rokers classic	2.65 ± 0.80	90	3.01 ± 1.10	94	8.02 ± 1.86	95	5.62 ± 1.24	94

a) Amount detected below the LOQ (S/N ratio of 10).
SD, Standard deviation obtained from addition standard calibration curve; ND, not detected.
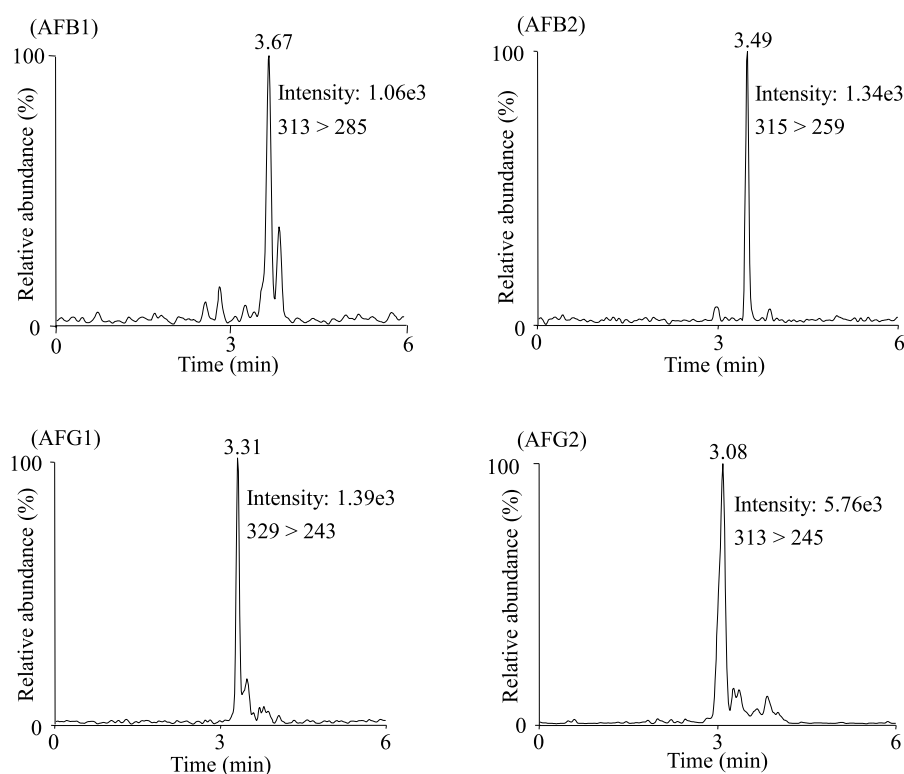
Figure 3. UPLC-MS/MS chromatograms of aflatoxins in Moussi beer sample.

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