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Analytical method for multimycotoxins in roasted coffee samples using liquid chromatography-tandem mass spectrometry

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Abstract

Mycotoxins are natural toxins that consist of secondary metabolites produced by fungal species of *Aspergillus*, *Fusarium*, and *Penicillium*. The present work aimed to validate the analytical method for detecting multimycotoxins (aflatoxin B_1, B_2, G_1, G_2, fumonisin B_1, B_2, ochratoxin A, and zearalenone) in roasted coffee samples using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Eight stable ^{13}C isotope-labelled internal standards were used for quantification, and an immunoaffinity column (IAC) was used for sample pre-treatment to eliminate interferences. Calibration curves showed good fitness (R^2 > 0.995) for all mycotoxins tested. The method detection limit (MDL) and method quantification limit (MQL) for eight mycotoxins were in the range of 0.002 - 0.2 and 0.005 - 0.5 ng/g, respectively. The recoveries ranged from 98.2 to 111% at three concentrations. The coefficients of variation (CVs) ranged from 1.2 to 14% intraday, and 1.4 to 13% interday. These results were within the acceptable range of the Codex Alimentarius Commission (CAC), thus indicating that the validated method could be suitable for multimycotoxin detection in roasted coffee samples.

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Introduction

Mycotoxins are natural toxins that are produced by various fungal species such as *Aspergillus*, *Fusarium*, and *Penicillium*, which are primarily generated in agricultural commodities under relatively humid and high temperature conditions (Yogendrarajah *et al*., 2013; Khayoon *et al*., 2014). These mycotoxins are classified into different group based on their chemical properties: aflatoxins (aflatoxins B_1, B_2, G_1, and G_2), fumonisins (fumonisins B_1 and B_2), ochratoxin A, zearalenone, *etc*. (Garcia-Moraleja *et al*., 2015a). The International Agency for Research on Cancer (IARC) classified aflatoxins as potential toxicants that are carcinogenic to humans (Group 1). Fumonisins and ochratoxin A are listed as probable human carcinogens (Group 2B), while zearalenone is classified in Group 3 because it is considered non-carcinogenic to humans (IARC, 2012). Different mycotoxins also have different known toxicological effects such as immunosuppressive, carcinogenicity, hepatoxicity, nephropathies, and oestrogenic effects (Koppen *et al*., 2010; Amezqueta *et al*., 2012; Afsah-Hejri *et al*., 2013; Da Rocha *et al*., 2014; Taroncher *et al*., 2021). These toxicities have been monitored and regulated to evaluate the risk exposure due to the negative effect of mycotoxins on humans and animals (Kim *et al*., 2017; Bessaire *et al*., 2019; Lee *et al*., 2019).

Coffee, one of the most widely consumed beverages in Korea, is known to have various bioactive components such as caffeine and phenolic

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compounds, and antioxidant activity (Vignoli *et al*., 2014; Oueslati *et al*., 2022). The consumption of coffee has increased rapidly in recent years as a favourite food. According to the International Coffee Organization (ICO), coffee consumption in Korea amounted to 2,758.12 tons in 2019 (ICO, 2021). Coffee beans could be contaminated by the growth of filamentous fungi under various conditions, including those encountered during manufacturing, harvest, transport, storage, and fermentation (Silva *et al*., 2008; Tolosa *et al*., 2021). The European Commission (EC) regulation has set the maximum limit of ochratoxin A as 5.0 ng/g for ground coffee bean and roasted coffee, and 10 ng/g in soluble coffee (EC, 2006). In Korea, the maximum limits for ochratoxin A (MFDS, 2021) set for roasted coffee bean and roasted coffee are 5 ng/g, and 10 ng/g for instant coffee; while those for other mycotoxins have not been established. Exposure to multiple mycotoxins through the dietary intake of roasted coffee could be a potential health concern in certain populations. Considering their deleterious health effects on humans, the development and validation of reliable methods for detecting multimycotoxins is urgently required to ensure the safety of roasted coffee. Due to the complexity of the coffee matrix which consists of carbohydrates, liquid, and proteins, a sample pre-treatment that reduces interferences is required to ensure the extraction yield and an accurate analysis.

Interferences with the complex matrix can cause problems in peak separation and MS detection. LC-MS/MS is regarded as a suitable analytical tool for detecting multimycotoxins due to its high selectivity and specificity. The disadvantage of LC-MS/MS is known as matrix effects, which are ion suppression and/or enhancement caused by co-eluting matrix compounds. In several studies (Fabregat-Cabello *et al*., 2015; Varga *et al*., 2012; Zhang *et al*., 2013; 2017; Rausch *et al*., 2021), stable isotope-labelled internal standards have been used to correct the matrix effect and extraction recoveries without matrix-matched calibration curves in various food matrices. There are some types of coffees such as roasted beans, instant coffee, and coffee beverages that contain various matrices. Therefore, if compound-matched internal standards are used, there is no need to make matrix-matched calibration curves for each sample. Additionally, appropriate sample pre-treatments can reduce problems such as matrix effects, signal interference, and instrument contamination from various components in food matrices (Zhang *et al*., 2019). Therefore, the LC-MS/MS method using internal standards and proper pre-treatments would yield accurate and reliable determinations of multimycotoxins in various coffee samples.

In previous studies, Vanesa and Ana (2013) published the simultaneous determination of multimycotoxins in roasted coffee beans and ground roasted coffee using solid phase extraction (SPE). Garcia-Moraleja *et al*. (2015a) developed a method based on solid phase extraction and liquid chromatography-tandem mass spectrometry to identify 21 mycotoxins in coffee beverages. QuEChERs (quick, easy, cheap, effective, rugged, and safe) were also used to achieve a satisfactory recovery of multimycotoxins (Carballo *et al*., 2018). However, most previous studies had a high detection limit for the simultaneous determination of multimycotoxins in coffee samples, or their methods were used for a specific matrix. The immunoaffinity column (IAC) has been considered the most effective purification technique for the targeting of mycotoxins in various food matrices due to its high specificity and efficiency, and low detection limit (Lattanzio *et al*., 2007; Vanesa and Ana, 2013; Ali *et al*., 2015; Kim *et al*., 2017; Gambacorta *et al*., 2018; Yang *et al*., 2020). The Ministry of Food and Drug Safety (MFDS) in Korea used an extraction of multimycotoxins (aflatoxin B_1, B_2, G_1, G_2, ochratoxin A, zearalenone, fumonisin B_1, and B_2) by 0.1% formic acid in 50% acetonitrile using Isolute Myco followed by LC-MS/MS for the analysis of plant-derived foodstuffs (MFDS, 2021). The European Committee for Standardisation (CEN) adopted an immunoaffinity column (IAC) as a clean-up for the simultaneous analysis of multimycotoxins using LC-MS/MS (Lattanzio *et al*., 2007). Therefore, it is useful to develop and validate a multimycotoxin determination method from roasted coffee samples using IAC and LC-MS/MS for risk assessment, which requires a low detection limit.

The present work aimed to validate an analytical method for the detection and determination of multimycotoxins in roasted coffee samples using an IAC and isotope-labelled internal standards in LC-MS/MS analysis. We determined multimycotoxins from different commercial coffee samples to confirm the reliability of the validated analytical method. To our knowledge, an analytical method using an IAC for sample clean-up and isotope-labelled internal

standards for each mycotoxin was applied and evaluated in roasted coffee samples for the first time in the present work.

Materials and methods

Chemicals and materials

Analytical standard solutions (aflatoxins B_1, B_2, G_1, and G_2, fumonisins B_1 and B_2, ochratoxin A, and zearalenone) and their isotopically labelled internal standards ($^{13}C_{17}$-aflatoxins B_1, B_2, G_1, and G_2, $^{13}C_{34}$-fumonisins B_1 and B_2, and $^{13}C_{20}$-zearalenone) were purchased from LGC Standards (Wesel, Germany). The internal standard of $^{13}C_{20}$-ochratoxin A was purchased from Sigma-Aldrich (St. Louis, MO, USA). For sample extraction, methanol, water, and acetonitrile were purchased from J. T. Bater (Center Valley, PA, USA). Phosphate-buffered saline (PBS) solution was purchased from Samchun Pure Chemical (Pyeongtaek, Korea). For the mobile phase, analytical MS grade methanol, water, and formic acid were purchased from Merck (Billerica, MA), and ammonium formate was purchased from Sigma-Aldrich. Immunoaffinity column (IAC, Myco6in1$^{®+}$) was purchased from Vicam (Watertown, MA, USA). The quality control (QC) material T17169QC (ochratoxin A in roasted coffee) was purchased from Fera Science Ltd. (York, United Kingdom), and we participated in the food chemistry proficiency test 17206 for ochratoxin A in roasted coffee served by the Food Analysis Performance Assessment Scheme (FAPAS) 2020 of Fera Science Ltd.

Stock and calibration solutions

Each stock solution was diluted with 50% acetonitrile to give 0.1, 1, and 10 µg/mL, and a working solution was prepared by mixing eight mycotoxin standards with 50% acetonitrile. The working solutions were prepared as follows: 0.003 µg/mL for aflatoxins B_1 and B_2; 0.018 µg/mL for aflatoxins G_1 and G_2; 0.12 µg/mL for fumonisins B_1 and B_2; 0.06 µg/mL for ochratoxin A; and 0.16 µg/mL for zearalenone. For calibration solutions, the working solutions were diluted with 50% acetonitrile. The mixture of internal standards was prepared to the following concentrations: $^{13}C_{17}$-aflatoxin B_1 and B_2 (0.02 µg/mL), aflatoxin G_1 and G_2 (0.08 µg/mL), $^{13}C_{34}$-fumonisin B_1 and B_2 (0.5 µg/mL), $^{13}C_{20}$-ochratoxin A (0.1 µg/mL), and $^{13}C_{18}$-zearalenone (0.2 µg/mL). For validation studies, working solution mixtures of eight standards and their isotopically labelled internal standards were spiked into each sample.

Samples

Two types of roasted coffee samples – roasted (11 brands) and instant (4 brands) products – were purchased from a local supermarket based on Korean market share in 2017 (KFIRI, 2018). Roasted coffee is roasted without food additives or with additional sugar to increase the aroma and flavour. Instant coffee is the soluble coffee extract obtained by dehydration or drying of roasted coffee bean with additional sugar or milk. Samples were stick-type packaged powder for consumer convenience. Roasted and instant coffee samples were prepared following the brand recommendations. Briefly, stick samples (2 g) were put in the beaker and mixed with 100 mL of hot water at approximately 97.8°C. After cooled to below 40°C, the coffee beverages were kept in a conical tube in the refrigerator. All samples were analysed in triplicate.

Extraction and clean-up

Analytical procedures for the extraction and clean-up of multimycotoxins in coffee were performed according to Gambacorta *et al*. (2018) with slight modifications. Briefly, 5 g of coffee sample was spiked with an internal standard mixture (50 µL) and then extracted with 35 mL of PBS by shaking for 60 min using a shaker (C-SK-6, Changshin-Lab, Seoul, Korea). After centrifugation (1736MGR; Gyrozen, Daejeon, Korea) at 10,000 *g* for 10 min at 4°C, 25 mL of PBS extract was collected (extract A). The residue (remaining solid containing 10 mL of PBS) was again extracted with 25 mL of methanol and centrifuged. Then, 3 mL of methanol/PBS extract was diluted with 27 mL of PBS (extract B). Aliquots of the two extracts (A and B) were separately loaded for clean-up through the immunoaffinity column (IAC) Myco6in1$^{®+}$. Next, 20 mL of extract B was passed through Myco6in1$^{®+}$ at 1 - 2 drops per second by gravity. The column was washed with 20 mL of PBS to eliminate methanol residues. Then, 3 mL of extract A was passed and eluted at 1 - 2 drops per second, after which the column was again washed with 10 mL of water to remove PBS and matrix interference material. Multimycotoxins were eluted from the column with 1.5 mL of methanol twice, and then 2 mL of water. The methanol eluate was concentrated under nitrogen gas at 50°C (EYELA MG-2200, Tokyo, Japan). The residue was reconstituted with 200 µL of 50%

methanol, transferred to a microcentrifuge tube, and centrifuged at 13,000 *g* for 10 min at 4°C (Smart R17, Hanil Science, Incheon, Korea) for LC-MS/MS analysis.

LC-MS/MS analysis

The eight mycotoxins were analysed using a liquid chromatograph (1290 Infinity II, Agilent Technologies, Ratingen, Germany) coupled with a QTRAP® 6500+ mass spectrometer equipped with an electrospray ionisation (ESI) interface source (AB Sciex, Singapore). An Acquity UPLC BEH C_{18} column (2.1 × 100 mm, 1.7 µm) purchased from Waters (Wexford, Ireland) was used for chromatographic separation, which was connected to a Security Guard™ ULTRA cartridge C_{18} (2.1 × 2 mm) from Phenomenex (Torrance, CA) and maintained at 40°C. The injection volume was 10 µL. The multimycotoxins were eluted using a mobile phase consisting of solvent A (5 mM ammonium formate, 0.1% formic acid in water) and solvent B (5 mM ammonium formate, 0.1% formic acid in methanol) at a flow rate of 300 µL/min. The mobile phase (B) was programmed as follows: 0 min, 40%; 6 min, 90%; 7 min, 90%; 7.5 min, 10%; 8.5 min, 10%; 8.7 min, 40%; and 10.5 min, 40%.

The ionisation source parameters were as follows: the ion spray voltages for the positive and negative modes were 4500 and -4500 V, respectively; the curtain gas was pressurised to 35 psi; the nebulising gas was pressurised to 60 psi; the heating gas was pressurised to 55 psi; the nitrogen collision gas was high; and the source temperature was 550°C. The time window of scheduled multiple reaction monitoring was 30 s, and the polarity switching time was 30 ms. The multiple reaction monitoring (MRM) transitions for quantification and qualification were monitored, and all MS/MS parameters for multiple mycotoxins are summarised in Table 1.

Table 1. Optimised MS/MS parameters for eight multimycotoxins and their internal standards.

Mycotoxin	Molecular weight	MRM transition[a]	DP[c] (eV)	CE[d] (eV)	CXP[e] (eV)
Aflatoxin B_1	312.90	312.9→285.2/241.3[b]	97/95	32/51	4/20
$^{13}C_{-17}$-aflatoxin B_1	330.08	330.1→301.1/255.2	16/16	35/53	12/18
Aflatoxin B_2	314.90	314.9→287.1/259.1	130/118	37/41	14/25
$^{13}C_{-17}$-aflatoxin B_2	332.10	332.1→303.3/273.0	160/132	33/40	3/31
Aflatoxin G_1	329.00	329.0→242.9/200.0	80/99	36/54	10/20
$^{13}C_{-17}$-aflatoxin G_1	346.20	346.2→257.1/212.0	116/70	34/57	4/52
Aflatoxin G_2	331.20	331.2→313.3/245.0	104/112	36/41	13/10
$^{13}C_{-17}$-aflatoxin G_2	348.10	348.1→259.2/330.0	130/104	40/30	4/21
Fumonisin B_1	722.50	722.5→334.3/352.3	110/126	50/55	18/16
$^{13}C_{-34}$-fumonisin B_1	756.50	756.5→374.5/356.6	22/104	51/59	17/8
Fumonisin B_2	706.40	706.4→336.1/318.2	154/154	52/54	38/38
$^{13}C_{-34}$-fumonisin B_2	740.50	740.5→358.2/340.3	136/92	55/55	31/16
Ochratoxin A	404.10	404.1→239.0/221.0	66/69	31/47	29/34
$^{13}C_{-20}$-ochratoxin A	424.10	424.1→250.1/110.1	54/66	22/101	15/16
Zearalenone	317.00	317.0→175.0/130.9	-116/-154	-33/-40	-26/-26
$^{13}C_{-18}$-zearalenone	335.10	335.1→185.1/140.1	-98/-109	-31/-38	-8/-14

[a]MRM transition was used or quantification; [b]MRM transition was used for qualification; [c]DP: declustering potential; [d]CE: collision energy; and [e]CXP: collision exit potential.

Method validation

Validation of the analytical method for multimycotoxins was performed based on the guidelines of the Codex Alimentarius Commission (CAC/GL 71-2009) (CAC, 2014). Method validation included selectivity, linearity, method detection limit (MDL), method quantification limit (MQL), matrix effect, accuracy (recovery), precision (intraday and interday repeatability), and proficiency tests. An instant coffee was chosen as a representative matrix for validation.

The linearity of the matrix-spiked calibration curve was made with six points of each mycotoxin at different concentrations. The ranges of the calibration

curve are shown in Table 2. The MDL and MQL were determined from the analysis of seven replicates of a 10-fold diluted working solution fortified into the coffee matrices. The MDL and MQL values were calculated as 3.3- and 10-fold the standard deviation (SD) in seven replicate experiments divided by the slope of the calibration curve, respectively. For accuracy, we evaluated two recoveries from the standard spiked samples and the QC sample ochratoxin A in roasted coffee (T17169QC; Fera Science Ltd, United Kingdom). Spike recovery was calculated as the percentage of each mycotoxin spiked in coffee samples, and QC recovery was calculated from the equation: (concentration of the experiment/assigned concentration) × 100%; then, they were compared with an acceptable level of the CAC guideline (CAC, 2014). For precision, the coefficient variation (CV) is the percentage of the SD divided by the mean. Precision (intraday and interday repeatability) was evaluated by the analysis of spiked coffee samples at three concentrations. The intraday and interday CVs were calculated from five analyses on a day, and five different days, respectively. The matrix effect was calculated as follows: (area of the post-spiked analyte in the coffee extract / area of the spiked analyte in the solvent) × 100 (Lee *et al*., 2019). A proficiency test was performed using the roasted coffee samples contributed by the FAPAS scheme October 2020 (No. 17206).

Table 2. Linearity, method detection limit (MDL), method quantification limit (MQL), and matrix effect of multimycotoxin analysis in instant coffee.

Mycotoxin	Calibration range (ng/g)	Linear regression equation	R^2	MDL (ng/g)	MQL (ng/g)	Matrix effect (%)
Aflatoxin B_1	0.005 - 0.15	$y = 0.9568x$	0.9986	0.002	0.005	80
Aflatoxin B_2	0.005 - 0.15	$y = 2.0996x$	0.9989	0.002	0.005	79
Aflatoxin G_1	0.04 - 0.9	$y = 1.2416x$	0.9951	0.01	0.04	20
Aflatoxin G_2	0.07 - 0.9	$y = 2.0586x$	0.9973	0.02	0.07	37
Fumonisin B_1	0.5 - 6	$y = 1.1650x$	0.9997	0.2	0.5	182
Fumonisin B_2	0.3 - 6	$y = 0.7173x$	0.9991	0.1	0.3	182
Ochratoxin A	0.09 - 3	$y = 2.2057x$	0.9997	0.03	0.09	76
Zearalenone	0.16 - 4	$y = 1.0726x$	0.9992	0.05	0.16	100

Results and discussion

Method validation

In the present work, we modified the IAC Myco6in1 method for multimycotoxins to optimise the extraction efficiency and clean-up procedure in a coffee matrix. The amount of sample and volume of extraction solvent were optimised. The following modifications were applied to the methods: decreased weight of sample from 10 to 5 g, decreased volume of PBS and methanol for extraction from 50 to 25 mL, and 2 mL of water was used after methanol elution to completely elute the fumonisins from the IAC. Samples were fortified with eight ^{13}C isotope-labelled mycotoxins as internal standards to correct the recoveries affected by the matrix.

The chromatograms of multimycotoxins are shown in Figure 1, which describes eight standards and their ^{13}C-labelled internal standards spiked in the roasted coffee sample. To evaluate selectivity, non-spiked and spiked samples were analysed following previously described methods, and the corresponding chromatograms were compared. All peaks for mycotoxins were separated from the interferences, thus indicating the good selectivity of this method. The linearity, MDL, and MQL are shown in Table 2. The linearity of the standard curves was determined by the determination coefficients (R^2), which were more than 0.997 for all mycotoxins in the coffee matrix. The MDL and MQL for eight mycotoxins ranged from 0.002 to 0.2 ng/g and 0.005 to 0.5 ng/g, respectively. Vanesa and Ana (2013) reported that the LOD and LOQ determined by IAC (Ochraprep®) in coffee beans, namely ground roasted coffee and soluble coffee were 0.02 and 0.05 ng/g for ochratoxin A, respectively. Garcia-Moraleja *et al*. (2015b) found that the MDL and MQL determined by ultra turrax extraction (UTE) in coffee beverages were 0.02 - 18.70 and 0.05 - 39.64 ng/g, respectively. Although UTE provided a faster extraction with less solvent

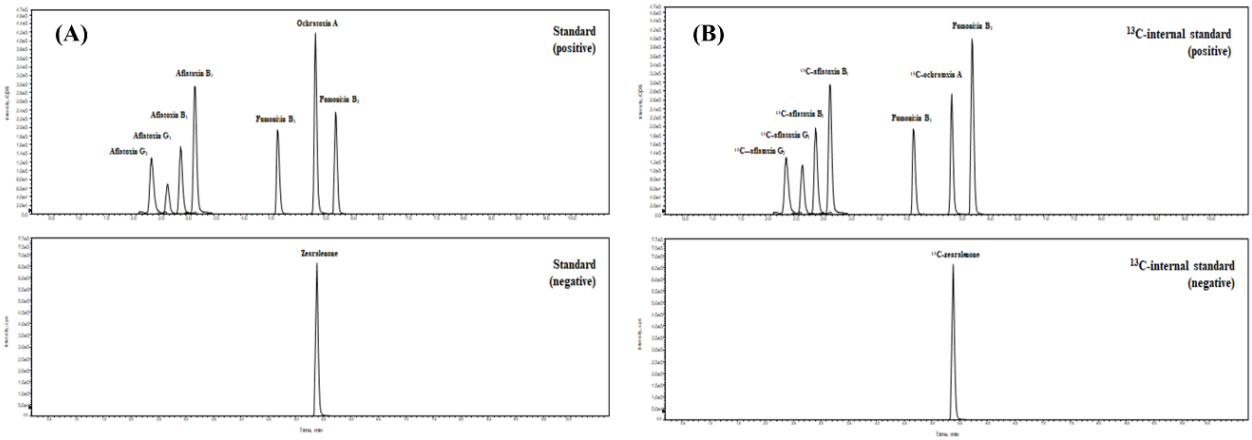

Figure 1. Extracted ion chromatograms of LC-MS/MS for multiple mycotoxins in coffee samples **(A)**, standards and 13C-balled internal standards **(B)**.

consumption than the SPE method, the LOD and LOQ were higher than this result. The matrix effect (Table 2) ranged from 20 to 80% (aflatoxin B_1, B_2, G_1, and G_2) and 182% (fumonisin B_1 and B_2). These results suggested that the coffee matrix appeared to be relatively complex, and required the use of internal standards to correct the matrix effect.

As shown in Table 3, the recoveries of all mycotoxins in the spiked coffee matrix ranged from 98.2 to 111%. The quality control (QC) sample (ochratoxin A in roasted coffee) was prepared and analysed in triplicate. The concentration (5.8 ng/g) was within the satisfactory range for $|z| \leq 2$ (ochratoxin A, 2.81 - 7.21 ng/g), and recovery calculated from the assigned value was 86.6%. Khayoon *et al*. (2014) reported aflatoxin recoveries ranging from 86.0 to 109.5% in coffee beverages. Nielsen *et al*. (2015) found that the recoveries of ochratoxin A and fumonisins in coffee using UPLC-MS/MS were 76 - 93% and 36 - 64%, respectively. The acceptable criteria of CAC (CAC/GL 71-2009) varied depending on the spiked levels, which were as follows: 60 - 120% for 1 - 10 ng/g, 70 - 120% for 10 - 100 ng/g, and 70 - 120% for 100 - 1,000 ng/g (CAC, 2014). The recoveries in the present work were within the acceptable range of CAC, thus indicating that the analytical method was reliable for the determination of multimycotoxins.

The precision data are expressed as CV%, and shown in Table 4. The CV values were acceptable in the range of 1.2 - 14% intraday and 1.4 - 13% interday. Garcia-Moraleja *et al*. (2015b) reported that the CVs were 4 - 12% intraday and 5 - 15% interday in coffee beverages. Codex guidelines for acceptable precision range (within-lab) are 30 - 35%. We participated in the proficiency test for instant coffee provided by FAPAS in 2020 (scheme No. 17206). Ochratoxin A was analysed in instant coffee using the validated method, and the z score was 0.2 within $|z| \leq 2$ (ochratoxin A, 10.9 ng/g). These results demonstrated that the validated analytical method had good reliability and repeatability in determining multiple mycotoxins in different coffee samples.

Method application to roasted and instant coffee

To evaluate the applicability of the multimycotoxin analysis method in different coffee samples, 15 commercial coffee samples from the Korean market (four roasted and 11 instant coffee samples) were analysed using the validated method. Six mycotoxins (aflatoxin B_1, B_2, G_1, and G_2, fumonisin B_1 and B_2) were not detected (ND) in any of the coffee samples. The concentrations of ochratoxin A ranged from ND to 0.08 ng/g in roasted coffee, and from ND to 2.23 ng/g in instant coffee. These values were lower than the established maximum limits of 10 ng/g in instant coffee, and 5 ng/g in roasted coffee (EC1881/2006). Vanesa and Ana (2013) detected ochratoxin A in instant coffee at concentrations ranging from 0.22 to 13.66 ng/g. Benites *et al*. (2017) detected ochratoxin A in roasted coffee at concentrations ranging from 1.56 to 32.40 ng/g. The concentrations of zearalenone in roasted and instant coffee ranged from 0.15 to 0.89 ng/g and ND to 0.46 ng/g, respectively. No specific maximum limit is established for zearalenone in coffee samples in countries outside of the European Union.

Table 3. Accuracy percentage of recoveries for instant coffee and roasted coffee samples.

Mycotoxin		Fortification (ng/g)	Instant coffee (mean ± SD)	Roasted coffee (mean ± SD)	Quality control[a] (ng/g)
Aflatoxin B_1	Conc. (ng/g)	0.375	0.385 ± 0.012	0.407 ± 0.017	-[b]
	Accuracy, %		103 ± 3.6	108 ± 4.5	-
Aflatoxin B_2	Conc. (ng/g)	0.375	0.376 ± 0.003	0.400 ± 0.015	-
	Accuracy, %		101 ± 0.8	107 ± 4.4	-
Aflatoxin G_1	Conc. (ng/g)	2.25	2.26 ± 0.03	2.38 ± 0.2	-
	Accuracy, %		100 ± 1.4	106 ± 7.6	-
Aflatoxin G_2	Conc. (ng/g)	2.25	2.22 ± 0.11	2.28 ± 0.3	-
	Accuracy, %		98.8 ± 4.9	102 ± 13.5	-
Fumonisin B_1	Conc. (ng/g)	15	14.7 ± 0.3	14.0 ± 0.8	-
	Accuracy, %		98.2 ± 2.4	98.7 ± 5.7	-
Fumonisin B_2	Conc. (ng/g)	15	14.8 ± 0.2	14.8 ± 0.7	-
	Accuracy, %		98.4 ± 1.0	98.7 ± 4.4	-
Ochratoxin A	Conc. (ng/g)	7.5	7.4 ± 0.14	7.8 ± 0.1	5.8 ± 0.4
	Accuracy, %		99.0 ± 1.9	103 ± 1.4	86.6 ± 5.6
Zearalenone	Conc. (ng/g)	10.0	10.3 ± 0.4	11.6 ± 0.2	-
	Accuracy, %		103 ± 4.4	111 ± 2.0	-

[a]Quality control (QC) sample was not fortified; [b]Not available.

Table 4. Precisions of the multiple mycotoxins in instant coffee at three concentrations.

Mycotoxin	Spiked conc. (ng/g)	CV (%, *n* = 5)	
		Intraday	Interday
Aflatoxin B_1	0.015	9.1	9.5
	0.075	3.4	3.5
	0.113	3.1	4.3
Aflatoxin B_2	0.015	14	13
	0.075	3.1	2.3
	0.113	1.2	2.7
Aflatoxin G_1	0.09	7.5	7.2
	0.45	5.7	7.8
	0.675	5.2	11
Aflatoxin G_2	0.09	10	11
	0.45	5.6	8.8
	0.675	6.9	9.0
Fumonisin B_1	0.6	3.0	3.0
	3	2.4	2.7
	4.5	1.2	4.6
Fumonisin B_2	0.6	3.5	1.9
	3	3.8	7.2
	4.5	3.3	3.3
Ochratoxin A	0.3	7.0	4.9
	1.5	1.8	1.8
	2.25	1.7	2.5
Zearalenone	0.4	4.3	4.1
	2	1.8	1.4
	3	2.5	3.1

Conclusion

The analytical method for multimycotoxin determination in roasted coffee samples was validated. The selectivity, linearity, MDL, MQL, recovery, and precision using LC-MS/MS after IAC clean-up showed that this method could be reliable and suitable for multimycotoxin determination from coffee matrices. Low detection limits and few interferences as compared to conventional solid phase extraction or other methods could make the risk assessment of mycotoxins from coffee intake accurate and reliable. When applying the validated method to analyse 15 commercial coffee samples, ochratoxin A and zearalenone were detected in some samples, but their levels were lower than the established maximum limits or previously reported concentrations. However, the number of coffee samples considered in the present work was insufficient; thus, additional studies involving larger sample sizes are required.

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