Comparing of two different detection methods (HPLC and LC-MS/MS) for the determination and confirmation of ochratoxin A in liquorice extract powder

M.H. Shojaee AliAbadi[1], R.Karami osboo[2], M.Mazaheri [3,*]

1. Iran Toxicology Department, Faroogh Life Sciences Research Laboratory, Tehran, Iran
2. Mycotoxins Research Laboratory, Agricultural Research Education and Extension Organization (AREEO), Iranian Research Institute of Plant Protection, Iran
3. Standard Research Institute, Research Center of Food Technology and Agricultural Products, Department of Food Toxicology, Karaj, Iran

Mycotoxins in food and feed are important risk factor for public health. There are many test methods for determination mycotoxins in food and feed and their products. The most common mycotoxins test method is based on high-performance liquid chromatography, but depend on the matrix, it is essential to develop validated methods of analysis and detection of them in food products. This study evaluated two different detection methods for the determination and confirmation of ochratoxin A (OTA) in liquorice extract powder. The detection of OTA was accomplished with high-performance liquid chromatography (HPLC) combined with fluorescence detector and Liquid Chromatography with tandem mass spectrometry (LC-MS/MS). The results indicate that the LC-MS/MS method was more specific and sensitive for the analysis and confirmation of OTA in liquorice extract than HPLC method because of false positive in HPLC method in some sample. So to comply with OTA legislation and consumer health protection, improvements in OTA analytical methodology is essential. [DOI: 10.22034/ASAS.2022.366043.1015]

Keywords: Mycotoxins; Ochratoxin A; Liquorice extract; HPLC; LC-MS/MS

Manuscript submitted October 17, 2022; Accepted December 14, 2022.

Introduction

Ochratoxin A (OTA) is a mycotoxin produced by some fungal species including Aspergillus ochraceus, A. carbonarius, A. niger and Penicillium verrucosum [1]. OTA is toxic molecule that is classified as probably carcinogenic to humans (Group 2B) by the International Agency for Research on Cancer [2]. Many studies have been shown that OTA causes nephrotoxicity [3]. Also neurotoxic, genotoxic properties and renal tumors were reported in a variety of animal species [4], OTA was reported in some agriculture products such as cereals [4], fig, raisin [5] coffee [6], and liquorice [7] due to the favorable weather conditions and/or to improper storage of food components [8]. Many countries set regulation for OTA in food and feed. In EU countries, maximum levels for OTA have been set in some foodstuffs such as infant food, roasted coffee, cereals, spices, liquorice root and liquorice extract [9]. Regulation of OTA in European Union are ranging from 0.5 µg/kg in baby food for infants and young children to 80 µg/kg for Liquorice extract for use in food in particular beverages and confectionary [9].

According to European Food Safety Authority (EFSA), a tolerable weekly intake of 120 ng per kg body weight for OTA was determined [10]. The Joint FAO/WHO Expert Committee on Food Additives (JECFA) established PTWI of 100 ng OTA/kg body weight [11]. OTA is relative stable during common food processing [12]. Liquorice (Glycyrrhizia glabra L.) is cultivated mainly in countries of the Mediterranean area, and also in India, Russia, China and Iran. Liquorice extract are currently used in food industries such as confectionery, beverages and chewing gum, functional foods, food supplements, and pharmaceutical products [13]. Presence of OTA in liquorice was reported in some studies [14, 15].

According to EC Regulation No. 1881/2006, maximum level for OTA in liquorice products at 20 µg/kg for root and 80 µg/kg for extract materials were have been set and liquorice products are monitored by imported countries in EU. So in order to control of OTA in products such as liquorice products validated methods should be used.

There are some analytical methods for detection and determination of OTA in various food and food products but the most widely used procedure for OTA determination is reversed phase liquid chromatography

* *e-mail: m_mazaheri@standard.ac.ir*

with detection by fluorescence. Liquid chromatography coupled with tandem mass spectrometry are other examples of methods for detecting and determination of OTA. For control of products, it is important to use a selective and reliable analytical method in order to protect consumer health.

The Institute for Reference Materials and Measurements (IRMM) of the Joint Research Centre (JRC) set a collaborative study to validate an analytical method for the determination of OTA in liquorice (root powder) and liquorice extracts using high performance liquid chromatography coupled to fluorescence detection with Immunoaffinity column for sample clean up [16]. They used 4 liquorice extract powder from 27.1, 6.5, 59.2 and 90.3 µg/kg of OTA for validation. The method described them was included the extraction of a portion of liquorice extract with a mixture of methanol and aqueous sodium bicarbonate solution. After filtration the extract, it is diluted with phosphate buffered saline and applied to an Immunoaffinity column containing specific antibodies of OTA for purification. Then purified eluate is dried, reconstituted, and quantified by reverse phase HPLC- fluorescence detection (HPLC-FLD) system. The results of injection showed that the method is satisfactory.

Also BS EN 17250:2020 has been published as a standard method for determination of OTA in spices, liquorice by Immunoaffinity column clean-up and HPLC-FLD. According to this standard, for spiking, 40 µl of OTA spiking solution with 0.4 µg/mL was added to 10 g test portion as a blank matrix. So the test portion is spiked at 1.6 µg/kg. With due attention to maximum limit of OTA in liquorice extract (80 µg/kg) [17].

It seems that the wrong concentration was chosen. But OTA peak of liquorice extract powder shown in annex A of this standard was mention related to mass fraction of ochratoxin A approximately 80 µg/kg.

In this study, we examined this method again on the liquorice extract powder with 10, 40, 80 µg/kg concentration of OTA and also an unknown sample and compared two detection method HPLC and LC-MS/MS.

Materials and Methods

Chemicals

OTA standard (1000 ng/ml in methanol) for the experiments was prepared from stock standard solution of Sigma Chemical Co. (Sigma, USA). Working standard solutions OTA at concentrations of 0.5- 15 µg/L in mobile phase were used to obtain the calibration curve. All solvents used for the experiments (Phosphate buffered saline solution (pH = 7,4), HPLC grade of methanol, acetonitrile, acetic acid solution and deionized water) were from Merck Company. Also sodium hydrogen bicarbonate (99,5 % purity) and tween 20 was prepared from Merck Company. Immunoaffinity column for OTA clean-up obtained from Libios, France.

Extraction

2 g of samples of liquorice extract powder with 10, 40, 80 µg/kg as QC samples and an unknown sample were extracted with a 40 mL of mixture of 50% of methanol and 50% of aqueous sodium bicarbonate solution (30 g/ L). After shaking by hand for a few seconds to obtain a homogeneous suspension, they were shacked for 40 min with a laboratory shaker. The extracts were filtered through the 150 mm glass fiber and centrifuged at 3000 g for 30 min. 15 mL of phosphate buffered saline (PBS with pH = 7.4) was added to 0.5 mL of the upper methanol layer from each and mixed and then applied to an Immunoaffinity column containing antibodies specific to OTA. The mixture was passed through the column (at dropping speed of 1 drop/s) until all extract has passed the column. The column was washed by PBS – tween 20 (2%) and then by 10 mL of deionized water. After drying the column, 1 ml methanol / acetic acid (98/2 v/v) was passed from column and collected in a vial. After 1 minute, 1 ml of methanol / acetic acid (98/2 v/v) was passed and collected in the vial again. The eluents were dried at 40°C under a gentle flow of nitrogen. Then 0.5 ml of mobile phase (methanol - water - glacial acetic (1800-1485-30, v/v) was added to them and the extracted was dissolved in it by using ultrasonic bath and served as the sample solution for direct injection into the HPLC-fluorescence detection (FLD) and LC-MS/MS system.

Sample fortification procedure

The recovery of the method was determined by samples fortification. In this regard, 2 g of liquorice extract powder was fortified 1 h before extraction with a solution of OTA in acetonitrile at 40 µg/kg, equal to half of maximum limit of OTA in liquorice extract powder according to EU regulation [9], then all of them analyzed according to 2.2 section.

Quantitative analysis by HPLC and LC-MS/MS

Waters HPLC system (USA) consisted of binary pumps 1525, florescence detector 2475, analytical column, Novapack-C18, 250 - 4.6 mm: 4µm) by reverse-phase and fluorescence detector was used for OTA quantification. The mixture of methanol - water - glacial acetic (1800+1485+30, v+v+v) at 2 mL/min flow rate was used as a mobile phase. Six standard calibration solutions of OTA from 0.5-15 ng/mL were prepared by dilution of 1000 ng/mL of OTA in mobile phase. The fluorescence detector was operated at excitation wavelength and emission wavelength of 333 and 460 nm respectively. The column heater was set at 40 ºC. The extracted samples were injected to HPLC. For identification of OTA peak in the samples chromatogram, their retention time were compared with that of the analyzed

OTA standards under the same conditions. Using the equation of calibration curve, the area under the curve of sample chromatogram was calculated for quantitation of OTA. Figs 1-3 show the OTA chromatogram of quality control, spiked sample and unknown sample detected by HPLC.

For LC/MSS detection, a Waters® ACQUITY UPLC I-Class System coupled to a Xevo TQ-S was used for rapid, high quality, and ultra-sensitive analysis of OTA, a HSS-T3 column (2.1 mm × 100 mm, 1.8 µm particle size, Waters, Milford, MA, USA) was used to separate the analytes. Gradient UPLC elution was carried out with 0.1% formic acid in water (containing 5 mmol/L ammonium formate) as mobile phase A and 0.1% formic acid in methanol (containing 5 mmol/L ammonium formate) as mobile phase B. The flow rate was set as 0.5 mL min−1 to separate the target compounds at 40 °C. The gradient program was started with 95% mobile phase A at injection time and increased linearly to 60% A in 3.5 min, and continuously ramped to 15 % over 5 min. Then, the mobile phase was restored to the initial conditions 2%B in the following 0.1 min, and kept for 1 min to re-equilibrate for the next injection. The column oven temperature was maintained at 40 °C in order to decrease viscosity. The samples were kept at 10 °C in the auto-sampler manager, and the injection volume was 10 µL. Figs 4-6 are related to OTA chromatogram of samples detected by LC-MS/MS.

Validation

Linearity, limit of quantification, precision and accuracy of the method of extraction and detection were evaluated. The accepted linearity of the calibration of R2 > 0.99 was obtained at the working range. For accuracy, recovery test was performed by spiking of the

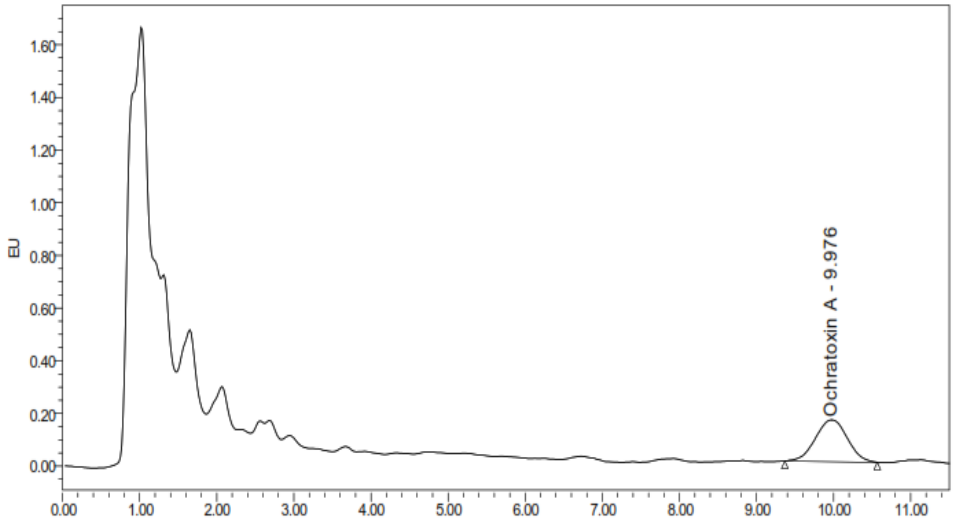

Figure 1- OTA chromatogram of quality control (10 µg/kg).

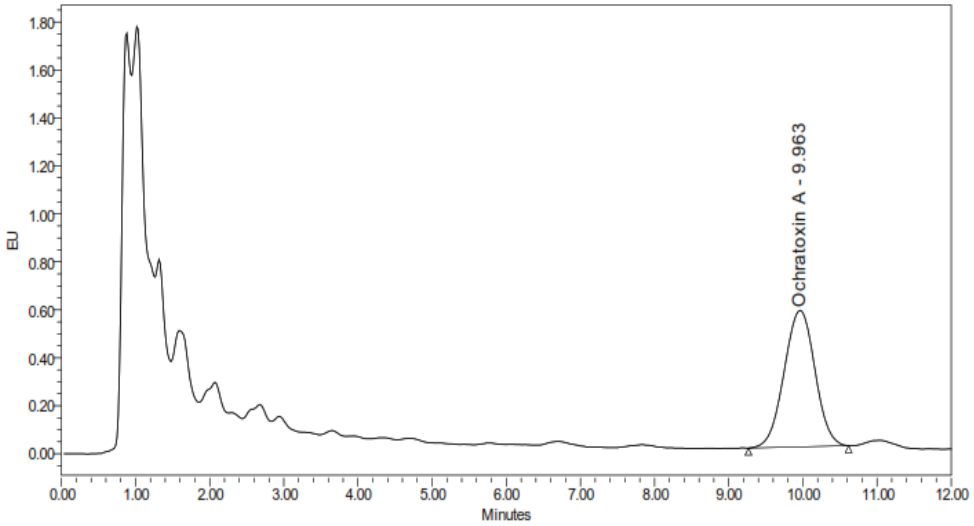

Figure 2- OTA chromatogram of Spiked sample at 40 µg/kg .

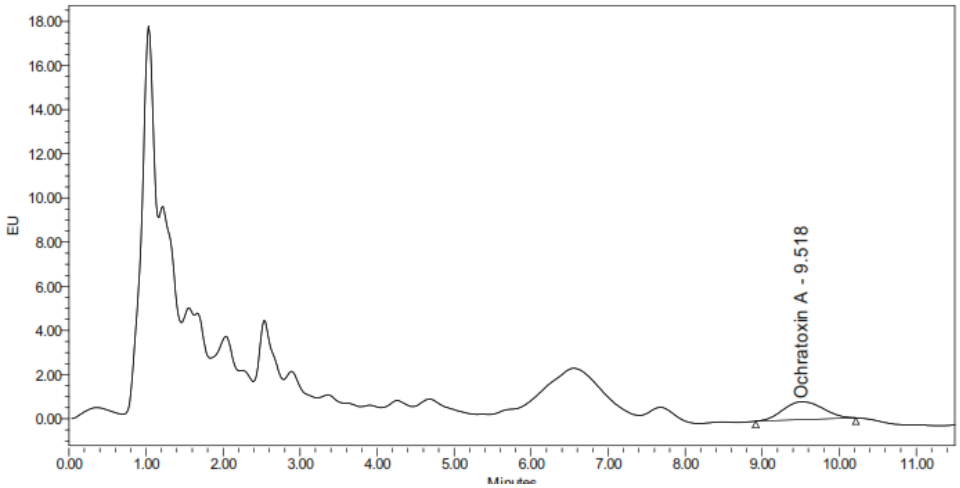

Figure 3- OTA chromatogram of unknown sample .

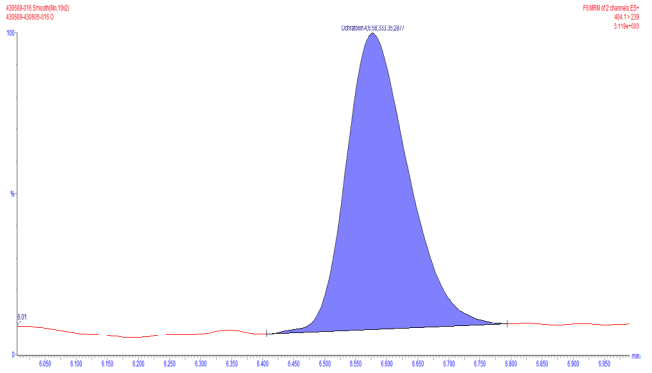

Figure 4- OTA chromatogram of unknown sample detected by LC-MS/MS

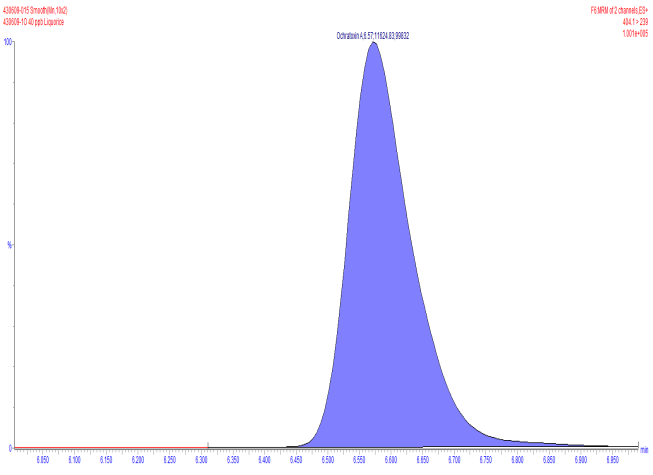

Figure 5- OTA chromatogram of spiked of unknown sample at 40 µg/kg detected by LC-MS/MS

one of the QC sample in all working days. Mean recovery rates was 79.5±7.8% for spike sample at 40 µg/kg. Also limit of quantification and detection were 0.3 and 0.1 µg/kg, respectively for LC-MS/MS detection.

Results and Discussion

In this study, three liquorice extract powder samples with certain concentration (about 10, 40 and 80 µg/kg) of OTA analyzed by HPLC-FLD and LC-MS/MS. They were tested six times by HPLC-FLD in different days and their results confirmed by LC-MS/MS too. So they were used as QC samples. It means they showed the same results by two instruments and their results were repeatable. For an unknown and random sample, result by HPLC-FLD was positive and repeatable. It was about 184 µg/kg, but its result by LC-MS/MS was < 6 µg/kg.

It means that we obtained false positive result in a sample by HPLC-FLD. So HPLC-FLD is not proper for deciding about these samples with positive result and there is a need for confirmation the positive result of OTA in liquorice extract. So to validate the positive results of OTA in liquorice extract powder samples obtained by HPLC purified by IAC, the LC-MS/MS method should be used.

The results for control samples with a certain concentration of OTA contamination were completely consistent with the HPLC results, but in an unknown sample that analyzed by HPLC, which showed contamination at high level, there was no considerable contamination in the LC-MS /MS method. On the other word, the LC-MS/ MS results confirmed that there was no detectable ochratoxin A in this sample.

Conclusion

Up to now, many different methods of extractions and detections have been tested and used to analyze mycotoxins in food and feed. HPLC in combination with fluorescence detector is common detection method for OTA in food and agriculture products and it is reli-

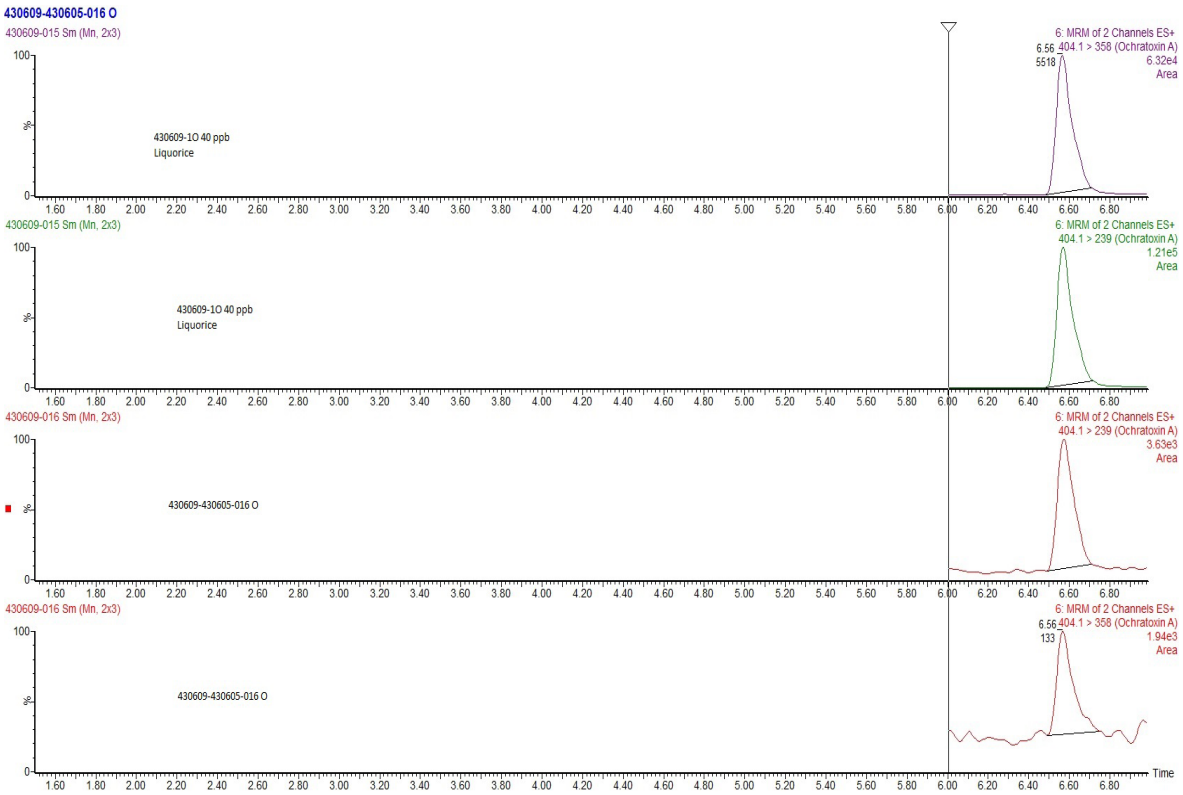

Figure 6- OTA chromatogram of spiked of unknown sample and its spike at 40 µg/kg detected by LC-MS/MS .

able and sensitive method for determination of OTA in many matrixes. But in this research, we showed that there may be a false positive for OTA detection in blank liquorice extract samples. The main limitations of HPLC technique for detection of OTA in liquorice extract sample may be because of the matrix effect and sample type and it doesn't have proper sensitivity and reliability for liquorice extract powder samples that showed positive result. Therefore, LC-MS/MS should be used as a confirmation tool to deal with the analytical challenges in liquorice extract powder samples and it is better to add to standard method of BS EN 17250.

Acknowledgments

The authors gratefully acknowledge the support of Toxicology Department, Standard Research Institute, Karaj, Iran and Faroogh Life Sciences Research Laboratory. We also thank our colleague, Mr. Kazemi, for his collaboration with performing the laboratory experiments.

Funding

This work was supported by the Standard Research Institutes and Faroogh Life Sciences Research Laboratory.

Conflict of interest

The authors declare that there are no conflicts of interest.

References

[1] Frisvad, J.C.; Thrane, U.; Samson, R.A., Mycotoxin producers. In Food Mycology. A Multifaceted Approach to Fungi and Food; Dijksterhuis, J., Samson, R.A., (Eds.); Taylor & Francis Group CRC Press, Boca Raton, FL, USA, 2007, pp. 135–159.

[2] Ostry, V. ; Malir, F. ; Toman, J. ; Grosse, Y. Mycotoxins as human carcinogens-the IARC Monographs classification, Mycotoxin Res, 2017, 33(1), 65-73. doi: 10.1007/s12550-016-0265-7.

[3] Joint FAO/WHO Expert Committee on Food Additives, Ochratoxin A, In Safety Evaluation of Certain Mycotoxins, Prepared by the Fifty-sixth Meeting of JEFCA. WHO food additives series 47/FAO food and nutrition 74, International Programme on Chemial Safety (IPCS), WHO: Geneva, Switzerland, 2001, pp. 281.

[4] Shehzad, A.; Muhammad, R.; Muhammad, S., Ochratoxin A in cereal products, Potential hazards and prevention strategies: A Review. Journal of Food Sciences, 2013, 23, 52-61. https://bit.ly/3oApioU

[5] Bircan, C., Incidence of ochratoxin A in dried fruits and co-ocurrence with aflatoxins in dried figs. Food and chemical toxicology, 2009, 47(8),1996-2001. DOI: 10.1016/j.fct.2009.05.008.

[6] Leitão, A., Occurrence of ochratoxin A in coffee: Threads and solutions, A Mini-Review. Beverages, 2019, 5(2), 36.

[7] Khalesi, M., Ochratoxin A in liquorice products, a review. Food Additives & Contaminants: Part A 2015, 12 (32), 2086-2092. https://doi.org/10.1080/19440049.2015.1094708.

[8] Majeed, S.; Iqbal, M.; Rafique Asi, M.; Iqbal, S. Z., Aflatoxins and ochratoxin A contamination in rice, corn and corn products from Punjab, Pakistan. J. Cereal Sci., 2013, 58 (3), 446-450.
[9] Commission Regulation (EC) No. 1881, setting maximum levels for certain contaminants in foodstuff, J. Eur. Union L 364, 2006, 5–24. http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=C.
[10] Schrenk, D.; Bodin, L.; Chipman, J.K.; del Mazo, J.; Grasl-Kraupp, B.; Hogstrand, C.; Hoogenboom, L.; Leblanc, J.; Nebbia, C.S.; Nielsen, E.; et al., Risk assessment of ochratoxin A in food. EFSA J., 2020. 18, e06113.
[11] Joint FAO/WHO Committee On Food Additivies, Evaluation of certain food additives and contaminants (Sixty-eighth report) of the JECFA Technical Report Series. 2007.
[12] Boudra, H.; Le Bars, P.; Le Bars, J., Thermostability of Ochratoxin A in wheat under two moisture conditions. Appl. Environ. Microbiol. 1995, 61, 1156–1158.
[13] Gabriele, D.; Curcio, S.; Cindio, B., Optimal design of single-screw extruder for 7 liquorice candy production: a rheology based approach. Journal of Food Engineering 2001, 8 (48): 33-44.
[14] Herrera, M.; Herrera, A.; Arino, A., Estimation of dietary intake of ochratoxin A from liquorice confectionery. Food Chem. Toxicol. 2009, 47, 2002–2006.
[15] Arino, A.; Herrera, M.; Estopañan, G.; Juan, T., High levels of ochratoxin A in licorice and derived products. Int. J. Microbiol. 2007, 114, 366–369.
[16] Lerda, D.; Ambrosio, M.; Kunsagi, Z.; Stroka, J., Determination of ochratoxin A in licorice and licorice extracts by high-performance liquid chromatography coupled with fluorescence detection: Collaborative study. Journal of AOAC international, 2013, 96 (2); 331-340. JRC73112.
[17] BS EN 17250, Foodstuffs Determination of ochratoxin A in spices, liquorice, cocoa and cocoa products by IAC clean-up and HPLC-FLD, 2020.