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Original research

# Validation of an analytical method to determine the content of ochratoxin A in malt extract and malt beverage

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ABSTRACT

This study aims to develop a method for the determination of ochratoxin A in malt extract and malt beverage samples based on the extraction of the samples with acetonitrile solution, followed by immunoaffinity cleanup and determination by high-performance liquid chromatography with fluorescence detection at an excitation of 333 nm and emission of 477 nm. The proposed method was validated to assess the linearity, limit of detection, limit of quantitation, precision, and recovery. The results demonstrated that the procedure was suitable for determining ochratoxin A in these samples and could be performed for their routine analysis in mycotoxin laboratories. The study involved 6 participants representing a cross-section of research, private, and official control laboratories from accredited laboratories in Iran. The method showed acceptable results within-laboratory and between-laboratory precision for each matrix, as required by the codex manual.

Keywords: Mycotoxins; Ochratoxin A; Malt extract; Malt beverage; HPLC

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## 1. Introduction

Malt is a barley grain that has been made to germinate by soaking in water and then stopped from germinating further by drying with hot air. It is made with the help of enzymes, then filtering and concentrating while under vacuum evaporation. In this process, barley converts into malt. It uses in foods and beverage (Hübner & Arendt, 2013).

Alcohol-free malt beverage is a cereal-based beverage (Habschied, Živković, Krstanović, & Mastanjević, 2020; Hager, Taylor, Waters, & Arendt, 2014; Marinova, Mileva, & Batchvarov, 2017). In the production of malt beverage, the main ingredients include water and grain malt (mainly barley and to a lesser extent wheat) (Gupta, Abu-Ghannam, & Gallaghar, 2010; Habschied et al., 2020).

A product made from malt, known as a "malt beverage" (ma'al-sha'ir), has become increasingly popular in recent years. Statistics show that the world's per capita consumption of malt beverages especially those without alcohol is rising. The majority of consumers of this food product are teenagers and young people. Due to the increase of consuming this drink in the household food basket, it is very important to pay attention to the safety aspects of this product as well as malt extract, which is the main basis of this beverage.

Ochratoxin is a mycotoxin produced by species of Aspergillus and Penicillium (Alshannaq & Yu, 2017). There are three different forms of ochratoxin. They are ochratoxin A, B, and C (Kőszegi & Poór, 2016). Ochratoxin A (OTA) is the most important of these because it is susceptible to contamination of agricultural products and its impact on the food chain. It is a widespread contaminant of types of foodstuff, such as cereals and their products (Lim, Yoshinari, Layne, & Chan, 2015; Sun, Su, & Shan, 2017), coffee, figs, grapes, and dried grapes (Freire et al., 2017).

OTA is a potential carcinogen and nephrotoxic. The International Agency for Research on Cancer (IARC) has classified OTA as a group 2B carcinogen (possible human carcinogen) (WHO, 1993).

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Besides health issues, OTA exposure results in economic losses via increasing the cost of treating the disease, the mortality rate of animals, and the costs related to the control or detoxification of OTA (Pfliegler, Pusztahelyi, & Pócsi, 2015). OTA accumulates in the organs of animals through the consumption of contaminated animal feed and is subjected to products such as milk and meat. Therefore, sensitive and reliable detection and quantification methods for the determination of OTA in foods are essential for effective contaminant control (Kumar et al., 2020). Determination of OTA in food is a priority for industry and competent authorities to check compliance with national regulations restrictions. Many countries have set maximum limits in national standards due to the risk that the consumption of contaminated foods poses to human health. The maximum level for OTA was established at the EU level for a wide range of foodstuffs. For non-alcoholic malt beverages, that level is 3 µg/kg (Sorbo et al., 2022).

Fungal contamination of barley (especially in the post-harvest stage) affects the quality of malt used in the malt industry and the amount of OTA in beverages (Maham, Kiarostami, Waqif-Husain, Karami-Osboo, & Mirabolfathy, 2013).

During seed preparation, high temperature and seed moisture level (Agu & Palmer, 1998; Djameh et al., 2015) and metabolites of enzymatic activity during the seed immersion stage can lead to mold growth and activation of inactive spores (Djameh et al., 2015).

There are a number of analytical methods to analyze OTA. Most methods are based on chromatographic techniques such as high performance liquid chromatography fluorescence detection or HPLC-FLD (Schummer, Brune, & Moris, 2018). Based on the matrix effects, a susceptible, specific, and reliable tool for detecting contaminants in food is required.

In a study conducted in 2016, a nanoparticle-modified chemical electrode carbon method applied as a sensitive electrochemical sensor to OTA determination in cereal products such as malt beverage samples. The minimum and maximum amounts of OTA in the drink samples were reported 4.13 and 6.11 nM respectively (Afzali, Fathirad, & Ghaseminezhad, 2016).

In 2009, OTA determination was done during the malting process using HPLC equipped with a fluorescence detector. The amount of OTA in 39 % of beer samples was between 0.001 ng/ml and 0.0544 ng/ml. Only in one sample 0.243 ng/ml of OTA detected (Běláková, Benešová, Mikulíková, & Svoboda, 2011).

In a study, the presence of OTA in barley, malt, and beer samples was investigated using the ELISA method. The amount of OTA in 23 samples (out of 24 samples) of malt was 0.5-6.6 µg/kg. 28 % of beer samples (42 out of 150 samples) had 0.1-1.8 µg/kg of OTA, and Only in one beer sample OTA contamination was more than 5 µg/kg (Gumus, Arici, & Demirci, 2004). In 2006, 70 malt beverage samples collected from Iran market analyzed for OTA determination by ELISA method. The results did not show remarkable contamination. But validation criteria of the method did not mention in the paper (Mahdavi, Khorrami, & Jabbari, 2007).

There are the national test standard methods for determination of OTA in cereals (INSO9238, 2011), dried fruits (INSO9237, 2011), roasted oats and coffee (INSO13122, 2010), and cereal-based foods for babies and children (INSO14556, 2011). Still there is no validated test method for determination OTA in malt extract and malt beverage in as the national standard. So, the incidence of OTA in these products has not been accessed based on validated methods in the country.

The aim of the present study is to develop a validated method for extraction and determination of OTA from malt extracts and malt beverages.

## 2. Material and Methods

### 2.1. Chemicals

OTA at a concentration of 10 µg/mL in methanol (originated from product NO. O1877) for the experiments was purchased from Sigma-Aldrich Chemie GmbH Company, Germany. To prepare the OTA stock solution, OTA was dissolved in methanol at a concentration of 1 µg/ml, which was then stored in glass-covered test tubes at 0 ◦C (Dahal, Lee, Gu, & Ryu, 2016).

The HPLC grade of methanol and acetonitrile from Merck Company were used for the experiments, and deionized water was prepared using a Milli-Q purification system. Analytical grade of acetic acid glacial 96 %, saline-phosphate buffer (PBS) at pH =7.4, and sodium chloride purchased from Merck Chemical Co. Six different malt extract samples were received from the manufacturing company, and six malt beverage samples were purchased from the local markets.

### 2.2. Sample preparation

Samples of malt extract and samples of malt beverage were homogenized and stored in original bottles or containers and stored at 4 ◦C.

### 2.3. Extraction

Before extraction, samples subjected to analysis were thoroughly degassed in an ultrasonic bath for 45 minutes. 25 g ± 0.01 g of each homogenized sample was extracted with 100 ml of acetonitrile/ water 84: 16 (v/v) in a screw-cap flask, 250 mL using a shaker for 30 min. The extract was filtered through a paper filter. Then, 10 mL of the extract was diluted with 50 mL of PBS to reach 60 mL mixture.

The resulting solution was passed through a glass microfiber filter Whatman GF/A 1.6 µm (47 mm). 55 mL of the filtered solution was passed through a preconditioned immunoaffinity column (IAC) at a flow rate of 2-3 mL/min. The IAC was washed with 10 mL water and dried under positive air pressure. The OTA was eluted from the column by 1500 µL of HPLC grade methanol/ acetic acid 98:2, (v/v). The eluted was collected into a clean amber vial and diluted to a final volume of 3 mL with 1500 µL of deionized water. The solution was thoroughly mixed with a vortex mixer. 100 µL of this solution served as the sample solution for direct injection into the HPLC-fluorescence detection (FLD) system (INSO9238, 2011).

### 2.4. HPLC analysis

OTA quantification was done using a HPLC system by reverse-phase and fluorescence detector (Waters, pump 1525, auto sampler 717, fluorescence detector 2475, analytical column, C18 (ODS), 250 - 4.6 mm: 4µm).

The mixture of water, acetonitrile, and acetic acid 99:99:2 (v/v/v) at 1 mL/min flow rate was used as a mobile phase. OTA stock solution was used to prepare the working standard solution. To prepare a working standard solution, 50 µL of the stock standard solution was poured into a suitable vial and dried at 40-50 ºC under nitrogen gas pressure. 2500 µL of mobile phase was added to a dried

vial and vortexed to obtain an OTA solution at 20 ng/ml (INSO9238, 2011).

A calibration curve was created using standard calibration solutions prepared from a 20 ng/mL OTA standard solution, resulting in seven OTA concentration levels from 0.5 to 15 ng/mL, with all standard stock solutions stored at 4 °C.

These standard calibration solutions were prepared by diluting 20 ng/mL of OTA in the mobile phase. The fluorescence detector was operated at an excitation wavelength of 333 nm and an emission wavelength of 477 nm. The column heater was set at 40 ºC. The calibration curve was built each working day for quantification of OTA in samples. Then, the extracted samples were injected into HPLC.

### *2.5. Method validation*

For validation of the method, the linearity of the calibration curves, limit of detection, and limit of quantification, repeatability, and reproducibility of the method were determined.

Repeatability and reproducibility of the method were defined with analysis of three fortified samples at the 3 levels of 5, 10, and 15 µg/kg for 3 days. Trueness was verified by a recovery experiment where blank samples were spiked with OTA at three concentration levels (5, 10, and 20 µg/kg) in triplicate. In this regard, 25 g of blank malt extract samples and malt beverage samples in triplicate were fortified 1 h before extraction with a solution of OTA. Blanks and fortified samples were extracted and analyzed as described in the previous section.

Also, the blank and the spiked samples of the malt extract and malt beverage with OTA, were distributed to six different accredited laboratories according to ISO 17025. The laboratories used a unique extraction and determination method (HPLC-FLD) for validation of the method according to a copy of the method.

## 3. Results and Discussion

The methods provide optimal performance in terms of linearity of calibration curves, trueness reproducibility, and repeatability. The detection and quantification limits were 0.5 µg/kg and 1.5 µg/kg for OTA respectively. RSD was equal to 0.52 %. The standard solution chromatograms in different concentrations are shown in Fig. 1. In each working day, the linearity of calibration curves was checked daily by injection of 100 µL of standard calibration solutions (Fig. 2) and $R^2$ was reported more than 0.99.

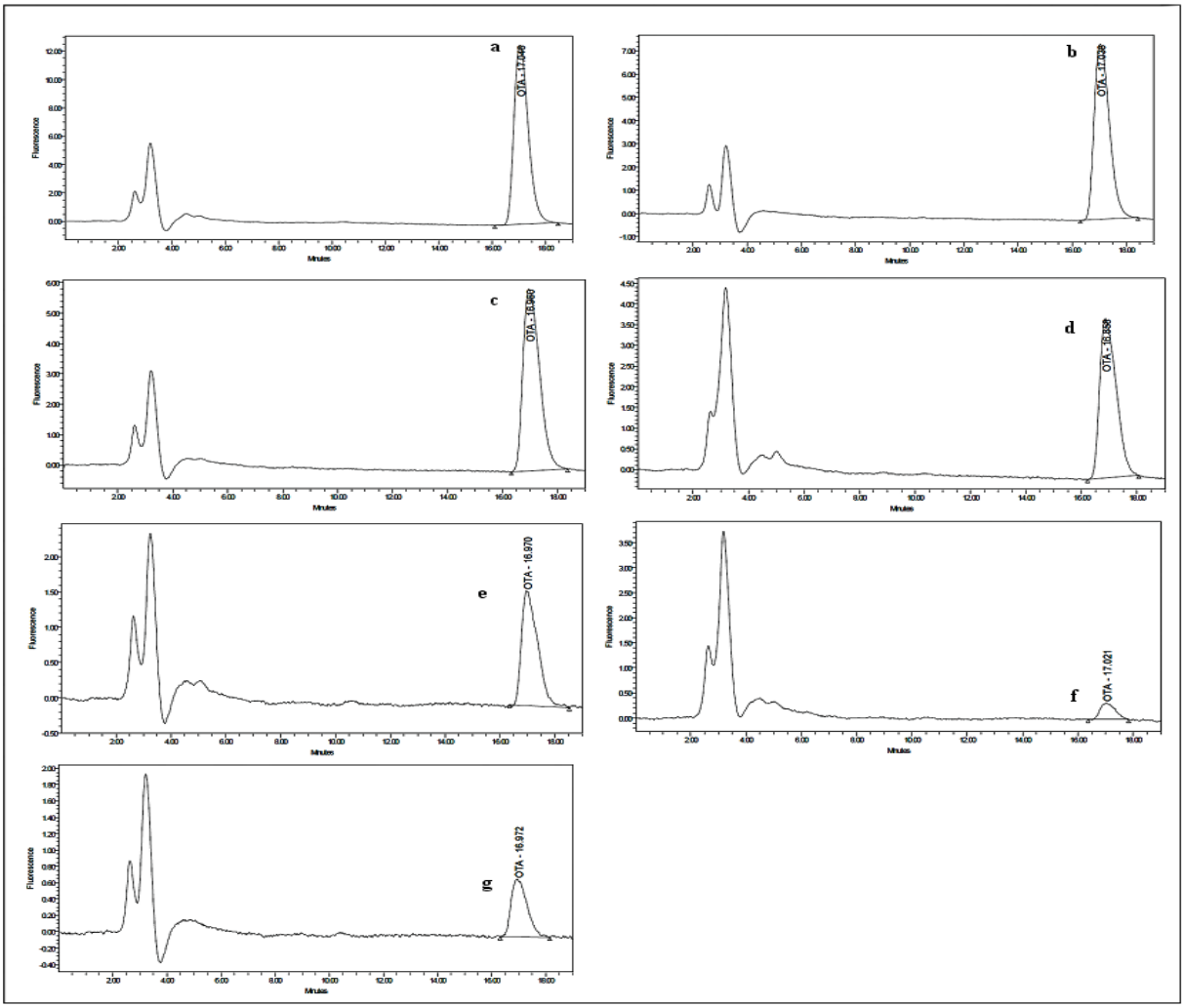


Fig. 1. Standard chromatogram of OTA with a concentration of (a): 15 µg/kg, (b): 10 µg/kg, (c): 7.5 µg/kg, (d): 5 µg/kg, (e): 2.5 µg/kg, (f): 1 µg/kg, (g): 0.5 µg/kg

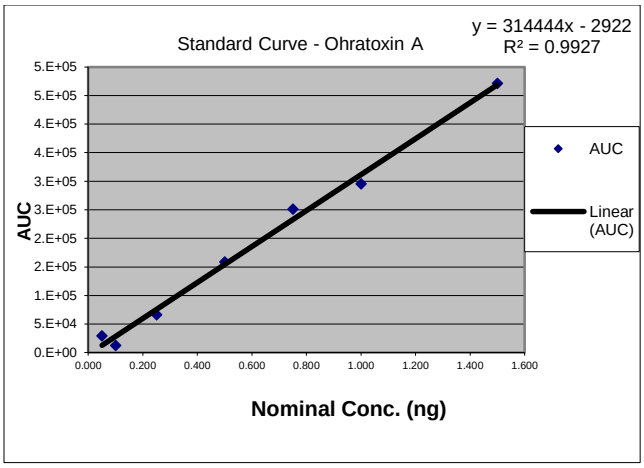


Fig.2. Calibration curve of OTA standards solutions

Also, the selectivity of the method was determined by adding standard solution of OTA to the control sample and injecting to HPLC.

The relative standard deviation of repeatability and reproducibility of the method showed the good precision. The relative standard deviation for reproducibility ranged from 0.89 % – 9.37 %. The recoveries of results for all spiked samples were determined in the range of 75 %. –120 %.

Chromatograms of blank samples and spiked samples at 5 µg/kg are shown in Fig 3.

The range of recovery values reported in the collaborative study spanned from 65 % – 101 % with a mean of 84 %. The relative standard deviation for reproducibility of laboratory results ranged from 13.3 % – 17.6 %. This method therefore showed acceptable within-laboratory and between-laboratory precision for each matrix.

The method performance in terms of recommended recovery for OTA set by the codex manual is 40–120 % for concentrations below 1 µg/kg, and 65–115 % for concentrations above 1 µg/kg, in comparison the maximum RSDr % is set to be 40 % for concentrations below 1 µg/kg, and 20 % for concentrations above 1 µg/kg (FAO/WHO, 2023). So, the results of this method validation were acceptable according to guidelines for establishing numeric values for the criteria of a fully validated method described in the codex manual.

OTA as a toxic and poisonous, can occur even with the very small quantities in food commodities, and consumption of such food products causes several health risks. Therefore, there is a need to analysis and quantification of it by sensitive and accurate methods. For the quantification and detection of OTA in cereals and cereals products, several analytical methods have been adopted. Most researchers employed liquid chromatography with fluorescence detection (Aresta, Palmisano, Vatinno, & Zambonin, 2006; Bertuzzi, Rastelli, Mulazzi, Donadini, & Pietri, 2011; Kawashima, Vieira, & Soares, 2007; Lhotská, Šatínský, Havlíková, & Solich, 2016; Odhav & Naicker, 2002), with limits of detection varying between 0.002 µg/kg and 1 µg/kg. Liquid chromatography with tandem mass detection (LC-MS-MS) or ultra-pressure liquid chromatography with mass detection (UPLC-MS) was also applied, with limits of detection varying between 0.75 µg/kg and 0.0003 µg/kg, respectively (Běláková et al., 2011; Rubert, Soler, Marín, James, & Mañes, 2013). Among the methods, using HPLC have been developed to overcome the constraints of the other methods and it is applied for the detection of mycotoxins (Singh & Mehta, 2020; Yang et al., 2020).

Because most of the laboratories are equipped with HPLC for mycotoxins determination and the Iranian national standards of test methods for mycotoxins are based on HPLC, therefore, it is important to generalize the existing national standard methods to determination of mycotoxins in different matrices by validating the

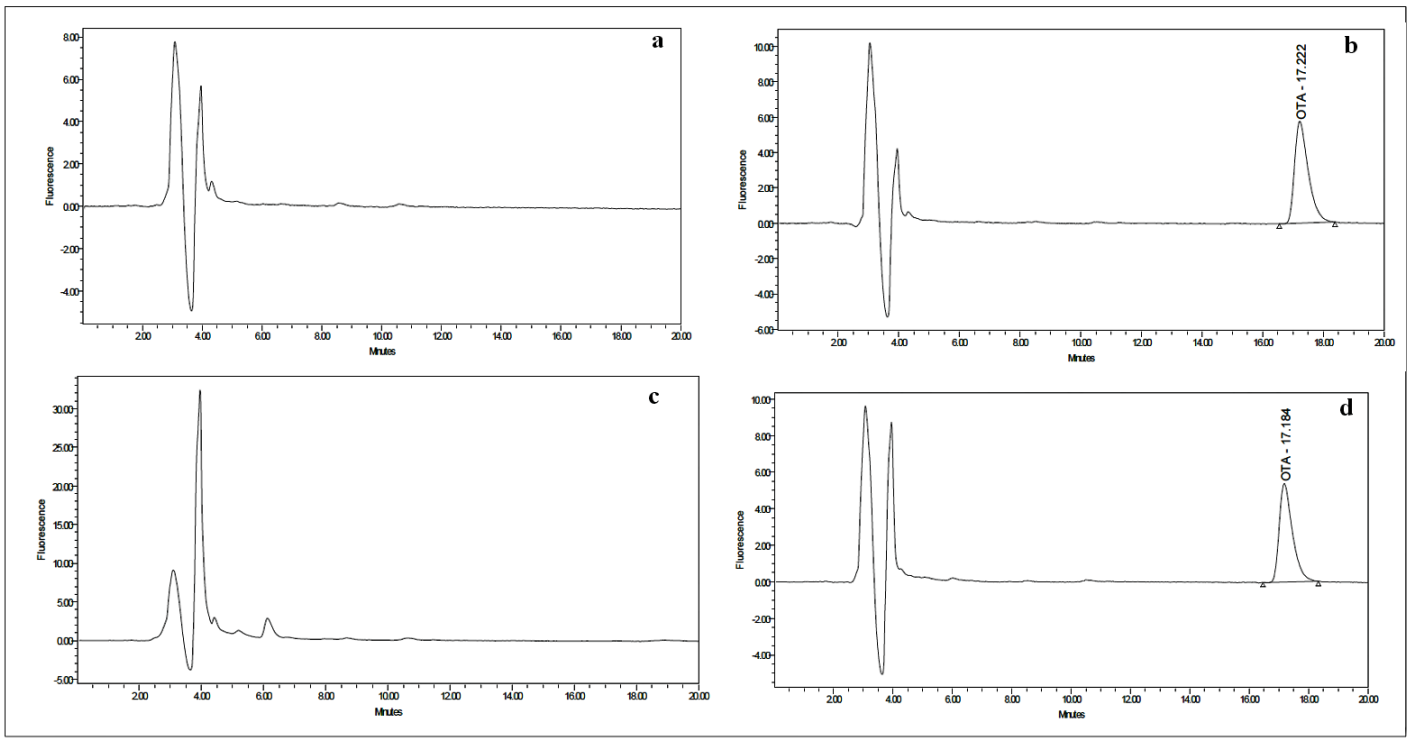


Fig. 3. Chromatogram of samples, (a): Blank malt beverage sample, (b): Spiked malt beverage sample at 5 µg/kg, (c): Blank malt extract sample, (d): spike malt extract sample at 5 µg/kg

methods. In this regards, the need to use different equipment for different products has been eliminated, and toxins can be identified in different food products with one equipment.

In this study, the method of determination of OTA in malt extract and malt beverage was validated using the HPLC. Therefore, it is possible to add the determination of this mycotoxin in these two matrices in the national standards, and any investigation should be done using a valid national method so that its data is reliable.

## 4. Conclusion

In this study, a method for the determination of OTA in the malt extract and malt beverage was developed by using HPLC/fluorescence and clean-up with an immunoaffinity column. This validated method is simple and reliable. Satisfactory recoveries and precision were observed. The results confirm the general criteria for the selection of analytical methods according to the Codex manual and can be used by accredited laboratories to check the presence of OTA in malt products. Therefore, it is possible to investigate the OTA contamination of these two products with a reliable method and, if necessary, national limits for this toxin in these two products will be developed based on the risk assessment.

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## Author contributions

Mazaheri and Alaei wrote the manuscript and searched the databases. Mahmoodi, Mazaheri and Ali akbarzadeh did this project.

## Conflict of interest

The authors declare that there is no conflict of interest.

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