A preliminary survey of citrinin contamination in dried fruits, molasses and liquorice products in Turkey

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Summary
Citrinin, a nephrotoxic mycotoxin, is mainly produced by some species of *Penicillium, Aspergillus* and *Monascus* genera. Citrinin can be found as a contaminant in various grains, fruits, juices, plants, spices and dairy products, resulting in chronic human exposure. The aim of this study was to investigate the presence of citrinin in several dried fruits, molasses and liquorice products collected from local markets in Istanbul, Turkey. Citrinin analysis was performed by high-performance liquid chromatography equipped with fluorescence detection after liquid-liquid extraction and immunoaffinity column clean-up. Among 41 samples, citrinin was determined in one out of 5 dried grape samples at a content of 5.56 $\mu g \cdot kg^{-1}$ and in three out of 5 dried white mulberry samples at a mean content of 5.29 $\mu g \cdot kg^{-1}$, whereas two out of 3 liquorice products contained citrinin at a mean content of 19.14 $\mu g \cdot kg^{-1}$.

Keywords
citrinin; dried fruit; molasses; liquorice product; immunoaffinity extraction; chromatography

Mycotoxins are secondary metabolites of various fungi commonly present in foods and feeds due to poor cultivation and storage conditions. Mycotoxins cause human, animal and environmental health as well as economic problems, the latter due to affecting their export value. Thus, many countries implemented regulations and set the limit values for mycotoxin contamination in foodstuffs [1]. Citrinin (Fig. 1) is naturally produced by some species of *Penicillium, Aspergillus* and *Monascus* genera. Citrinin contaminates commonly several food products including maize, rice, wheat and nuts [2] and can be found in food commodities. Therefore, it is important to monitor citrinin levels in commercial products such as breakfast cereals, fermented maize, flour, cheese, meat products, dried fruits and fruit juices [3–6].

Citrinin was shown to have nephrotoxic, hepatotoxic, neurotoxic, immunotoxic and teratogenic effects to several animal species [7–9]. The compound showed mutagenic effects in hepatocytes and was implicated to be a potential cause of human Balkan Endemic Nephropathy as well as porcine nephropathy. Citrinin is classified by International Agency for Research on Cancer as Group 3 carcinogen, i. e. not classifiable as to its carcinogenicity to humans, for which a minimum risk level is not yet determined [10]. In 2014, European Commission Regulation (EU) No. 212/2014 amending Regulation No. 1881/2006 set the maximum allowed level of citrinin to 2000 $\mu g \cdot kg^{-1}$ in red yeast rice-based food supplements [11]. However, in the light of the latest EU legislation on citrinin, the Commission Regulation (EU) No. 2019/1901 is to set citrinin level on 100 $\mu g \cdot kg^{-1}$ for the mentioned supplements in which the regulation shall be applied from 1 April 2020 [12]. Furthermore, no regulation regarding citrinin levels in dried fruits, molasses and liquorice products has been published within both European Union and Turkey. Overall, there is a lack of studies on contamination of dried fruits and, particularly, molasses and liquorice products with citrinin. Therefore, in the present study, we aimed to de-

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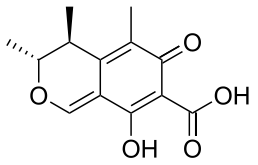

Fig. 1. Chemical structure of citrinin.

IUPAC name (3R,4S)-6-hydroxy-3,4,5-trimethyl-8-oxo-3,4-dihydroisochromene-7-carboxylic acid, CAS Number 518-75-2, molecular formula $C_{13}H_{14}O_5$, weight 250.25 g·mol^{-1}.

termine the citrinin levels using a validated analytical method in several dried fruits, molasses and liquorice products obtained from local markets in Istanbul, Turkey.

MATERIALS AND METHODS

Chemicals

Citrinin was obtained from Sigma-Aldrich (St. Louis, Missouri, USA) at purity of ≥ 98 %. A standard stock solution was prepared at a concentration of 50 μg·ml^{-1} in methanol in a glass-stoppered tube and kept at 4 °C. Standard solutions were prepared by serial dilutions in the concentration range of 0.25–100 ng·ml^{-1} in the high-performance liquid chromatography (HPLC) mobile phase and stored at 4 °C. The mobile phase was prepared by mixing water, acetonitrile and 2-propanol (65 : 30 : 5, v/v/v). pH was adjusted to 2.95 with ortho-phosphoric acid. CitriTest immunoaffinity columns were obtained from VICAM (Watertown, Massachusetts, USA). HPLC-grade methanol, acetonitrile and other analytical grade reagents were obtained from Riedel-de Haën (Seelze, Germany) or Merck (Darmstadt, Germany).

Samples

A total of 41 samples including 5 dried grape, 5 dried white mulberry, 5 dried fig, 5 dried plumb, 4 grape molasses, 4 white mulberry molasses, 4 carob molasses, 3 liquorice root, 3 liquorice powder and 3 liquorice extract were collected randomly from various supermarkets, traditional bazaars and homemade products in Istanbul during May–October 2018. The samples were aliquoted of 10 g each, and stored in a polyethylene bag or bottle at 2–8 °C for maximum two weeks.

Extraction and immunoaffinity clean-up

The extraction and clean-up procedures for citrinin were carried out using CitriTest immunoaffinity columns according to the manufacturer's instructions with minor modifications. Each sample of 10 g was extracted with 20 ml methanol-water (70 : 30, v/v) using an Ultra-Turrax disperser homogenizer (IKA Werke, Staufen, Germany) at high speed in short periods to avoid excess heating. Then they were centrifuged at 1500 ×g for 10 min. A portion of 1 ml supernatant was diluted with 49 ml of 10 mmol·l^{-1} phosphoric acid (pH 7.5) and mixed well. The diluted extract was filtered through a microfibre filter as indicated by the manufacturer. Then, 10 ml of filtrate (i.e. 0.04 g sample equivalent) was passed through a CitriTest immunoaffinity column attached to a vacuum manifold (VacElut 20 Manifold; Agilent Biotechnologies, Santa Clara, California, USA) at a flow rate of 1 drop per second until air came through the column. The column was washed with 5 ml of 10 mmol·l^{-1} phosphoric acid (pH 7.5), dried under vacuum and citrinin was eluted with 1 ml of methanol : 10 mmol·l^{-1} phosphoric acid (70 : 30, v/v) into a glass syringe barrel at a rate of less than 1 drop per second. Finally, 100 μl of the eluate was injected to an HPLC apparatus with a fluorecence detector.

HPLC with fluorecence detection

The analysis was conducted on an HPLC instrument LC-20A (Shimadzu, Kyoto, Japan) coupled to a fluorescence detector RF-10AXL (Shimadzu). The chromatographic separation was performed using a Phenomenex C_{18} column (250 mm × 4.6 mm, 5 μm particle size; Phenomenex, Torrance, California, USA) attached to a Phenomenex guard C_{18} column (4 mm × 3 mm, 5 μm particle size; Phenomenex). The retention time was approximately 9.5 ± 0.5 min with water, acetonitrile and 2-propanol (65 : 30 : 5, v/v/v) used as a mobile phase at a flow rate of 1 ml·min^{-1}. The column temperature was kept at 25 °C. The fluorescence detection was carried out using excitation at 330 nm and emission at 500 nm.

Method validation

Method selectivity was evaluated by analysing the extracts of citrinin-free blank and the same spiked samples. Linearity of the method was assessed using citrinin-free samples spiked with a citrinin standard at levels of 5, 10, 25, 50 and 100 μg·kg^{-1}. The spiked samples were prepared in triplicates and the injections were done in triplicates for each. The calibration curve established by plotted peak areas versus concentration was used for linearity determination. Recovery was determined using citrinin-spiked samples at levels of 5 μg·kg^{-1} and 10 μg·kg^{-1}, and the percentage of

the detected citrinin amount was used for recovery determination. The relative standard deviation of replicate results (*RSDr*) was used to evaluate the precision.

RESULTS AND DISCUSSION

Method performance

For method selectivity, no interfering peaks were observed at the retention time of citrinin in the dried fruits, molasses and liquorice products. Fig. 2 shows the chromatographic profiles of the citrinin standard solution. The calibration curve was linear in the concentration range of 5–100 $\mu g \cdot kg^{-1}$ with correlation coefficient $r^2 = 0.9993$. Limit of detection (*LOD*; signal-to-noise ratio = 3) was calculated to be 1.25 $\mu g \cdot kg^{-1}$, and limit of quantification (*LOQ*; signal-to-noise ratio = 10) was 4 $\mu g \cdot kg^{-1}$ for citrinin in dried fruits, molasses and liquorice products.

The mean recoveries at 5 $\mu g \cdot kg^{-1}$ spiking level were 69.6–99.7 % with *RSDr* lower than 10.2 %, and at 10 $\mu g \cdot kg^{-1}$ spiking level were 54.5–101.9 % with *RSDr* lower than 15.6 % in dried fruits, molasses and liquorice products (Tab. 1). Commission Regulation (EU) No 519/2014 [13] states that, for all levels of citrinin, the recoveries are acceptable in the range of 70–120 %. In our study, the recoveries exceeded 70 % in dried fruits and liquorice products, whereas, for molasses, recoveries were in the range of 54.5–68.6 %. Since molasses represent more concentrated samples, relatively low recoveries could be caused due to blocking of antibody sites during the purification through immunoaffinity columns [4]. According to the validation results, for the dried fruits and liquorice products citrinin analysis utilizing liquid-liquid extraction and immunoaffinity column cleansing (LLE/IAC) extraction followed by the HPLC with fluorescence detection (HPLC-FLD) method can be qualified as "acceptable" according to the EU criteria.

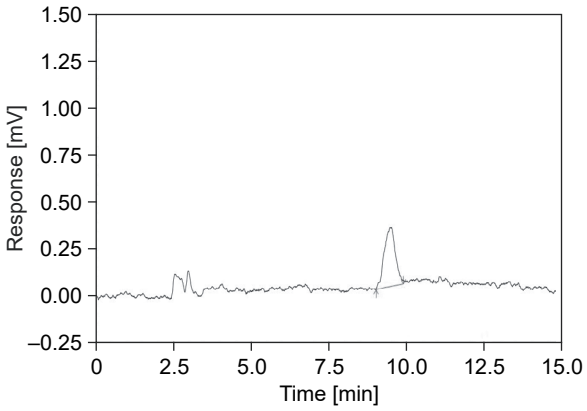

Fig. 2. Chromatogram of 10 $\mu g \cdot kg^{-1}$ citrinin standard solution.

Citrinin in the samples

Grains and grain products, fruits and fruit juices, black olive, cheese, nuts, several spices and food supplements containing *Monascus purpureus* fermented red rice were listed as dietary sources of citrinin [14, 15]. Sun-drying has been used for centuries in Mediterranean regions to preserve fruits by reducing the water content. It is expected that the reduction in the water content could provide inhibition of microbial growth and enzymatic activities. However, in poor conditions, fungal contamination can occur and this

Tab. 1. Recovery data for various dried fruit samples and molasses spiked with citrinin.

Sample	Spiking level 5 $\mu g \cdot kg^{-1}$		Spiking level 10 $\mu g \cdot kg^{-1}$	
	Recovery [%]	*RSDr* [%]	Recovery [%]	*RSDr* [%]
Dried grape	72.6 ± 3.8	< 5.2	73.4 ± 11.5	< 15.6
Dried white mulberry	93.5 ± 5.5	< 5.9	87.5 ± 8.5	< 9.6
Dried fig	74.6 ± 6.2	< 8.4	71.6 ± 4.2	< 5.8
Dried plumb	80.2 ± 8.1	< 10.2	76.9 ± 6.2	< 8.0
Grape molasses	68.6 ± 2.0	< 2.9	54.5 ± 3.5	< 6.4
White mulberry molasses	61.5 ± 1.8	< 2.8	60.4 ± 1.6	< 2.7
Carob molasses	66.6 ± 4.6	< 6.8	65.4 ± 4.4	< 6.7
Liquorice root	82.3 ± 2.7	< 3.3	101.9 ± 3.7	< 3.6
Liquorice root extract	99.8 ± 4.6	< 4.7	97.8 ± 6.4	< 6.5

Recovery values represent mean ± standard deviation ($n = 3$).
RSDr – relative standard deviation of replicate results.

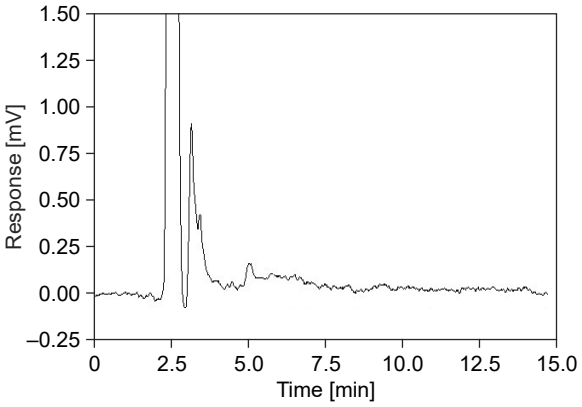

Fig. 3. Chromatogram of an uncontaminated molasses sample.

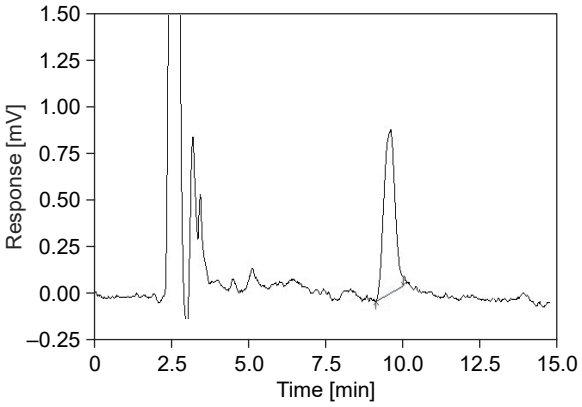

Fig. 4. Chromatogram of a naturally contaminated liquorice sample.

Content of citrinin was found at a level of 23.62 μg·kg-1.

may lead to mycotoxin contamination [16]. In the present study, we focused on the citrinin contamination in several dried fruits, molasses and liquorice products. Representative chromatograms of an uncontaminated sample of molasses and naturally contaminated liquorice samples are shown in Fig. 3 and Fig. 4, respectively. As shown in Tab. 2, citrinin was detected in only one out of five dried grapes at a level of 5.56 μg·kg^{-1}, and in three out of five dried white mulberry samples in the range of 4.26–7.02 μg·kg^{-1}, whereas citrinin was not detected in any of the fig and plumb samples. Our results are similar to those of Zohri and Abdel-Gawad [17], who reported that various dried fruits (4 samples of fig, 3 samples each of apricot, plumb and raisin) collected in Egypt did not contain citrinin by thin-layer chromatography (TLC). In Portugal, Martins et al. [18] analysed patulin and citrinin in 351 apples of seven varieties by TLC and reported that only 14 samples were contaminated with citrinin, while 69 samples were contaminated with both citrinin and patulin. Citrinin levels were reported as ranging from 320 μg·kg^{-1} to 920 μg·kg^{-1}, which is substantially higher than the values presented in previous reports [18]. Aziz and Moussa [19] showed that citrinin was found in the range of 50–70 μg·kg^{-1} in 4 out of 100 samples of grape, fig and apple samples by HPLC-FLD analysis. However, the often reported low frequencies of citrinin-contaminated samples may also be caused by the poor sensitivity of the used analytical methods. This is particularly true when TLC methods are applied. Ruan et al. [20] showed that none of the fruit samples (5 each of pear, grape and apple) purchased from local markets in China were contaminated with ochratoxin A or citrinin,

Tab. 2. Occurrence of citrinin in dried fruits, molasses and liquorice products.

Sample	Number of samples	Contaminated samples		Contamination level [μg·kg^{-1}]	
		Number	[%]	In individual samples	Mean
Dried grape	5	1	20	5.56	5.56
Dried white mulberry	5	3	60	4.26; 4.61; 7.02	5.29
Dried fig	5	0	0	< *LOD*	–
Dried plumb	5	0	0	< *LOD*	–
Grape molasses	4	0	0	< *LOD*	–
White mulberry molasses	4	0	0	< *LOD*	–
Carob molasses	4	0	0	< *LOD*	–
Liquorice powder	3	0	0	< *LOD*	–
Liquorice root	3	2	66.6	14.66; 23.62	19.14
Liquorice root extract	3	0	0	< *LOD*	–

LOD – limit of detection.

using combined ultrasound-assisted solvent extraction and dispersive liquid–liquid microextraction followed by HPLC-FLD for analysis.

Grape, mulberry, fig and apple are raw materials of molasses, which is a highly concentrated traditional and popular Turkish fruit juice, especially for children due to its nutritional properties [21]. Molasses may contain citrinin and other mycotoxins when rotten fruits used and low-quality process are utilized [22]. DIETRICH et al. [23] detected citrinin at a maximum level of 0.2 $\mu g.l^{-1}$ in fruit and vegetable juices utilizing LLE/IAC followed by indirect competitive enzyme immunoassay. In the same study, data are not suitable to make a precise comparison, however, recovery rate of the applied method was comparatively low. In the present study, for the first time, citrinin residue levels were evaluated in molasses and we observed that none out of the 12 analysed molasses of grape, white mulberry and carob were contaminated with citrinin. The recent research focused on the instability of citrinin during food processing [15]. Citrinin is heat-sensitive and decomposes during heat treatment to form other complex compounds [15]. As molasses are produced by heating of grape juices, the negative results found for theses samples are therefore not surprising.

Liquorice is a commonly consumed medicinal plant. Dried roots and extracts of liquorice are used in the preparation of numerous dietary supplements, confectionery and other food commodities. Liquorice products are prone to be contaminated by mycotoxins during harvesting, handling, storage and distribution [24]. In the present study, we observed that citrinin was found at levels of 14.66 $\mu g \cdot kg^{-1}$ and 23.62 $\mu g \cdot kg^{-1}$ in two out of three liquorice root samples. Our results are consistent with those of HUANG et al. [25], who showed that two out of thirty liquorice samples contained citrinin at levels of 6.75–20.44 $\mu g \cdot kg^{-1}$. Concerning citrinin contamination in raw foods and their products, good agricultural practices and appropriate storage conditions from harvest to consumption are needed. Wearing suitable protective clothes and storing food products in well-ventilated and strictly-controlled food containers were suggested to minimize contamination with citrinin [7].

CONCLUSIONS

Literature data on citrinin are conflicting, most researchers reporting no or low-level contamination of raw and dried fruits with citrinin. However, it has to be kept in mind that citrinin has moderate to severe toxic effects in human. Although, the studies conducted to detect citrinin contamination alone or with other mycotoxins are still ongoing, neither European Commission nor the Turkish Food Codex Infection Regulation sets limit values for citrinin in foodstuffs except the Commission Regulation No. (EU) 2019/1901 [12], in which the maximum allowed content is 100 $\mu g \cdot kg^{-1}$ for citrinin in food supplements based on rice fermented with *Monascus purpureus*. Therefore, there is an urgent need to detect citrinin residue levels in various agricultural foods worldwide. Additionally, our study is the first that evaluated citrinin contamination in various dried fruits, molasses and liquorice products. A survey of a large number of suspected food materials including raw materials marketed in Turkey is needed for the exact evaluation of human health risks due to citrinin exposure.

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