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Measuring the Level of Main Mycotoxins in Wheat Samples Collected from Flour Factories Silos in Alborz and Tehran Provinces Using LC-MS/MS

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ABSTRACT

Aim: Cereals and cereal-based products are prone to be infected by mycotoxin-producing fungi. The aim of this study was to investigate the level of contamination caused by 11 major mycotoxins in wheat samples collected from wheat silos in Tehran and Alborz provinces using UHPLC-MS/MS device.

Materials & Methods: Samples preparation was performed based on the extraction and purification procedures using acetonitrile/water/acetic acid solvents and Myco6in1 immunoaffinity columns, respectively. Selected mycotoxins were detected simultaneously using reversed phase ultra-high performance liquid chromatography coupled with tandem mass spectrometry (UHPLC-MS/MS) with electrospray ionization technique in positive-ion mode in a 15-minute run in the MRM program. Spiked samples calibration curve was used to overcome the matrix effects and to determine the residual mycotoxins.

Findings: Quantification and detection limits for AFB_1 and OTA mycotoxins were 2 and 0.7 ppb; for DON, FB_1, and FB_2 were 100 and 33.3 ppb; for ZER were 50 and 16.6 ppb: for AFB_2, AFG_1, AFG_2, and T-2 were 5 and 1.6 ppb; and for HT-2 were 20 and 6.6 ppb, respectively. Good precision and linearity was observed for mycotoxins. The average recovery rate of mycotoxins was in the range of 72-123 %, and the relative standard deviation (RSDr), indicating the method accuracy, was between 0.6-24.2 %. The validated method for analyzing the 30 wheat samples was used to evaluate the residual mycotoxins. OTA, T-2, and HT-2 mycotoxins were found in wheat samples. Only in one sample, the level of residual OTA exceeded the allowable limit set by the Iranian National Standards Organization.

Conclusion: The present study results highlighted the need for monitoring wheat and wheat-based products and the implementation of control and preventive measures in wheat fields, storage warehouses, and flour factories.

Keywords: Mycotoxins, Wheat, UHPLC-MS/MS, Myco6in1.

CITATION LINKS

[1] Bhat R, Rai RV, Karim AA. Mycotoxins ... [2] Cheng CT, Perak M. Mass poisoning tale of ... [3] Bryden WL. Mycotoxin contamination of the feed supply chain: Implications for animal productivity and ... [4] Marquardt RR. Effects of molds and their toxins on livestock performance: A western Canadian perspective. Anim Feed ... [5] Richard JL. Some major mycotoxins and their mycotoxicoses- An overview. Int J Food ... [6] Kim DH, Hong SY, Kang JW, Cho SM, Lee KR, An TK, et al. Simultaneous determination of ... [7] Vaclavikova M, MacMahon S, Zhang K, Begley TH. Application of single immunoaffinity clean-up for ... [8] Park J, Kim DH, Moon JY, An JA, Kim YW, Chung SH, et al. Distribution analysis of ... [9] Sarrafian MR. Country report: Iran. Thailand, Chiang Rai: Fourth session of the technical ... [10] Reddy CS. Mycotoxin ... [11] Zamani-Zadeh HR, Khoursandi H. Occurrence of ... [12] Sulyok M, Berthiller F, Krska R, Schuhmacher R. Development and... [13] Rasmussen RR, Storm IMLD, Rasmussen PH, Smedsgaard J, Nielsen K F. Multi-mycotoxin analysis of ... [14] Sorensen LK, Elbaek TH. Determination... [15] Frenich AG, Vidal JLM, Romero-Gonzalez R, Aguilera-Luiz MM. Simple and ... [16] Razzazi-Fazeli E. A review of recent trends in applications of liquid chromatography-mass spectrometry for determination of mycotoxins. J Liq Chromatogr ... [17] Di Mavungu JD, Monbaliu S, Scippo ML, Maghuin Rogister G, Schneider YJ, Larondelle Y, et al. LC-MS/MS multi-analyte method for ... [18] Monbaliu S, Van Poucke C, van Peteghem C, Van Poucke K, Heungens K, de Saeger S. Development of ... [19] Boyd RK, Basic C, Bethem RA. Trace ... [20] Ediage EN, Di Mavungu JD, Monbaliu S, Van Peteghem C, De Saeger S. A validated multianalyte LC–MS/MS method for quantification of 25 mycotoxins in cassava flour, peanut cake and ... [21] Ediage EN, Van Poucke C, De Saeger S. A multi-analyte... [22] Commission Regulation ... [23] Soleimany F, Jinap S, Abas FJ. Determination of... [24] Kumar R, Ansari KM, Saxena N, Dwivedi PD, Jain SK, Das M. Detection ... [25] Zhou Q, Li F, Chen L, Jiang D. Quantitative... [26] Hussein HS, Brasel JM. Toxicity, metabolism, and ... [27] Yazdanpanah H, Miraglia M, Calfapietra FR, Brera C. Natural... [28] Zinedine A, Brera C, Elakhdari S, Catano C, Debegnach F, Angelinib S, et al. ...

Introduction

Mycotoxins are secondary metabolites often produced by filamentous fungi of the genera *Aspergillus*, *Fusarium,* and *Penicillium* and other genera such as *Claviceps* and *Alternaria* [1]. Nowadays, it has been well established that fungi toxic metabolites or mycotoxins are responsible for many epidemics in human and livestock communities, especially in recent years [2]. Mycotoxins could infect cereal plants and cereal-based products during the plant growth, harvest, or improper storage through environmental factors [3-4]. Among the *Fusarium* produced mycotoxins, deoxynivalenol (as the main contaminant of wheat, corn, barley, oats, and rye), T-2 toxin (contaminant of wheat, barley, and oats), and zearalenone (contaminant of corn and wheat) and among the *Aspergillus* produced mycotoxins, aflatoxins (AFs) (contaminant of corn and wheat) are the most important mycotoxins contaminating cereals and cereal-related products. Exposure of humans and animals to these natural contaminants leads to acute and chronic diseases, and in some cases even death [5].

In a study by Kim et al. (2017), conducted on 5 types of cereals (brown rice, corn, cluster corn, millet, and breakfast cereals), 13 types of mycotoxins [Deoxynivalenol (DON), Nivalenol (NIV), 3-acetyl nivalenol, Aflatoxin B_1 (AFB_1), Aflatoxin B_2 (AFB_2), Aflatoxin G_1 (AFG_1), Aflatoxin G_2 (AFG_2), Fumonisin B_1 (FB_1), Fumonisin B_2 (FB_2), Zearalenone (ZEN), T-2 toxin (T-2), HT-2 toxin (HT-2), and Ochratoxin A (OTA)] were detected and measured simultaneously using LC/MS/MS after a purification step using immunoaffinity columns [6]. In another study, to simultaneously analyze 12 types of mycotoxins (AFs, FBs, ZEN, DON, OTA, T-2, and HT-2) in corn, barley, peanuts, and corn-based breakfast cereals, an immunoaffinity column was used for purification of samples, then UHPLC–MS/MS technique was applied for analysis; the immunoaffinity column used in this study contained antibodies against all the mentioned mycotoxins. Their study results showed that using this method not only reduced the preparation time and the volume of solvents used but also reduced the analysis time to 10 minutes. In their study, method trueness was examined using spiked samples and certified reference materials (CRM); the recovery rate of all mycotoxins was in the range of 71-112 % [7]. In another study by Park et al. (2018), conducted to investigate the amount of 12 mycotoxins (DON, NIV, AFB_1, AFB_2, AFG_1, AFG_2, FB_1, FB_2, ZEN, T-2, HT-2, and OTA) in corn and corn-based foodstuffs in South Korea, purification was performed by immunoaffinity columns, and analysis was done by LC-MS/MS device. The results showed that the use of this method, compared to the old methods of purification, significantly improved the recovery rate; however, no significant effect was observed on the limit of detection (LOD) and limit of quantification (LOQ) [8].

According to the World Food and Agriculture Organization, about 20% of food sources produced in each year in the world are contaminated with fungal toxins. The normal fungal flora in human food sources mainly includes the three genera *Aspergillus*, *Fusarium,* and *Penicillium*. These fungi are able to produce mycotoxin on grains before or immediately after harvest. These toxins could be formed during the grains growth, maturation, storage, or transfer. Due to the fact that mycotoxicogenic fungi are usually able to produce more than one mycotoxin and that agricultural products are prone to be infected by several types of fungi at the same time, the study of each mycotoxin contamination alone provides incomplete information regarding their risk assessment in food [9-11]. Mycotoxin contamination of wheat as a result of bioaccumulation may

cause large amounts of toxic substances to be transmitted through the food chain eventually to humans.

Objectives: This study was designed to investigate the simultaneous contamination of wheat samples, collected from flour factories silos in Tehran and Alborz provinces, with 11 major mycotoxins, for which the European Union has set a limit (AFB_1, AFB_2, AFG_1, AFG_2, FB_1, FB_2, ZEN, DON, OTA, T-2, and HT-2).

Materials and Methods

Chemicals and solvents: All chemical solvents purchased from Merck Company (Merck, Germany) had laboratory grade (HPLC grade). All mycotoxins standards were purchased from Sigma Company, except for T-2 and HT-2 toxins, which were prepared from LGC. The Myco6in1 immunoaffinity column was prepared from Vicam. PBS solution was made in the laboratory and adjusted at pH = 7.4.

Samples: Wheat samples were collected from the warehouses of flour factories in Tehran and Alborz provinces during February to June 2019. Among the flour factories of Tehran and Alborz provinces, 6 factories were selected randomly. Of each factory, 5 wheat samples were taken on different months and transferred to the laboratory. Totally, 30 samples were examined for the presence and amount of mycotoxins.

Preparation of the standards:

The stock standard solution of all mycotoxins at a concentration of 200 µg/mL was made from the reference standard material in methanol, acetonitrile, and water solvents. All the standards were maintained at -20 °C, except for fumonisins (4 °C). Then to optimize the MS parameters for each compound, the tests were operated by direct injection of standard solutions at 1µg/mL concentration in methanol: water (50:50 v/v) solvent containing 5mM ammonium format+0.1% formic acid into the mass spectrometer. Mixed standard solution was prepared from the mycotoxins under study using methanol solvent. This standard was used to prepare working standards in order to draw spiked samples calibration curve.

UHPLC–MS/MS equipment: Dionex 3000 ultra-high performance liquid chromatography machine coupled with Applied Biosystems API 3200 tandem mass spectrometry device, with electrospray ionization technique and triple quadrupole mass analyzers along with the Ultimate 3000 Autosampler (LC-MS/MS) was purchased from Dionex American company. Kinetex 2.6 µm XB-C18 100 mm × 2.1 mm i.d. chromatography column was applied to separate mycotoxins through the soluble mobile phase of Solvent A (0.1% formic acid with 10 mM ammonium formate) and Solvent B (0.1% formic acid with 10 mM ammonium formate in methanol). The liquid chromatography gradient program is shown in Table 1.

Samples extraction and purification: About 5 g of homogenized wheat flour sample was weighed and transferred to a 50 mL falcon tube. Then 20 mL of acetonitrile (79.5%): water (20%): acetic acid (0.5%) solvent was added to the samples. The samples were placed on an orbital shaker and mixed at 250 rpm for 60 min. After mixing, the samples were centrifuged at 5000 rpm for 2 min at -5 °C. Then 2 mL of the supernatant was removed and transferred to a 15 mL falcon tube and dried under nitrogen gas. Then 10 mL of PBS solution was added to the dried samples and vortexed for 3 min. The resuspended extract solution containing the analytes was passed over the Myco6in1 columns with a flow rate of one drop per second and rinsed with 10 mL of Myco6in1 columns deionized water. The analytes were collected from Myco6in1 columns with 3 mL

Table 1) Gradient program of the LC system.

Step	Time (min)	Solvent A (%)	Solvent B (%)	Fluorite (min/mL)
1	0	95	5	0.3
2	2	60	40	0.3
3	10	0	100	0.3
4	11.5	0	100	0.3
5	12	95	5	0.3
6	15	95	5	0.3

methanol in two steps (1.5 mL in each step). Finally, the analytes were transferred into a 5 mL vial. The collected extract was dried under nitrogen gas at 50 °C. Then 1 mL of acetonitrile: water (1:1) solvent containing 0.1% acetic acid was added to the dried extract and vortexed for 3 min. The resulting solution was filtered using 0.45 µm PTFE syringe filters. Finally, 20 µL of the diluted extract was injected into the UHPLC-MS/MS device.

Method validation: The validity of the method was examined based on the parameters such as linearity, accuracy, precision, and specificity as well as determination of the LOD, LOQ, and matrix effect. In order to overcome the matrix effect, the calibration curve was drawn using spiked samples. For this purpose, in 7 falcon tubes, different concentrations of AFs, T-2 toxin, and OTA (2, 5, 10, 20, 50, 100, 150 ppb) as well as FBs, DON, ZEN, and HT-2 (20, 50, 100, 200, 500, 1000, 1500 ppb) were poured on 5 g of homogenized wheat flour without contamination (blank), and the extraction and purification stages were performed according to the extraction method described in Part 2. 5. This test was repeated in 3 different working days. Finally, the calibration curve was calculated and plotted using the mean of 3 points by specific software for LC-MS/MS device, called Analyst, based on the following formula: the area under the analyte peak versus the analyte concentration. To investigate the recovery rate and the method precision and accuracy, wheat blank samples were spiked at three levels of 3, 15, 75 ppb for AFB_1, AFB_2, T-2, and OTA; 15, 75, 120 ppb for AFG_1 and AFG_2; and 150, 750, 1200 ppb for FBs, DON, ZEN, and HT-2 while spiking three samples at each level (9 samples in total). Then extraction was performed according to the method described in Part 2. 5. The samples were analyzed, and the recovery rate was determined using the calibration curve. This test was repeated in 3 working days, and the average recovery rate and RSDr (%) were determined to evaluate the accuracy and precision of the method used. In this study, Excel 2016 and Analyst software were used for statistical analysis. Analyst software is the single LC-MS/MS software that is used for data acquisition and data processing.

Findings

The obtained results showed that the calibration curves of all the desired compounds were in the ranges of 2-150 ppb for AFB_1, AFB_2, and OTA; 5-150 ppb for AFG_1,

AFG_2, and T-2; 20-1500 ppb for HT-2; 50-1500 ppb for ZER linearity; and 100-1500 ppb for FB_1, FB_2, and DON. Considering three decimal places, the correlation coefficient (R^2) was calculated as greater than 0.990. As shown in Table 2, the accuracy (recovery rate) and precision (repeatability) of the points calculated by the calibration curve were in the ranges of 93.1-105.4 and 0.8-26 %, respectively. Limits of quantification and detection of mycotoxins were in the ranges of 2-100 and 0.7-33.3 ppb, respectively. To calculate the accuracy and precision of the method, wheat blank samples were spiked at 3 levels (Table 3). As shown in Table 3, the average recovery rate was in the range of 72-123%, and the relative standard deviation (RSDr) was in the range of 0.6-24.2 %.

After validating the analysis method in this study, 30 wheat samples collected from flour factories in Tehran and Alborz provinces were analyzed in the spring of 2019 before processing. The results of this study were compared, in terms of the level of mycotoxins, with the Iranian National Standard No. 5925 under the title of "Maximum tolerated limits of mycotoxins in food and feed". The amount of mycotoxins was calculated using the calibration curve. OTA, T-2, and HT-2 mycotoxins were identified in wheat samples. It was determined that the level of OTA in one sample (3.3%) exceeded the allowable limit (5 ppb for OTA) set by the Iranian National Standards Organization. Also, HT-2 and T-2 toxins were observed in 2 and 1 sample, respectively; however, their amount was lower than LOQ of the validated method (Table 4). According to the obtained results, none of the samples exhibited simultaneous contamination with mycotoxins. Due to the regular control of temperature and humidity as well as proper

Table 2) Results obtained from the spiked samples (n=3) calibration curve.

Analyte	Calibration Curve	Correlation Coefficient (R^2)	Recovery Rate (%)	RSDr (%)	LOQ(ppb)
AFB_1	Y=874.63x-404.47	0.9996	93.1	21.1-5.2	2
AFB_2	Y=407.21x-572.5	0.9998	102.6	2-17.8	2
AFG_1	Y=492.47x-1915.2	0.9928	103.5	0.8-18.9	5
AFG_2	Y=107.54x+502.37	0.9984	94.8	3-14.3	5
OTA	Y=149.57x-23.596	0.9992	102.1	2-18.1	2
T-2	Y=107.81x+46.204	0.9988	97.6	1.1-13.4	5
HT-2	Y=24.431x-202.56	0.9961	105.4	4.2-21.4	20
ZER	Y=41.851x-457.25	0.9984	101.6	2.7-17.9	50
DON	Y=2.318x+29.291	0.9947	105	1.7-20.9	100
FB_1	Y=8.641x-637.26	0.9909	105	3.4-14.3	100
FB_2	Y=5.1x-281.8	0.9998	100	2.7-26	100

Table 3) Results of mycotoxins recovery from the wheat samples (n=3) at three levels.

Mycotoxins	Average Recovery (%) (n=3) (Repeatability)		
	Level 1	Level 2	Level 3
AFB1	119.3(15.5)	120(7.3)	108(10.7)
AFB2	118.(2.5)	114(5.2)	107(13.7)
AFG1	105(0.6)	110(13.2)	118(6.1)
AFG2	123(7.1)	72(18.3)	104(20)
OTA	87(14.4)	74(13.1)	108(8.5)
DON	100(12.4)	100(11)	99(13.9)
FB1	116(18.7)	98(20)	118(10.6)
FB2	115(0.6)	110(13.2)	118(6.1)
ZER	112(9.3)	117(13.2)	86(18.7)
T-2 toxin	122(16.4)	106(12.3)	77(7.2)
HT-2 toxin	103(5.6)	112(10.2)	87(24.2)

Table 4) Contamination rate of wheat flour samples collected from flour factories silos in Alborz and Tehran provinces.

Test System	Mycotoxins										
	AFB_1	AFB_2	AFG_1	AFG_2	OTA	ZER	DON	T-2 TOXIN	HT-2 toxin	FB_1	FB_2
Sample size	30	30	30	30	30	30	30	30	30	30	30
Positive (%)	0	0	0	0	1(3.3%)	0	0	1	2	0	0
Range (ppb)	N.D	N.D	N.D	N.D	11	N.D	N.D	<LOQ	<LOQ-23	N.D	N.D
MTL[c]	5		15[a]		5	200	1000	-	-	1000[b]	

[a]Sum of AF_S
[b]Sum of FB_1 + FB_2
[c] Maximum tolerated limit

aeration of the silos, the sampling season did not affect the contamination rate. Figure 1 and 2 shows the chromatogram of OTA identification in contaminated and blank wheat samples.

Discussion

In this study, it was made use of Dionex 3000 ultra-high performance liquid chromatography machine coupled with

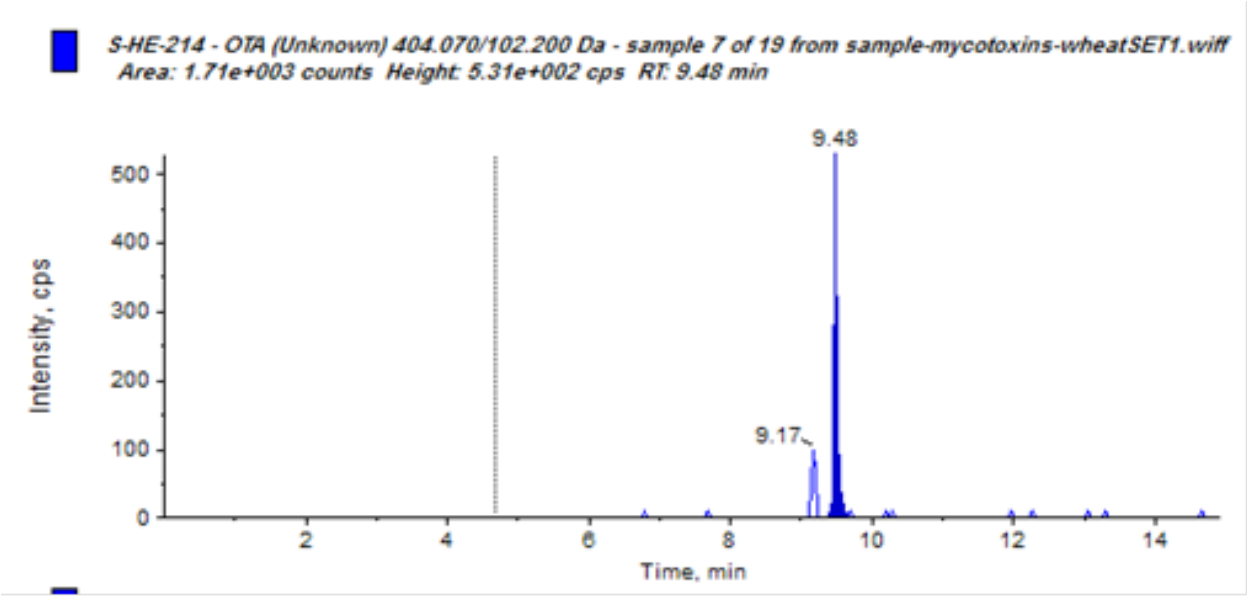

Figure 1) Chromatogram of OTA in a contaminated wheat sample.

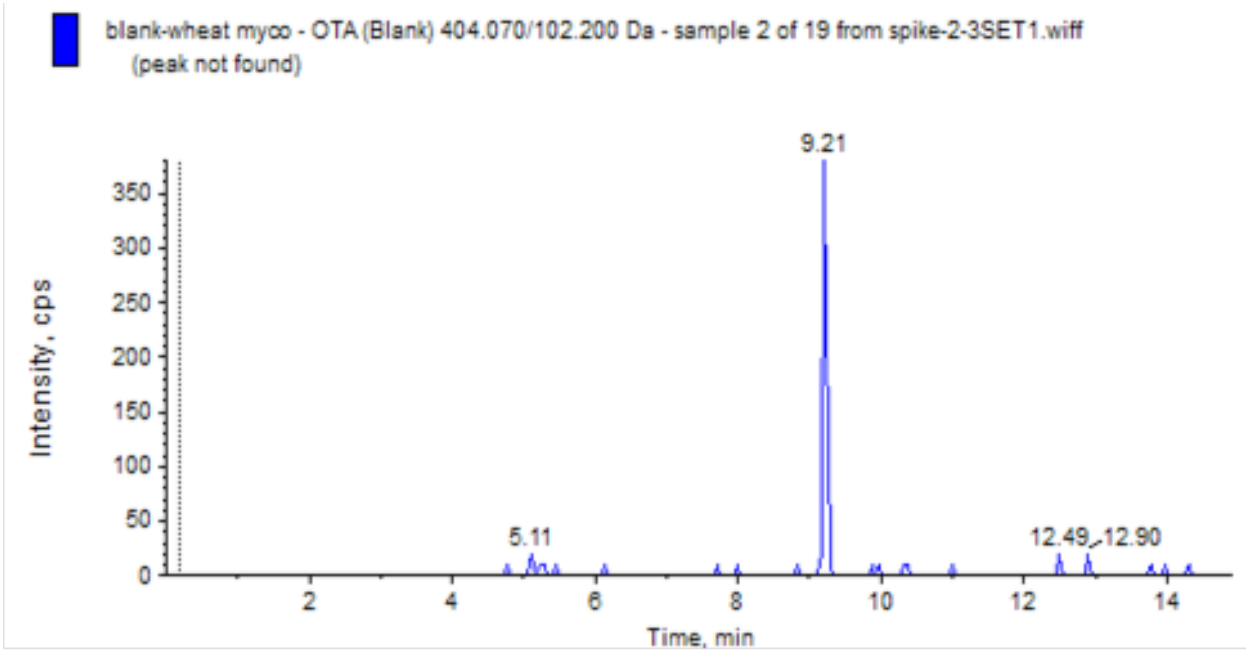

Figure 2) Chromatogram of a blank wheat sample (OTA).

Applied Biosystems API 3200 tandem mass spectrometry device with electrospray ionization technique and triple quadrupole mass analyzers along with the Ultimate 3000 Autosampler (LC-MS/MS). In this method, ionization was performed using electrospray technique. This technique is based on the production of ions before the analyte reaches the mass electrospray. Examination of the mycotoxins under study showed that all of them were able to produce parent and product ions in positive mode and some in negative mode. In some studies, deoxynivalenol and zearalenone were optimized in negative mode [12-14]; however, in a few studies, optimization of ions was done in positive mode, which had poor signalization and inappropriate peak shape [15]. By switching from positive to negative mode and vice versa, due to the increase

in analysis time, measurement sensitivity is reduced, and two separate analysis steps are implemented, once for toxins producing anions, such as deoxynivalenol, and once again for toxins producing cations, and the parent ions of each toxin are determined. Therefore, due to the above reasons, it was decided to optimize all ions in one (positive) mode. In positive mode, ions are resulted from the addition of a proton ion $[M + H]^+$ and an ammonium ion $[M + NH_4]^+$. When hydrogen adduct is formed, one unit, and when ammonium adduct is formed, 18 units are added to the mass of the substance. In this study, the compounds such as T-2 and HT-2 toxins were found to be able to form ammonium adduct. In his published article, Razzazi attributed this finding to the presence of ester groups in the T-2 toxin structure [16]. In addition to improving the separation process, selecting a suitable mobile phase affects the analytes ionization before entering the mass spectrometry system and increases method sensitivity. In simultaneous analysis of mycotoxins, acetonitrile and methanol are the most commonly used solvents due to the solubility of most mycotoxins in these two solvents, volatility, and the ability to adapt well to the reverse phase of chromatography and mass spectrometry [17]. Although better separation could be achieved with other solvents and mixtures, methanol has been used as mobile phase in most simultaneous analysis methods [17-18]. This could be due to the weak dissolution of methanol in the C18 column, which causes the rinsing power of methanol in the column to be more increased. It should also be noted that at the start of gradient washing with the same percentage of two solvents for highly polar mycotoxins, methanol performs better than acetonitrile, given the increased inhibition time. Therefore, there is no need to increase the water ratio, causing problems in mass spectrometry ionization process [19]. Figure 3 shows the simultaneous identification of mycotoxins under study in a 15-minute analysis.

In simultaneous analysis of mycotoxins, the extraction and purification steps are the most sensitive steps. In this study, the combination of acetonitrile, water, and acetic

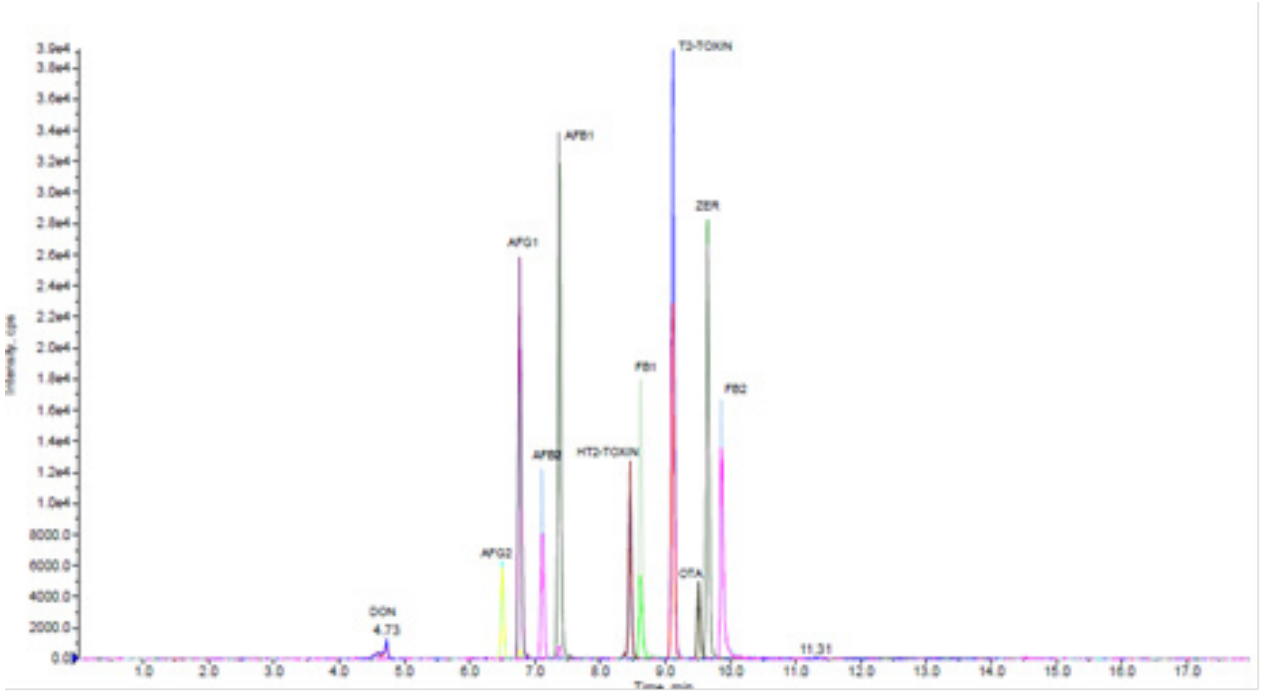

Figure 3) Chromatogram of simultaneous identification of 11 mycotoxins in wheat flour matrix.

acid was used as the extraction solvent, and Myco6in1 immunoaffinity columns were used to purify the samples. This was the first time in Iran that these columns were used to purify samples in the simultaneous analysis of 11 mycotoxins. In a study in 2011, in order to improve preparation and validation methods, a method was designed for simultaneous analysis of 25 mycotoxins in peanuts, corn, and starch samples, in which with one purification step (using cartridge), sample became ready to be injected into the LC-MS/MS device. Their study results showed that LOQ was less than 2 µg/kg for all AFs and OTA, about 421 µg/kg for FB_1, and less than 12 µg/kg for ZEN [20].

In a study conducted in Belgium by Ediage et al. (2015) on various types of corn, SPE method and LC-MS/MS technique were used to identify and measure 23 types of mycotoxins; in their study, the repeatability rate (RSDr), the average accuracy (RSD_R), and the recovery rate were calculated in the ranges of 7-22, 14-44, and 0.2-11 %, respectively [21]. In the present study, mycotoxins were collected from among the columns using methanol in two stages. The collected extract was dried under nitrogen gas at a suitable temperature, recovered in a suitable solvent, and identified by the LC-ESI/MSMS device. In this study, spiked wheat samples were used to draw the calibration curve. In this method, after spiking and analyzing samples by validated method, the calibration curve was plotted based on the values related to the peak area of the analyte (in spiked samples) versus the analyte concentration. The resulting curve was used to determine the amount and concentration of mycotoxins under study and unknown ones. The recovery rate, relative standard deviation (RSDr), and quantification limit of most toxins were in the acceptable ranges of 72-123%, 0.6-24.2%, and 2-100 ng/g, respectively; the results were within the allowable limits set by the European Union [22]. In a study by Soleimani et al. (2012) in Malaysia on cereal samples, the average recovery rate and quantification limit were reported in the ranges of 76.5-108.4 % and 20-40 ppb, respectively. In their study, 77% of the samples were infected by at least one mycotoxin, and the simultaneous contamination with OTA, ZEA, DON, FB1, FB2, T-2, and HT-2 mycotoxins was reported [23]. In another study in India, the recovery rate and relative standard deviation in wheat samples were reported in the ranges of 71-92 and 1.7-8.3%, respectively [24]. Zhou et al. (2016) determined the recovering rate, relative standard deviation, and quantification limit of all mycotoxins under study in wheat samples in the ranges of 70-116 %, 2-13%, and 7 µg/kg using QuChERS extraction method, respectively [25]. The information about the wheat samples contamination with various mycotoxins in Tehran and Alborz provinces is shown in Figure 4. The results showed that a small number of wheat samples were contaminated with mycotoxins under study. The level of OTA in one sample (11 ng/g) exceeded the allowable limit set by the Iranian Standards Institute. The concentration of HT-2 toxin in one sample was determined as 23 ppb, while in another sample; it was lower than LOQ of the identification method. Also, the amount of T-2 toxin in one sample was lower than LOQ. As shown in Figure 5, among the flour factories warehouses under study, the highest level of mycotoxins detection (with 3 cases) was related to the factory warehouse Code HE. Ochratoxin A is a nephrotoxic and nephrocarcinogenic mycotoxins [26]. Yazdanpanah et al. (2001) reported that

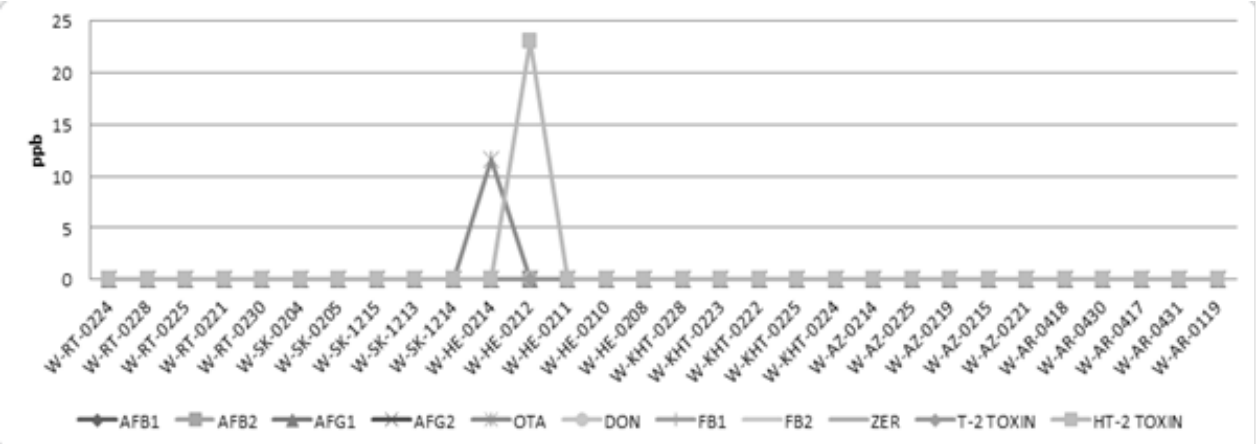

Figure 4) Contamination of 30 collected wheat samples with mycotoxins.

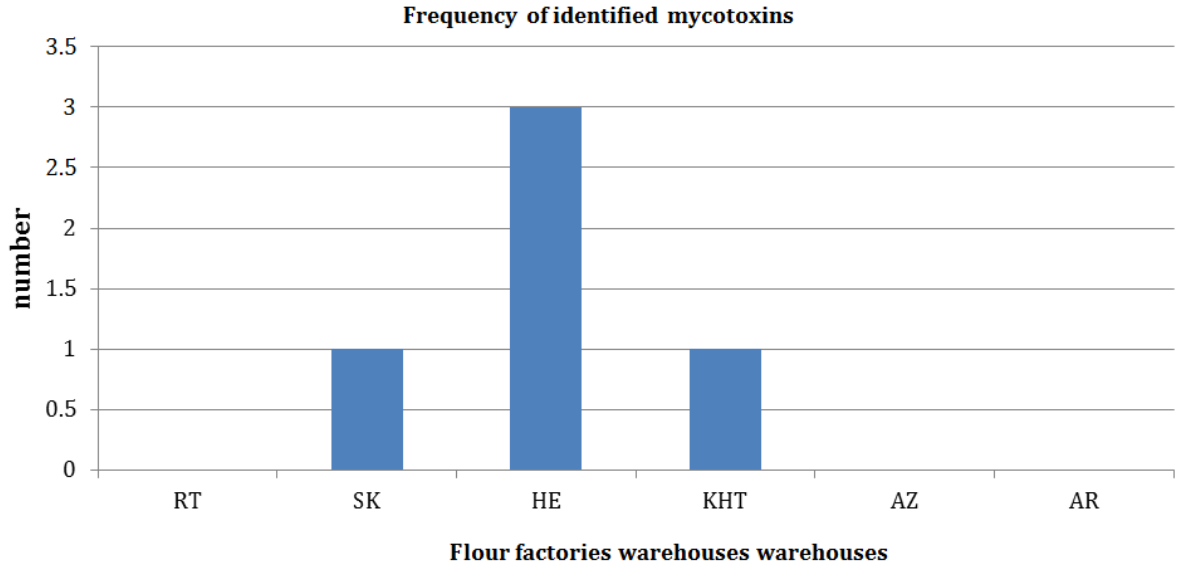

Figure 5) Frequency of identified mycotoxins based on the sampling location.

among the 40 barley and 9 corn samples collected from Golestan and Mazandaran provinces, no contamination was observed in barley samples; however, in one corn sample, simultaneous contamination with AFB_1 and OTA at 0.35 ppb was reported. Their study was the first report about the simultaneous prevalence of AFB_1 and OTA in northern Iran [27]. In a study by Zinedine et al. (2006), conducted on 20 wheat samples in Morocco, contamination with various types of mycotoxins was reported in wheat samples; the level of OTA contamination was 0.42 ppb in wheat, 1.08 ppb in corn, and 0.17 ppb in barley. In their study, the level of ZEN and FB_1 contamination was reported to be 14 and 1930 ppb in average, respectively [28]. In a study by Kumar et al. (2012) in India on wheat samples, 58% of the samples were contaminated with OTA in the range of 1.3-21.1 ppb [24]. The present study results showed that the use of this method had a higher level of linearity, sensitivity, specificity, and accuracy compared to the previous methods used. On the other hand, this method was very fast and easy to perform and made it possible to simultaneously analyze 11 types of mycotoxins.

Conclusion

Based on the obtained results, it could be concluded that this method is able to identify and measure very small amounts of mycotoxins, compared to the previous studies and other commonly used methods. Also, according to obtained results, the contamination rate in the samples was very low and in most cases was zero or lower than the standard and allowable limits set for mycotoxins in foodstuffs. However, continuous consumption of food contaminated with mycotoxins could cause complications for human health over time. It is recommended to observe a hygienic control cycle from the production stage to transportation and wheat storage in factories warehouses to reduce wheat waste in flour factories by creating special silos for wheat storing, controlling moisture, and ventilating silos properly.

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References

1. Bhat R, Rai RV, Karim AA. Mycotoxins in food and feed: Present status and future concerns. Compr Rev Food Sci Food Saf. 2010; 9(1):57-81.
2. Cheng CT, Perak M. Mass poisoning tale of the 9 emperor gods and rat tail noodles. Am J Forensic Med Pathol. 1992; 3:13-26
3. Bryden WL. Mycotoxin contamination of the feed supply chain: Implications for animal productivity and feed security. Anim Feed Sci Technol. 2012; 173(1-2):134–58.
4. Marquardt RR. Effects of molds and their toxins on livestock performance: A western Canadian perspective. Anim Feed Sci Technol. 1996; 58(1-2):77–89.
5. Richard JL. Some major mycotoxins and their mycotoxicoses- An overview. Int J Food Microbial. 2007; 119(1-2):3-10.
6. Kim DH, Hong SY, Kang JW, Cho SM, Lee KR, An TK, et al. Simultaneous determination of multi-mycotoxins in cereal grains collected from South Korea by LC/MS/MS. Toxins. 2017; 9(3):106.
7. Vaclavikova M, MacMahon S, Zhang K, Begley TH. Application of single immunoaffinity clean-up for simultaneous determination of regulated mycotoxins in cereals and nuts. Talanta. 2013; 117:345-51.
8. Park J, Kim DH, Moon JY, An JA, Kim YW, Chung SH, et al. Distribution analysis of twelve mycotoxins in corn and corn-derived products by LC-MS/MS to evaluate the carry-over ratio during wet-milling. Toxins. 2018; 10(8):319.
9. Sarrafian MR. Country report: Iran. Thailand, Chiang Rai: Fourth session of the technical committee of APCAEM; 2009.

10. Reddy CS. Mycotoxin contamination of rice. Rice knowledge management portal(RKMP), http://www.rkmp.com.in
11. Zamani-Zadeh HR, Khoursandi H. Occurrence of Fusarium species and their mycotoxins in wheat in Mazandaran province. Iran J Plant Pathol, 1995; 31:23-37.
12. Sulyok M, Berthiller F, Krska R, Schuhmacher R. Development and validation of a liquid chromatography/ tandem mass spectrometric method for the determination of 39 mycotoxins in wheat and maize. Rapid Commun Mass Spectrom. 2006; 20(18): 2649-59.
13. Rasmussen RR, Storm IMLD, Rasmussen PH, Smedsgaard J, Nielsen K F. Multi-mycotoxin analysis of maize silage by LC-MS/MS. Anal Bioanal Chem. 2010; 397(2):765-76.
14. Sorensen LK, Elbaek TH. Determination of mycotoxins in bovine milk by liquid chromatography tandem mass spectrometry. J Chromatogr B. 2005; 820(2): 183–96.
15. Frenich AG, Vidal JLM, Romero-Gonzalez R, Aguilera-Luiz MM. Simple and high-throughput method for the multi mycotoxin analysis in cereals and related foods by ultra-highperformance liquid chromatography/tandem mass spectrometry. Food Chem. 2009; 117(4):705-12.
16. Razzazi-Fazeli E. A review of recent trends in applications of liquid chromatography-mass spectrometry for determination of mycotoxins. J Liq Chromatogr Relat Technol. 2008; 31(11):1641 – 86.
17. Di Mavungu JD, Monbaliu S, Scippo ML, Maghuin Rogister G, Schneider YJ, Larondelle Y, et al. LC-MS/MS multi-analyte method for mycotoxin determination in food supplements. Food Addit Contam Part A. 2009; 26(6):885-95.
18. Monbaliu S, Van Poucke C, van Peteghem C, Van Poucke K, Heungens K, de Saeger S. Development of a multi-mycotoxin liquid chromatography/ tandem mass spectrometry method for sweet pepper analysis. Rapid Commun Mass Spectrom. 2009; 23(1):3-11.
19. Boyd RK, Basic C, Bethem RA. Trace quantitative analysis by mass spectrometry. Chichester: John Wiley & Sons; 2008.
20. Ediage EN, Di Mavungu JD, Monbaliu S, Van Peteghem C, De Saeger S. A validated multianalyte LC–MS/MS method for quantification of 25 mycotoxins in cassava flour, peanut cake and maize samples. J Agric Food Chem. 2011; 59(10):5173-80.
21. Ediage EN, Van Poucke C, De Saeger S. A multi-analyte LC–MS/MS method for the analysis of 23 mycotoxins in different sorghum varieties: The forgotten sample matrix. Food C hem. 2015; 177:397-404.
22. Commission Regulation (EC) No 1881/2006, Commission Directive 2006/1881/EC of 19 December 2006. Setting maximum levels for certain contaminants in food stuffs. OJEC. 2006; L364: 5–24.
23. Soleimany F, Jinap S, Abas FJ. Determination of mycotoxins in cereals by liquid chromatography tandem mass spectrometry. Food Chem. 2012; 130(4):1055-60.
24. Kumar R, Ansari KM, Saxena N, Dwivedi PD, Jain SK, Das M. Detection of Ochratoxin A in wheat samples in different regions of India. Food Control. 2012; 26(1): 63–7.
25. Zhou Q, Li F, Chen L, Jiang D. Quantitative analysis of 10 mycotoxins in wheat flour by ultrahigh performance liquid chromatography-tandem mass spectrometry with a modified QuEChERS strategy. J Food Sci. 2016; 81(11):2886–90.
26. Hussein HS, Brasel JM. Toxicity,

metabolism, and impact of mycotoxins on humans and animals. Toxicology. 2001; 167(2):101-34.

27. Yazdanpanah H, Miraglia M, Calfapietra FR, Brera C. Natural occurrence of aflatoxins and Ochratoxin A in corn and barley from Mazandaran and Golestan in north provinces of Iran. Mycotoxin Res. 2001; 17(1):21-30.
28. Zinedine A, Brera C, Elakhdari S, Catano C, Debegnach F, Angelinib S, et al. Natural occurrence of mycotoxins in cereals and spices commercialized in Morocc*o*, Food Control. 2006; 17(11): 868-74.