FOOD CHEMICAL CONTAMINANTS

# Method Validation for the Quantitative Analysis of Aflatoxins (B1, B2, G1, and G2) and Ochratoxin A in Processed Cereal-Based Foods by HPLC with Fluorescence Detection

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**Modified AOAC 991.31 and AOAC 2000.03 methods for the simultaneous determination of total aflatoxins (AFs), aflatoxin B1, and ochratoxin A (OTA) in processed cereal-based foods by RP-HPLC coupled with fluorescence detection were validated. A KOBRA® Cell derivatization system was used to analyze total AFs. One of the modifications was the extraction procedure of mycotoxins. Both AFs and OTA were extracted with methanol–water (75 + 25, v/v) and purified with an immunoaffinity column before HPLC analysis. The modified methods were validated by measuring the specificity, selectivity, linearity, sensitivity, accuracy, repeatability, reproducibility, recovery, LOD, and LOQ parameters. The validated methods were successfully applied for the simultaneous determination of mycotoxins in 81 processed cereal-based foods purchased in Turkey. These rapid, sensitive, simple, and validated methods are suitable for the simultaneous determination of AFs and OTA in the processed cereal-based foods.**

Mycotoxins of different chemical structures and modes of action are produced as secondary metabolites by various fungal species (1). There are more than 300 known mycotoxins classified as hepatotoxins, nephrotoxins, neurotoxins, and immunotoxins by clinicians, and as teratogens, mutagens, carcinogens, and allergens by cell biologists (1, 2). Common mycotoxins include aflatoxins, ochratoxin A, ergot alkaloids, fumonisins, patulin, trichothecenes, and zearalenone (3). Aflatoxins (AFs) have the most acute toxic effects in humans and carcinogenic effects in susceptible animals among all mycotoxins (4).

AFs, which are difuranocoumarin derivatives, are the toxic metabolites generated by the genus *Aspergillus* that include *A. flavus, A. parasiticus, A. nomius, A. tamari*, and *A. bombycis* (5, 6). The major AFs, B1 (AFB1), B2 (AFB2), G1 (AFG1), and G2 (AFG2), are mainly present in cereals, peanuts, corn, nuts, and cottonseeds, and the order of their toxicity is AFB1>AFG1>AFB2>AFG2. The International Agency for Research on Cancer (IARC) classified AFB1 as a human carcinogen (Group 1), and AFB2, AFG1, and AFG2 as possibly carcinogenic to humans (Group 2B). AFs also have toxigenic, mutagenic, and teratogenic effects (2).

Ochratoxin A (OTA), which is produced mainly by *A. ochraceus* and *Penicillium verrucosum*, has the most toxic effects among all ochratoxins. Because OTA biosynthesis requires complex fungi-substrate interactions, its production and accumulation are difficult under normal conditions (7). Scientific investigations indicate that food may be mainly contaminated with OTA during storage, and it is stable during most food processing stages (8–10). OTA has been commonly found in cereals and starch rich foods with spices, coffee, dried fruits, grapes, wines, beer, and meat (11). OTA can be nephrotoxic, hepatotoxic, teratogenic, mutagenic, and carcinogenic and can show fertility inhibition effects of an immunosuppressive nature in a variety of laboratory animals. It was considered to be responsible for a chronic kidney disease that had been observed in Balkans' people (Balkan Endemic Nephropathy). OTA was classified by the IARC as a possible carcinogen for humans (Group 2B; 12). Recent studies indicated that neurodegenerative diseases such as Parkinson's and Alzheimer's could be related to OTA (13, 14).

Foods can be contaminated with mycotoxins preharvest or postharvest, during processing or preparation, or in storage (12, 15). Cereals and processed cereal-based products represent a serious health risk for consumers because of their sensitivity against mycotoxin contamination (16). The European Union has established maximum legal limits as 4 µg/kg for total AFs, 2 µg/kg for AFB1, and 5 µg/kg for OTA in cereals (17). Numerous methods based on HPLC analysis with either precolumn or postcolumn derivatization have been developed for determination of AFs and OTA in cereals (18–21).

There are several methods for simultaneous determination of AFs and OTA. For example, EN 15851 and EN ISO 16050 methods specify an RP-HPLC method with immunoaffinity column cleanup and postcolumn derivatization for the determination of aflatoxins in cereals, nuts, and their derived products, and EN 15835 method specifies determination of OTA in cereal-based foods for infants and young children using HPLC with immunoaffinity column cleanup and fluorescence detection (FLD). Unlike these methods, the aim of the present work was to validate the modified AOAC **991.31** and AOAC **2000.03** methods for the simultaneous determinations of total AFs (B1, B2, G1, and G2), AFB1, and OTA by RP-HPLC-FLD in 81 processed cereal-based food samples from Turkey. Total AF analysis of the samples was carried out using a KOBRA® Cell (KOk BRomine Apparatus Cell) derivatization system.

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The validation procedure was applied according to Eurachem Guide (22). In this study, the AOAC **991.31** (23) and AOAC **2000.03** (24) methods that have been currently used for the simultaneous analyses of AFs and OTA in cereal samples, respectively, were modified to obtain high sensitivity and reduce time of analysis and consumption of solvents. In addition, the extraction procedure that was developed in this study was used to determine both AFs and OTA in the samples.

## Experimental

### *Materials and Reagents*

The analytical standards of total AFs (AFB1, AFB2, AFG1, and AFG2), OTA, and Aflaprep and Ochraprep immunoaffinity columns were purchased from R-Biopharm (Darmstadt, Germany). The catalog numbers of total AFs, OTA and Aflaprep and Ochraprep immunoaffinity columns are RBRP22, RBRP11, RBRP04, and RBRP14B, respectively. Total AF and OTA standards were produced and certified in accordance with ISO Guide 34:2009 and ISO/IEC 17025:2005). Potassium bromide (≥99 purity, 104907), HPLC grade acetonitrile (≥99.93% purity, 600030.2500), HPLC grade methanol (≥99.9% purity, 106007.2500), nitric acid (65% purity, 100456), and glacial acetic acid (100% purity, 100056) were purchased from Merck (Darmstadt, Germany). All eluents were filtered through 0.45 µm filters (Chromafil, Düren, Germany). Whatman No. 4 filters were purchased from GE Healthcare (Buckinghamshire, UK). An ultrasonic cleaner bath (24 × 14 × 10 cm) was purchased from Bandelin Sonorex (Berlin, Germany). Phosphate-buffered saline (PBS) was prepared by dissolving PBS tablets (79382, Sigma-Aldrich, Steinheim, Germany) in distilled water. Deionized distilled water was obtained from a Human water purification system (Seoul, South Korea).

### *Safety Precautions*

AFs and OTA are toxic substances; therefore, were always manipulated in solution, avoiding the formation of dust and aerosols. Nitrile gloves were used for all procedures.

### *Stock and Working Standard Preparation*

Stock solutions that contained 1000 ng/mL total AFs (AFB1, AFB2, AFG1, and AFG2) and 1000 ng/mL OTA were prepared from the purchased AF and OTA certified standards. The working standard solutions were prepared by diluting these stock solutions with methanol or methanol–water (60 + 40, v/v) to achieve different concentrations of mycotoxin mixtures. The calibration curve ranged from 0.08 to 10.00 ng/mL for total AFs and from 0.125 to 10.00 ng/mL for OTA. All prepared solutions were stored at –4°C and kept at room temperature in the dark for 30 min before their use.

### *Instrumentation*

A Shimadzu LC-20AT liquid chromatographic system (Shimadzu, Kyoto, Japan) equipped with a fluorescence detector (RF-20A), autosampler system (SIL-20A), pump (LC-20AT), and column oven (CTO-10AS) and controlled by Lab Solutions software was used. Separation was achieved on an RP C18 column Cronusil-S ODS2 (4.6 × 250 mm, 100 Å, and 5 µm particle size; Gloucester, UK).

To enhance the fluorescence activity of AFs, a KOBRA Cell electrochemical postcolumn derivatization system (R-Biopharm) was applied before the fluorescence detector. This derivatization method includes the addition of potassium bromide and nitric acid to the mobile phase. Once they reach the KOBRA cell, electrolysis occurs and bromine is released. Bromine reacts with AFB1 and gives derivatives that fluoresce in the RP solvents (25).

All samples were ground using a blender (Waring 8011, Stamford, CT).

### *Chromatographic Conditions*

Acetonitrile–methanol–water for AFs, and methanol–water–acetic acid for OTA were used as mobile phases to equilibrate RP HPLC columns before analyses. The wavelengths of excitation and emission were 365 and 435 nm for AFs, and 333 and 460 nm for OTA, respectively. The injection volume was 100 µL, and flow rate was 1.00 mL/min. Separation was achieved at 30°C under isocratic elution with the following mobile phases: acetonitrile–methanol–water (8 + 38 + 54, v/v/v) with 0.2 g/L potassium bromide, acidified with nitric acid (300 µL/L, 65%) for AF analysis and methanol–water–acetic acid (68.5 + 29 + 2.5, v/v/v) for OTA analysis.

### *Samples*

Eighty-one processed cereal-based foods consisting of wheat ($n$ = 12), bread ($n$ = 9), starch ($n$ = 9), semolina ($n$ = 5), cake flour ($n$ = 7), pasta ($n$ = 7), cake ($n$ = 8), biscuit ($n$ = 13), chips ($n$ = 5), and rusk ($n$ = 6) were purchased from different markets in Istanbul (Turkey) in February 2013. All samples were kept in suitable containers and stored at +4°C until initial sample preparation. In this study, one of the wheat samples not contaminated with mycotoxins was used as a blank sample for spiking.

### *Sample Preparation for Analysis*

Analyses of replicate spiked samples provided applicability and verification of the method. Blank wheat samples were spiked with a suitable amount of mycotoxins to achieve 6.0 and 8.0 µg/kg total AFs, and 3.0 and 8.0 µg/kg OTA. After 30 min at room temperature, the spiked samples were extracted, cleaned up, and analyzed using the modified AOAC **991.31** and AOAC **2000.03** methods. All samples were ground with a blender. Each ground and spiked sample (25 g) was extracted with 125 mL methanol (75%). After blending vigorously for 30 min, the extract was filtered through Whatman No. 1 filter paper. For AF analysis, 15 mL filtrate was diluted with 30 mL distilled water and 15 mL of the supernatant was passed through an immunoaffinity column without preconditioning. For OTA analysis, 5 mL of the filtrate was diluted with 40 mL distilled water and 45 mL of the supernatant was passed through an immunoaffinity column without preconditioning. After the sample passed, the column was washed with 10 mL distilled water. Then, the column was air-dried and AFs were eluted with 1 mL methanol and OTA was eluted with 1.5 mL of methanol–acetic acid (98 + 2, v/v). Finally, 1 mL and 1.5 mL distilled water were added for AFs and

**Table 1. Linearity and sensitivity data of AFs and OTA using the optimal HPLC conditions**

| Analytes | Range, µg/kg | Slope ± SD | Intercept ± SD | $R^2$ | In spiked wheat, µg/kg | | U, %[a] |
|---|---|---|---|---|---|---|---|
| | | | | | LOD | LOQ | |
| AFB1 | 0.02–2.50 | 1.9747e-006 | 0.00664189 ± 0.004 | 0.9995828 | 0.019 | 0.078 | 6.9 |
| AFB2 | 0.02–2.50 | 1.33944e-006 | 0.00393259 ± 0.005 | 0.9995975 | 0.016 | 0.064 | 6.2 |
| AFG1 | 0.02–2.50 | 3.30819e-006 | 0.0117839 ± 0.005 | 0.9997416 | 0.021 | 0.085 | 5.2 |
| AFG2 | 0.02–2.50 | 3.0326e-006 | 0.0107589 ± 0.006 | 0.9997093 | 0.021 | 0.086 | 8.0 |
| OTA | 0.25–10.00 | 1.23559e-005 | 0.0763268 ± 0.003 | 0.9997438 | 0.230 | 0.922 | 8.2 |

[a] U = Percentage relative uncertainty at the 95% confidence level (k = 2; k= coverage factor).

OTA, respectively, into a glass vial, and 100 µL of these eluates was directly injected for HPLC.

### *Modifications of AOAC **991.31** and AOAC **2000.03** Methods*

In this study, the AOAC **991.31** and AOAC **2000.03** methods were modified by changing sample preparation steps in the simultaneous determination of AFs and OTA. The same extraction process was used for both AF and OTA analysis. The modified AOAC **991.31** and AOAC **2000.03** methods consisted of a solvent mixture (methanol–water) for extraction, and an extract cleanup with an immunoaffinity column. This modification provided some advantages in the analysis, i.e., reduction of the extraction solvent volume, analysis time, and sample amount. In addition, the compositions of mobile phases, flow rates, and column temperatures were changed in these methods. All of the changes reduced the analysis time. The new mobile phases [acetonitrile–water–methanol (8 + 54 + 38, v/v/v) and methanol–water–acetic acid (68.5 + 29 + 2.5, v/v/v)] provided the best peak resolution in AF and OTA analysis, respectively. Potassium bromide was added into the mobile phase for derivatization of AFs with the KOBRA Cell. When the amount of potassium bromide (0.2 g/L) increased, AF peaks got sharper. Since the recovery values were found to be <70% in the spiked samples below 30 min, the optimum incubation time was determined as 30 min in this study.

### *Method Validation*

The modified AOAC **991.31** and AOAC **2000.03** methods were validated according to Eurachem guidelines. Validation of HPLC-FLD methods was based on the following criteria: specificity, selectivity, linearity, sensitivity, precision (intraday and interday, and analyst variability), accuracy, LOD, LOQ, and recovery.

(**a**) *Specificity.*—The retention times of standards were compared with those of the samples to indicate the specificity of the modified methods.

(**b**) *Selectivity.*—One of the wheat samples was spiked with total AFs, and OTA at different concentrations (8.0 and 5.0 µg/kg for total AFs, 8.0 and 3.0 µg/kg for OTA) to indicate the selectivity of the modified methods. After the spiked samples were analyzed by the modified methods, their recoveries were calculated.

(**c**) *Linearity.*—Calibration curves were used to determine linearity. For this purpose, working standard solutions of total AFs and OTA were prepared in three replicates at different concentrations of 0.08–10.0 ng/mL for total AFs, and 0.125–10.0 ng/mL for OTA. As a result of HPLC measurements, the peak area ratio of the mycotoxin versus the nominal concentration of the analyte was used to obtain the calibration standard of each concentration (Table 1). The linearity was evaluated by the correlation coefficient, *y*-intercept, and slope of the calibration curve. Additionally, the use of external standards allowed us to evaluate the originality of the method. Also, it provided removal of the interferences that come from matrix. An attempted validation of the method in wheat samples, in the concentration ranges 0.08–10.0 µg/kg for total AFs and 0.25–10.0 µg/kg for OTA, gave the data presented in Table 2, and the chromatographic parameters are given in Table 3.

(**d**) *Sensitivity.*—LOD and LOQ values were used to determine sensitivity of the methods. LOD was calculated by the concentration of the analyte that produced a peak whose height was 3x the height of the noise from a blank sample (S/N = 3). LOQ was the lowest concentration at which the analyte could not only be reliably detected but at which some predefined goals for bias and imprecision were met. LOQ was calculated by S/N = 10. Spiked sample that was used to calculate LOD and LOQ values contained a small amount of mycotoxin that could be detected but not to be quantified.

(**e**) *Precision*.—The precision of the modified methods was demonstrated as repeatability ($RSD_r$) and reproducibility ($RSD_R$). $RSD_r$ and $RSD_R$ (Horwitz values) were determined by analyzing duplicates of each spiked sample at two different levels (6.0 and 8.0 µg/kg for total AFs, and 3.0 and 8.0 µg/kg for OTA). Within-day repeatability was determined by duplicate determination on the same day by the same analyst. Between-day repeatability was evaluated by using the same method on 5 different days (Table 4). The spiked samples at the respective concentration levels were used in the methods.

(**f**) *Accuracy and repeatability.*—Accuracy and repeatability

**Table 2. Precision and accuracy for AF and OTA determinations with matrix-matched method for the calibration curves**

| Analytes | Nominal concn, µg/kg | Mean calculated concn, µg/kg | Accuracy, % | Precision, % |
|---|---|---|---|---|
| Total AFs | 0.08–10 | 0.082–10.87 | 102.5–108.7 | 101.98–110.54 |
| OTA | 0.25–10 | 0.26–9.98 | 104–99.8 | 97.6–100.02 |

**Table 3. Chromatography parameters of AFB1, AFB2, AFG1, AFG2, and OTA in spiked samples at 3 µg/kg**

| | AFB1 | AFB2 | AFG1 | AFG2 | OTA |
|---|---|---|---|---|---|
| Retention time ($t_R$), min | 11.84 | 10.26 | 8.31 | 7.30 | 6.77 |
| Tailing factor | 1.383 | 1.380 | 1.384 | 1.402 | 1.244 |
| Resolution ($R_s$) | 2.077 | 2.854 | 1.624 | 3.024 | 6.916 |
| Retention factor (k') | 3.095 | 2.549 | 1.875 | 1.527 | 0.794 |
| Number of theoretical plates (N) | 3578 | 3161 | 2750 | 2333 | 4558 |
| Peak width at half height ($W_h$) | 0.274 | 0.257 | 0.239 | 0.227 | 0.230 |
| HETP[a] | 25.256 | 30.543 | 35.444 | 42.658 | 32.909 |

[a] HETP = Height equivalent to a theoretical plate.

were determined by analyzing spiked samples at low and high levels of each calibration curve (8.0 and 5.0 µg/kg for total AFs, 8.0 and 3.0 µg/kg for OTA) in duplicate on 5 different days and by two different analysts. A 25 g portion of each sample was spiked with adequate volumes of stock and working standard solutions. They were extracted for 30 min with methanol–water (75 + 25, v/v). Recovery was determined from the peak areas of mycotoxins. The accuracy has been calculated as the SE of the mean of the data obtained during the precision study, and the repeatability was RSD (Table 5).

The calculated and expected concentrations (C) of the spiked sample were compared to determine the recovery values using following equation:

$$\text{Recovery, \%} = [C_{\text{spiked sample}}/C_{\text{expected}}] \times 100$$

### *Statistical Analysis*

Concentration levels and analysis results were expressed as the average of AFs and OTA values (µg/kg) ± SD. The precision parameters $RSD_r$, $RSD_R$ (Horwitz values) were calculated according to the International Union of Pure and Applied Chemistry/AOAC Harmonized Protocol using an Excel® template (26). Horwitz values were used to compare the between-analysts variability ($RSD_R$) at different levels. The difference of the mean of the sample and the most extreme data considering the SD were based on the Grubbs' test using Grubbs' critical value table. The statistical significance was set at the level of 95% ($P = 0.05$).

## Results and Discussion

### *Selectivity and Specificity*

AFB1, AFB2, AFG1, AFG2, and OTA showed good chromatography with an acceptable baseline and resolution of each mycotoxin (Figure 1). In the chromatograms, the AF and OTA peaks of blank and spiked samples were well separated from each other. There were no foreign peaks that interfered with analytes at the retention times of the AFs and OTA, which were 7.0, 7.9, 9.8, and 11.4 min for AFG1, AFG2, AFB1, and AFB2, respectively, and 6.7 min for OTA. The modified methods exhibited good selectivity and specificity.

### *Linearity and Sensitivity*

Three replicates of eight calibration samples were analyzed for each mycotoxin and range. Correlation coefficient of determination ($R^2$) was > 0.9995 for all calibration curves. The slope of the linear calibration curve was statistically different from 0 ($P$ = 95%), and the intercept was not statistically different from 0 ($P$ = 95%). LODs of the spiked samples were 0.019, 0.016, 0.021, 0.021, and 0.230 µg/kg for AFB1, AFB2, AFG1, AFG2, and OTA, respectively, and LOQs of the spiked samples were 0.078, 0.064, 0.085, 0.086, and 0.922 µg/kg for

**Table 4. Precision for AF and OTA determinations in the optimal HPLC conditions for spiked wheat**

| | | Spiked wheat | | | |
|---|---|---|---|---|---|
| | | Within-day ($n$ = 2) | | Between-day ($n$ = 10) | |
| Mycotoxins | Spiked concn, µg/kg | Recovery, % | RSD, % | Recovery, % | RSD, % |
| AFB1 | 1.25 | 84.16 | 6.20 | 105.32 | 1.03 |
| | 2.0 | 73.52 | 1.12 | 109.18 | 6.05 |
| AFB2 | 1.25 | 98.63 | 10.01 | 89.25 | 6.92 |
| | 2.0 | 97.27 | 9.43 | 98.42 | 4.04 |
| AFG1 | 1.25 | 73.36 | 7.56 | 111.14 | 6.18 |
| | 2.0 | 76.42 | 3.47 | 116.30 | 1.52 |
| AFG2 | 1.25 | 74.13 | 9.80 | 74.82 | 6.45 |
| | 2.0 | 85.17 | 6.43 | 82.76 | 9.40 |
| OTA | 3.0 | 100.30 | 1.85 | 104.13 | 12.00 |
| | 8.0 | 96.73 | 2.40 | 78.10 | 11.14 |

**Table 5. Accuracy for AF and OTA determinations in the optimal HPLC conditions for mycotoxin standard solution**

| | | Mycotoxin standard solution | | | |
|---|---|---|---|---|---|
| | | Within-day ($n$ = 2) | | Between-day ($n$ = 10) | |
| Mycotoxin | Spiked concn, µg/kg | Accuracy, % | RSD, % | Accuracy, % | RSD, % |
| AFB1 | 1.25 | 100.01 | 0.02 | 100.50 | 1.56 |
| | 2.0 | 99.67 | 0.50 | 108.42 | 1.67 |
| AFB2 | 1.25 | 101.24 | 0.45 | 100.24 | 2.34 |
| | 2.0 | 100.87 | 0.88 | 98.34 | 1.05 |
| AFG1 | 1.25 | 90.44 | 3.48 | 102.0 | 0.89 |
| | 2.0 | 93.26 | 4.20 | 106.52 | 2.45 |
| AFG2 | 1.25 | 87.48 | 2.56 | 94.45 | 2.56 |
| | 2.0 | 86.32 | 1.80 | 92.66 | 4.80 |
| OTA | 3.0 | 99.64 | 0.76 | 104.66 | 0.10 |
| | 8.0 | 97.80 | 1.00 | 98.10 | 1.44 |

AFB1, AFB2, AFG1, AFG2, and OTA, respectively. The $R^2$, LOD, and LOQ values are summarized in Table 1.

### *Accuracy and Precision*

The accuracy and precision of the methods were determined by analyzing duplicate samples of standards and spiked samples at various concentrations. The methods were applied on the same day and over 5 different days (Table 4). The precisions (RSD) were all less than 10% for AFs, and 15% for OTA.

Recoveries of all mycotoxins were determined by analysis of spiked wheat samples at two different concentrations. For total AFs the mean recovery at 8.0 and 5.0 µg/kg was 98%, and for OTA the mean recovery at 8.0 and 3.0 µg/kg was 91% (Table 5). The RSD for repeatability was found to be 1.03–9.34% for total AFs and 11.15–15.05% for OTA. The methods had acceptable within-laboratory and between-analyst precision for processed cereal-based products at two different levels.

### *Application of the Validated Methods to the Processed Cereal-Based Products*

In this work, the validated methods were successfully applied for the simultaneous determination of AF and OTA in the processed cereal-based products. AF and OTA levels were detected in 81 processed cereal-based products, and the results are exhibited in Table 6. Our results indicated that 31 (38.2%) of 81 analyzed samples were contaminated with AFs, and nine samples (11.1%) with OTA. Only pasta samples were found to be not contaminated with AFs or OTA.

### *Estimation of Uncertainty*

The Eurachem Guide report was used to evaluate and quantify the uncertainty sources of the applied methods (22). Purity of reference standards (pur), sample weights (w), sample volumes (vol), calibration curves (cal), repeatability (rep), and recoveries (rec) were used to calculate the uncertainties. Calibration curves and the purity of standards were defined as the main sources of uncertainty. The relative uncertainties were calculated at the 95% confidence level (k = 2). Calculated uncertainties are given in Table 1.

## Conclusions

In the present study, the rapid, reproducible, sensitive, and simple modified methods were validated for the simultaneous determinations of AFs and OTA in 81 processed cereal-based food samples. The modified AOAC **991.31** and AOAC **2000.03** methods consisted of a solvent mixture [methanol–water (75 + 25, v/v)] for extraction, and an extract cleanup with an immunoaffinity column in the sample preparation step. The LODs of the spiked samples were found to be 0.019, 0.016, 0.021, 0.021, and 0.230 µg/kg for AFB1, AFB2, AFG1, AFG2, and OTA, respectively, and their LOQ values were 0.078, 0.064, 0.085, 0.086, and 0.922 µg/kg for AFB1, AFB2, AFG1, AFG2, and OTA, respectively. The within-day and between-day accuracy study indicated 86.32–101.24 and 92.66–108.42%

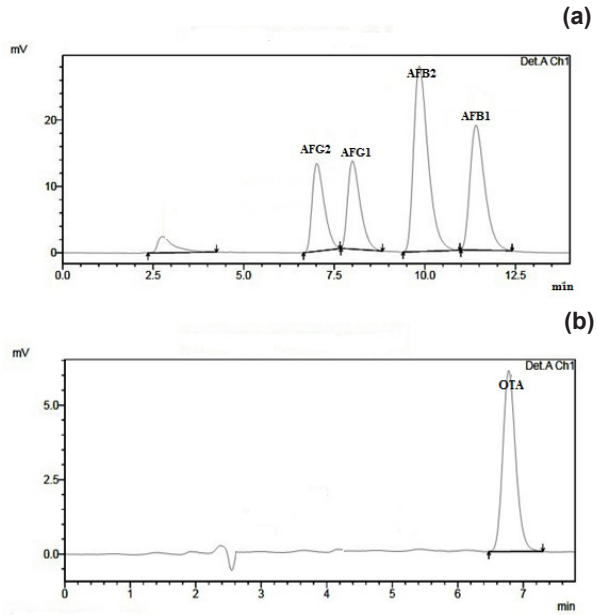


**Figure 1. Chromatograms of spiked wheat samples: (a) AFG2, AFG1, AFB2, and AFB1 and (b) OTA.**

**Table 6. Total AFs, AFB1, and OTA concentration ranges in processed cereal-based products**

| Sample groups | No. of analyzed samples | No. of contaminated samples by mycotoxin | | |
|---|---|---|---|---|
| | | Total aflatoxin | AFB1 | OTA |
| Wheat | 12 | 4 (0.033–3.606)[a] | 4 (0.016–3.250) | ND[b] |
| Bread | 9 | 2 (0.018–0.014) | 1 (0.018) | 3 (0.313–0.373) |
| Starch | 9 | 2 (0.116–0.191) | 2 (0.100–0.168) | ND |
| Semolina | 5 | 2 (0.056–0.132) | 2 (0.056–0.108) | 2 (0.361–0.409) |
| Cake flour | 7 | 4 (0.017–0.04) | 2 (0.017–0.027) | 1 (0.282) |
| Pasta | 7 | ND | ND | ND |
| Cake | 8 | 2 (0.018) | 2 (0.018) | ND |
| Biscuits | 13 | 10 (0.017–0.072) | 10 (0.017–0.072) | ND |
| Chips | 5 | 2 (0.052–0.137) | 2 (0.052–0.137) | ND |
| Rusk | 6 | 3 (0.017–0.041) | 3 (0.017–0.028) | 3 (0.380–1.157) |

[a] Values in parentheses represent the range of mycotoxin level (µg/kg).

[b] ND = Not detected.

**Table 7. Comparison of AOAC methods with the modified methods**

| | Aflatoxin analysis | | Ochratoxin A analysis | |
|---|---|---|---|---|
| | AOAC method | Modified method | AOAC method | Modified method |
| Extraction solvent | Methanol–water (70 + 30, v/v) | Methanol–water (75 + 25, v/v) | Acetonitrile–water (60 + 40, v/v) | Methanol–water (75 + 25, v/v) |
| Mobile phase | Water–methanol–acetonitrile (6 + 3 + 2, v/v/v) + 0.132 g KBr + 300 µL $HNO_3$ (for 1 L) | Water–methanol–acetonitrile (54 + 38 + 8, v/v/v) + 0.2 g KBr + 360 µL $HNO_3$ (for 1 L) | Acetonitrile–water–acetic acid (51 + 47 + 2, v/v/v) | Methanol–water–acetic acid (68.5 + 29 + 2.5, v/v/v) |
| Flow rate, mL/min | 1 | 1 | 1 | 1 |
| Column temperature, °C | 40 | 45 | 35 | 45 |
| Analysis time, min | 10 | 12 | 9 | 7.5 |

recoveries for AFS and OTA, respectively, in the spiked wheat samples, and the RSD values were 0.02–4.80%, respectively.

The modified methods were successfully applied to the processed cereal-based food samples purchased from different markets in Istanbul, Turkey. Among 81 processed cereal-based food samples, AFs were detected in 31 samples (38.2%) and OTA in nine samples (11.1%). The highest AF level was found in a wheat sample (3.606 µg/kg), the highest AFB1 level in a wheat sample (3.250 µg/kg), and the highest OTA level in a rusk sample (1.157 µg/kg), while no contamination was found in pasta samples.

The current AOAC and modified AOAC methods for the simultaneous determination of AFs and OTA in cereal-based products are compared in Table 7. Processes of these modified methods were economical because only one extract was prepared for both AF and OTA analyses, and their total analysis times were short. One of the most important advantages of these methods is their very low LOD and LOQ values. These modified methods are comparable in terms of sensitivity, linearity, and accuracy with the previous methods for the determination of AFs and OTA in the processed cereal-based products, and they are suitable for routine analyses of AFs and OTA in the processed cereal-based products and/or their official QC.

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## Conflict of Interest

Işıl Gazioğlu declares that she has no conflict of interest. Ufuk Kolak declares that she has no conflict of interest. This article does not contain any studies with human or animal subjects.

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