Aflatoxins in mesir paste
Özlem Çağindy

Department of Food Engineering, Celal Bayar University, Manisa, Turkey

ABSTRACT
In this research, 42 mesir paste samples produced by a limited number of manufacturers in Manisa, Turkey, were analysed for aflatoxin content by high-performance liquid chromatography with a fluorescence detector after pre-separation using immunoaffinity columns. A good correlation was found with good performance in terms of precision for the method. Limit of detection-limit of quantitation of AFG$_2$, AFB$_2$, and AFG$_1$, AFB$_1$, were 0.05/0.15 and 0.03/0.09 µg/kg, respectively. AFG$_2$, AFG$_1$ and AFB$_2$ were detected in 29%, 71% and 52% samples, whereas AFB$_1$ was not detected in any sample. AF$_{total}$, AFG$_2$, AFG$_1$ and AFB$_1$ content varied between 0.04/10.20 µg/kg, 0.07/0.42 µg/kg, 2.02/10.11 µg/kg and 0.04/0.11 µg/kg, respectively. About 9.5% of samples were found to be above the maximum limit (ML) for AF$_{total}$ (10 µg/kg), but none of samples exceeded the ML for AFB$_1$ (5 µg/kg) as set by the European Union for spices. According to the results, it was concluded that traditional products like mesir paste which contains different types of spices should be examined in terms of aflatoxins.

Aflatoxinas en pasta Mesir
RESUMEN
En este estudio se analizaron 42 muestras de pasta mesir por su contenido en aflatoxinas, producidas por un número concreto de industrias alimentarias en Manisa, Turquía, mediante HPLC-FLD después de una preseparación utilizando columnas de inmunoafinidad. Se encontró una correlación positiva con una actividad favorable en términos de precisión con este método. LOD-LOQ de AFG$_2$, AFB$_2$, y AFG$_1$, AFB$_1$, fueron 0,05/0,15 y 0,03/0,09 µg/kg. Se detectaron AFG$_2$, AFG$_1$ y AFB$_1$ en las muestras de 29%, 71% y 52%, mientras que no se detectó AFB$_1$ en ninguna de las muestras. El contenido de AF$_{total}$, AFG$_2$, AFG$_1$ y AFB$_1$ varió entre 0,04/10,20 µg/kg, 0,07/0,42 µg/kg, 2,02/10,11 µg/kg y 0,04/0,11 µg/kg, respectivamente. Se encontró que el 9,5% de las muestras estaba por encima de ML en AF$_{total}$ (10 µg/kg), aunque ninguna muestra excedió el ML en AFB$_1$ (5 µg/kg) según lo establecido por la UE en referencia a las especies. Según los resultados, se concluyó que los productos tradicionales como la pasta mesir que contiene diferentes tipos de especies deberían de ser examinados en términos de aflatoxinas.

1. Introduction
‘Mesir paste,’ also known as ‘meshir macun or putty,’ is a traditional confectionery product with characteristic aroma, taste, colour and high viscosity. It contains approximately 41 types of spices, different plant extracts, honey and sugar. Its beneficial effects on health have been reported for hundreds, and according to some resources, thousands of years (Artik, Poyrazoğlu, & Karkacier, 1999; Çekin & Şertoğlu, 2007; Hşyl, 1994; Oksel, Taneli, & Hakerlerler, 1997). The composition of mesir paste is 80–86% dry matter, and it contains 72–80% saccharide, 5–18% glucose, 0–8% honey, 1–2% spices, 0.07–0.20% lemon salt and 14–17% moisture. It is a Turkish folk medicinal product that has been used against diseases since the Ottoman era, and this confectionery product has been distributed to the public from Merkez Efendi Mosque in Manisa on every Newroz (March 21) since 1539 (Hşyl, Bagdatlioglu, & Otles, 1996; Karaman, Kesler, Kayacier, & Doğan, 2008; Oskay, Karayıldırım, Ay, & Ay, 2010; Sansal, 2015; Yahyaoğlu, 2005). Tons of mesir paste consumed in Turkey’s Manisa Province and in the Aegean region traditionally and exported to different European countries, Arabia, Far and Middle East countries. In 2012, Manisa’s Mesir Paste has entered into the UNESCO’s Representative List of the Intangible Cultural Heritage of Humanity (Sansal, 2015).
For mesir paste production, up to 42 different spices are weighed and grinded in a mortar. The mixture of spices is boiled with honey, sugar, glucose syrup and citric acid in boilers until a paste consistency is obtained (Giritlioglu, Avcikurt, & Savas, 2010; Hşyl, 1994). Differences were observed in spices used in the production of mesir paste in the recent years. The number of spices used was 20–25 in some products while it was 30–31 and 40–41 in some others. The composition of spices used in mesir paste gives the characteristic aroma and taste to the paste, while production, formulation and process parameters vary depending on the manufacturer. Mesir paste is produced by a limited number of manufacturers, only seven firms in Manisa and by a total of 10 firms in Turkey. Thus, it is difficult to speak of a standard production method for mesir paste. As a result, standardization in production carried out by the same firm at different times, or of mesir paste products produced by different companies, is recommended (Artik et al., 1999; Giritlioglu et al., 2010; Güven, 2010; Nergiz & Yıldız, 1995).
Spices that have a high possibility of containing mycotoxins are used in the preparation of mesir paste, which is a traditional concoctionery product. Drying the plants used as the spices in mesir paste production is a critical step in the course of mould growth and mycotoxin contamination. Cultivation of spices in warm and humid areas also increases the mould and bacterial contamination risks (Ali, Hashim, & Shuib, 2015; Romagnoli, Menna, Gruppiioni, & Bergamini, 2007). Mycotoxins are the secondary metabolites of mould subspecies growing on agricultural products, including spices at various stages of preparation from field to consumption, and depend for the growth on ecological conditions. Among 400 mycotoxins, aflatoxins (AF) are the most dangerous ones in terms of human health and are commonly seen in spices. Although 20 aflatoxin types have been identified, AFG₂, AFG₁, AFB₂ and AFB₁ are the most common ones and also, AFB₁ is the most toxic one. The International Agency for Research on Cancer has included AFB₁, one of the most dangerous AF, in its Group 1 classification owing to evidence of its carcinogenic effect in humans. (International Agency for Research on Cancer, 1993; Marin, Ramos, Cano-Sancho, & Sanchis, 2013; O’ Riordan & Wilkinson, 2008).

Previous studies on mesir paste gave information on the history of the paste (Asıl & Sar, 1984; Bayat, 1993, 1998a, 1998b; Çekin & Sertoğlu, 2007; Çokşar, Artik, Saltan, & Çoşkun, 2014; Đoğan, 2012; Nergiz, 1994; Nergiz & Yıldız, 1995; Şimşek, 2012), its chemical composition (Artik et al., 1999; Giritlioglu et al., 2010; Hışıl, 1994; Nergiz, 1994; Nergiz & Yıldız, 1995; Oksel et al., 1997; Oskay et al., 2010; Sauer, 1998), volatile compounds (Hışıl et al., 1996), antioxidative effects (Güven, 2010), antimicrobial effects (Oskay et al., 2010), texture (Ergönül, 2013) and rheological properties (Karaman et al., 2008). The evaluation of mesir paste containing various spices in terms of their mycotoxin content is of great importance, as there are no previous studies and/or reports on this subject. In this study, 42 mesir paste samples traditionally consumed in the Manisa province were collected from a limited number of manufacturers in order to evaluate their AFG₂, AFG₁, AFB₂ and AFB₁ content, contamination risk, and determine their compliance with maximum permitted limits and also contribute to the literature.

2. Materials and methods

2.1. Samples

A total of 42 samples of mesir paste were purchased from 7 firms in Manisa province from April 2014 to May 2015. The 6 different samples belonged to different batches were collected in each firm, and each sample weighed at least 1 kg. The samples were saved in airtight glass jars, and stored at 4°C until the analysis.

2.2. Chemicals and reagents

The mixed standard of AFG₂, AFG₁, AFB₂ and AFB₁ was supplied by Supelco (Bellefonte, PA, USA). The mixture in each bottle consists of 0.3 μg/mL AFG₂ and 1 μg/mL AFG₁, 0.3 μg/mL AFB₂ and 1 μg/mL AFB₁ in methanol. Working standard solutions were prepared daily from these standard solutions. The clean-up step immunoaffinity columns (AflaTest™) were purchased from VICAM, Watertown, MA, USA. Ultra-pure water was produced by a Milli-Q system from Thermo Scientific, Milford, MA, USA. Solvents like acetonitrile and methanol (HPLC grade) were purchased from Sigma Aldrich, St. Louis, MO, USA. All other chemicals and reagents were used at least of analytical grade. Due to the potential toxicity, all experimental supplies were disposable products.

2.3. Chromatographic apparatus

Chromatographic analysis was carried out by using an Agilent 1260 Infinity system (Palo Alto, CA, USA) with an auto sampler using a fluorescence detector. Chromatographic separations of AFs were achieved with 5 µm Thermo Scientific (Waltham, MA, USA) Hypersil™ ODS C₁₈, 250 × 4.6 mm column. The wavelengths for excitation and emission were 360 nm and 440 nm, respectively. The mobile phase was a mixture of water, methanol and acetonitrile (50:30:20%; v/v) with 350 μL of 4 M nitric acid and 120 mg KBr per 1 L of mobile phase. The flow rate was 1 mL min⁻¹, and column temperature was maintained at 25°C. The injection volume was 100 μL. Post-column derivatisation was carried out with electrochemically generated bromine in Cobra cell (Coring System Diagnostix, GmnH, Gernsheim, Germany) (AOAC, 2000).

2.4. Extraction of aflatoxins

The extraction of AFs from mesir paste was carried out as described in AOAC Official Method 999.07 (AOAC 2000) with some modifications. A 25 g of sample was mixed with 2.5 g of NaCl and blended with 150 mL methanol:water (80:20%; v/v) using a Waring blender at high speed for 10 min. The extract was filtered with filter paper (Whatman no: 4) and collected into clean flasks.

2.5. Separation with immunoaffinity columns

AFs’ clean-up was performed using immunoaffinity columns according to the method AOAC Official Method 999.07 (AOAC 2000) with slight modifications. A 5 mL of the filtrate was diluted with 20 mL phosphate buffered saline solution (pH: 7.3). The diluted extract (25 mL) was vortexed for 1 min and centrifuged at 4000 rpm/min at 4°C and then filtered through glass microfiber filter (1.5 μm, 110 mm, Vicam, Watertown, MA, USA). All filtrate was passed through an AflaTest™ immunoaffinity column attached onto a vacuum manifold at a rate of 2–3 mL min⁻¹ and washed with 15 mL ultrapure water at the same flow rate for cleaning. The column was quickly dried by passing air for 1.5 min. AFs were eluted from the column by applying 1 mL methanol and 1 mL ultra-pure water and collected in a HPLC vial and stored in a refrigerator at 4°C until analysis.

2.6. Method validation

The method validation parameters like linearity, limit of detections (LOD), and limit of quantifications (LOQ), recovery of AFG₂, AFG₁, AFB₂ and AFB₁ were determined (Iqbal, Rabbani, Asi, & Jina, 2014). Instrumental precision was demonstrated as instrumental repeatability.
(within-day precision) and intermediate precision (between-day precision). The linearity of the method was determined by five-point calibration curves. The calibration curves of AFG$_2$ and AFB$_2$ were linear between 0.058 and 5.184 µg kg$^{-1}$; AFG$_1$ and AFB$_1$ were linear between 0.192 and 17.28 µg kg$^{-1}$. The LODs based on a signal-to-noise S/N = 3/1 and LOQs were calculated with a signal-to-noise S/N = 10/1 as described in ISO11843 part 2 (British Standard, 2000). Relative standard deviation (RSD) percentages were calculated to determine for instrumental precision, in terms of instrumental repeatability and intermediate precision at one standard concentration on the same day with six-replicated and on the different days with three-replicated. The recoveries were ascertained by spiking 1–3 µg/kg of AFG$_2$ and AFB$_2$, 2–5 µg/kg of AFG$_1$ and AFB$_1$ in non-contaminated samples in two parallels at least an hour before the analysis. RSD percentages were calculated to determine for the method repeatability.

2.7. Statistical analysis

Experiments were performed triplicate using samples. The data of AFs were statistically analysed and presented as mean ± standard deviation (SD), and coefficient of determination (R$^2$) was determined by regression/correlation analysis using SPSS software (IBM, PASW Statistics 19, Armonk, NY, USA).

3. Results and discussion

3.1. Method validation

The method performance for AFs determination in mesir paste is summarised in Table 1. The results have shown good linear response with coefficient of determination $R^2 ≥ 0.99$ for both AFs. The calibration curves of AFG$_2$ and AFB$_2$ were linear between 0.058 and 5.184 µg kg$^{-1}$; AFG$_1$ and AFB$_1$ were linear between 0.192 and 17.28 µg kg$^{-1}$. Calculated LOD and LOQ were 0.05 and 0.15 µg kg$^{-1}$ for AFG$_2$ and AFB$_2$ and 0.03 and 0.09 µg kg$^{-1}$ for AFG$_1$ and AFB$_1$, respectively. They were well below the legal limits to be controlled. Instrumental precision was determined in terms of instrumental repeatability and intermediate precision at one standard concentration on the same day with six-replicated and on the different days with three-replicated. The % RSD value was calculated. % RSD of instrumental repeatability for AFG$_2$, AFG$_1$, AFB$_2$, and AFB$_1$ were 0.78, 0.95, 1.05 and 1.24, respectively. % RSD of intermediate precision for AFG$_2$, AFG$_1$, AFB$_2$ and AFB$_1$ were 2.22, 2.52, 2.67 and 3.06, respectively. Instrumental repeatability was under 2%, intermediate precision was under 5%. The retention times of AFG$_2$, AFG$_1$, AFB$_2$ and AFB$_1$ were 5.94, 6.64, 7.47 and 8.51 min, respectively. The recoveries were ascertained by spiking 1–3 µg/kg of AFG$_2$ and AFB$_2$, 2–5 µg/kg of AFG$_1$ and AFB$_1$ in non-contaminated samples in two parallels. For the first spike level: AFG$_2$ 88.40%, AFG$_1$ 96.96%, AFB$_2$ 118.66% and AFB$_1$ 68.00%. For the second spike level, recoveries were AFG$_2$ 92.77%, AFG$_1$ 70.09%, AFB$_2$ 90.39% and AFB$_1$ 69.43%. The method has shown good recoveries for AFs. The RSD percentages were ranged 2.42–10.91 for first spike level and ranged 5.57–8.41 for the second spike level for the four AF.

3.2. AFs in mesir paste

AFG$_2$, AFG$_1$, AFB$_2$ and AFB$_1$ analyses were conducted on a total of 42 mesir paste samples in 3 parallel samples. The occurrence and mean contamination of AF in mesir paste samples is shown in Table 1.

The results showed mediocre incidence of these mycotoxins in analysed samples. In mesir paste samples, AFG$_2$ values varied between 0.07 and 0.42 µg kg$^{-1}$, AFG$_1$ values varied between 2.02 and 10.11 µg kg$^{-1}$ and AFB$_1$ values varied between 0.04 and 0.11 µg kg$^{-1}$. AFB$_2$ was not detected in any of the samples. Total aflatoxin values in the samples ranged from 0.04 to 10.20 µg kg$^{-1}$, and the average was 5.52 µg kg$^{-1}$. Thirty of samples (71%) samples were below the AFG$_2$ detection limit (0.05 µg kg$^{-1}$), 12 (29%) samples were below the AFG$_1$ detection limit (0.03 µg kg$^{-1}$) and 20 (48%) samples were below the AFB$_1$ (0.03 µg kg$^{-1}$) detection limit, and also all of samples were below AFB$_2$ detection limit (0.05 µg kg$^{-1}$). In the light of the data obtained, it is seen that the results for the analysed samples, obtained

Table 1. Method performance for aflatoxins (AFs) determination in mesir paste.

| Analyte  | LOD (µg kg$^{-1}$) | LOQ (µg kg$^{-1}$) | Linearity         | $R^2$ | Range (µg kg$^{-1}$) | % RSD (instrumental repeatability) | % RSD (intermediate precision) |
|----------|------------------|------------------|------------------|------|---------------------|----------------------------------|--------------------------------|
| AFG$_2$  | 0.05             | 0.15             | $y = 98.0015x + 0.0033$ | 0.998| 0.058–5.184          | 0.78                             | 2.22                            |
| AFG$_1$  | 0.03             | 0.09             | $y = 22.7301x + 0.0178$ | 0.990| 0.192–17.28          | 0.95                             | 2.52                            |
| AFB$_2$  | 0.05             | 0.15             | $y = 88.4237x + 0.0118$ | 0.994| 0.058–5.184          | 1.05                             | 2.67                            |
| AFB$_1$  | 0.03             | 0.09             | $y = 44.6565x + 0.0334$ | 0.991| 0.192–17.28          | 1.24                             | 3.06                            |

Table 2. Occurrence and mean contamination of aflatoxins in mesir paste (n = 42).

| Parameter                          | AFG$_2$ | AFG$_1$ | AFB$_2$ | AFB$_1$ | AF$_{Total}$* |
|-----------------------------------|---------|---------|---------|---------|---------------|
| Positive samples** n (%)          | 12 (29%)| 30 (71%)| –       | 22 (52%)| 32 (76%)      |
| Samples above 10 µg kg$^{-1}$ n (%)| –       | –       | –       | –       | 4 (9.5)       |
| Range (µg kg$^{-1}$)              | LOD-0.42| LOD-10.11| <LOD   | LOD-0.11| LOD-10.20     |
| Mean ± SD (µg kg$^{-1}$)          | 0.24 ± 0.17 | 5.74 ± 2.67 | –       | 0.07 ± 0.02| 5.52 ± 3.01   |

*Sum of AFG$_2$, AFG$_1$, AFB$_2$, and AFB$_1$.
**AFs’ level > LOD.
*Suma de AFG$_2$, AFG$_1$, AFB$_2$ y AFB$_1$.
**Nivel de AF> LOD.
from different producers, and is related to the varying formulations in the preparation of mesir paste.

There are legal regulations in Turkey and in the European Union (EU) for levels of AF (total AF and AFB$_1$) permitted for nuts, cereals, dried fruits and some spices, but there are no official regulations in Turkey or in the European Union regarding the maximum allowable total AF and AFB$_1$ levels in mesir paste. The results of the samples tested here were below the maximum limit (ML) values determined for spices (5 µg kg$^{-1}$ for AFB$_1$) for comparison with the results obtained in this study. However, the mean value of total AF was found to be 5.52 µg kg$^{-1}$ in samples; 4 samples (9.5%) were above the EU MLs (10 µg kg$^{-1}$ for total AFs). In an extensive literature search, no studies or reports were found that examined AF contamination in mesir paste. Therefore, the other mycotoxin contamination must consider also traditional products like mesir paste.

It is believed that careful selection of raw materials especially spices during the preparation of mesir paste and fulfilling recommended production and storage requirements may prevent the emergence of AF, which have harmful effects on health. Also, the examination of traditional products in terms of mycotoxins will help raise awareness among both producers and consumers of these products about their possible contamination.

4. Conclusion

In this study, aflatoxin contamination risk for mesir paste was evaluated, considering the negative effects of AF on health. Mesir paste is a traditional confectionery product in Manisa and fondly consumed, having claimed functional properties, including antioxidant and energizing ones. This is the first study that focused on determining AFG$_2$, AFG$_1$, AFB$_2$, and AFB$_1$ contamination levels in mesir paste. Although mesir paste carries aflatoxin contamination risks, it was not previously investigated in any study in terms of AF and other mycotoxins. This study revealed positive results in terms of aflatoxin contamination, and these results suggest the necessity of investigating this kind of traditional product in terms of its mycotoxin content. Also, the examination of traditional products in terms of mycotoxin content will help raise awareness in both the producers and the consumers of these products. When the aflatoxin contamination were determined of the samples, it was obviously seen that some producers have high awareness because aflatoxin contamination was not found in all batches. Measuring AFG$_2$, AFG$_1$, AFB$_2$, and AFB$_1$ contamination in traditional mesir paste revealed the necessity of paying greater attention and care in the selection of spices used in mesir paste production and in their storage conditions, from procurement to production.

Disclosure statement

No potential conflict of interest was reported by the author.

ORCID

Özlem Çağındı http://orcid.org/0000-0002-6436-9208

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