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Validation of simultaneous analysis method for determination of aflatoxins in olive oil by high performance liquid chromatography-fluorescence detector

SamanehNabizadeh[a], Nabi shariatifar[a*], Shahram Shoeibi[b], Gholamreza Jahed Khaniki[a], Ramin Nabizadeh Nodeh[a], Esmail Shokoohi[c]

[a] *Department of Environmental Health Engineering, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran*
[b] *Food and Drug Laboratory Research Center, Food and Drug Organization, Ministry of Health and Medical Education, Tehran, Iran*
[c] *Department of Basic Science,Faculty of Science, Islamic Azad University of Ahar Branch, Ahar, Iran*

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ABSTRACT

Vegetable oils, especially olive oil, are used as healthy fats in modern nutrition. The study aim was to optimize and validate a methodology for the simultaneous analysis of aflatoxins B1, B2, G1 and G2 in olive oil by high performance liquid chromatography-fluorescence detector (HPLC-FLD). The procedure of the validation for the determination of aflatoxin B1, B_2, G1 and G_2 in traditional olive oil was performed. The recovery of AF_S (B_1, B_2, G_1 and G_2) in three spiking levels was in range of 84.45-109%. The repeatability of measurements, represented by the standard deviation (RSDr) was 8.11 %, 8.05%, and 3.76 % at the spiking levels of 0.5ng/g, 2 ng/g and 6 ng/g, respectively. The limit of quantification (LOQs) and limit of detection (LOD) were 0.5ng/g and 0.16 ng/g. The results of validation parameters show that the procedure is suitable and simple for the determination of aflatoxins B_1, B_2, G_1 and G_2 in traditional olive oil and can be implemented for the routine analysis.

1. Introduction

Mycotoxins are a group of fungal metabolites that are found in many plant foods. These toxins may accumulate in animal foods. Chronic ingestion of mycotoxins may lead to occurrence of genetic disorders in future generations. Physiological effects of mycotoxins (carcinogenic, acute hepatitis, nephrotoxic and immune system disorder) have been reported in many books and studies (1-3).

It is now well established that mycotoxins are responsible for major epidemics in human and animals, especially in recent years. Olives can be contaminated with a wide variety of molds (Aspergillus and/or Penicillium), also olives could support mycotoxin production, such as aflatoxin and ochratoxin (4-7) that often are stored for a long time in conditions that promote the growth of molds. The probability of aflatoxin production can be enhanced by parameters such as warm temperatures, high relative humidity and moisture, drought, insect activity, soil type and damaged kernels (8,9). Sometimes after picking olives, seeds accumulated on brief fermentation for 5-6 weeks and then extract the oil. This stage is also important in terms of appropriate conditions of molds' growth and production of mycotoxins. Mold can grow during the harvest, processing, transport or

*Corresponding author. Tel.: +989125091928
E-mail address: nshariati@tums.ac.ir(N. Shariatifar)

storage of olive. However, Thompson and Henke (2000) noted that aflatoxins can be produced without considering storage time, type of storage container and weather conditions (10).

Fanelli and Fabbri (1989)demonstrated the key role of lipids on fungal growth and lipid oxidation that also have an effect on AF biosynthesis (11). The seeds surface lipids represent a very important carbon source for fungal growth(12). Liquid chromatography is the technique of choice for AF separation (5, 13-15). Techniques such as thin-layer chromatography (TLC), enzyme-linked immunosorbant assay (ELISA), Capillary electrophoresis (CE), high performance liquid chromatography (HPLC) and liquid chromatography-tandem mass spectrometry (LC-MS/MS) are practical methods for detection and analysis of aflatoxins. All these methods dedicated extraction and clean-up procedures. HPLC and LC-MS/MS as advanced technique are commonly used.

Developed LC-MS/MS method is selective and accurate but is not as sensitive as HPLC coupled to fluorescence detection (FLD), which is expensive and requires special analyses education. Our objective in this study was investigation of efficiency of analytical method for detection and quantification of the four naturally produced aflatoxinsB1, G1, B2, and G2 in traditional olive oil.

2. Materials and Method

2.1.Chemicals

The solvents for obtaining the mobile phase and the mixture of the buffer, such as acetonitrile, methanol, toluene, NaCl, were supplied by Merck (Darmstadt, Germany). HPLC-grade water was obtained from Prime for Scientific Services, Laboratory Food Control, Tehran, Iran.

The standard of aflatoxinB1, B2, G1, and G2 were obtained from Sigma; A 6636 (AFB_1), A 9887 (AFB_2), A0138 (AFG_1) and A0263 (AFG_2).

2.2. Sample preparation

Five gram of sample was suspended in 10 mL of 80% methanol and vortexed for few minutes. After filtration, 1 mL of the mixture were added to 4 mL of phosphate buffered saline solution and passed through the immunoaffinity columns for the sample clean-up.

2.3. Apparatus

a) Shaker - Heidolphunimax 2010

b) Centrifuge - Thermo (Heraeus Bioguge stratus)

c) Spectrophotometer (CECIL, 7500)

d) Filter paper - grade 597, 125 mm (Schleicher & Microscience)

e) Glass microfiber filter paper, 124mm (Schleicher &Schull)

f) Immunoaffinity column, Aflatest WB^{SR} (G 1068, Vicam)

g) Column manifold (vicam)

h) HPLC system (waters 600) with a fluorescence detector (waters 2475), excitation wave lengths 365 nm, emission wave lengths 435 nm; LC column,C_{18}Chromolith, 100×4.6 mm; mobile phase, methanol - acetonitrile – water (29 + 4 + 67 %); run time, 19 min; flow rate, 1 ml/min. Post column derivatization device - with electrochemically generated bromine eg. Kobra cell.

2.4. Preparation of aflatoxin standards

Aflatoxin stock standard solutions were prepared by dissolving 1mg of each aflatoxin standard (B_1, B_2, G_1 and G_2) with toluene: acetonitrile (90:10, v/v),concentration of each AFs was, 200 µg/ml. Final concentration of each aflatoxin solution was determined by absorption spectrophotometer reading at 200 to 300 nm and following formula.

$C_{AFs} = (A_{max}. MW. 1000)/\varepsilon$

C_{AFs} =Aflatoxin concentration

A_{max} = Maximum absorbance

MW = Molecular weight for each aflatoxin

ε = Molecular absorptivity

Spike aflatoxin standards were prepared at four concentrations: AFB_1 and AFG_1 ,1 µg/ml; AFB_2 and AFG_2, 0.25 µg/ml. Intermediate aflatoxin standards were prepared from spike aflatoxin standards at four concentrations: AFB_1 and AFG_1 ,100 ng/ml; AFB_2 and AFG_2, 25 ng/ml. Six working standard solutions were prepared by dissolving each aflatoxin standard (B_1, B_2, G_1 and G_2) with water: methanol (60:40, v/v), concentration of each AFs according to Table 1.

Standard solutions wrapped in aluminum foil to prevent gradual break down of aflatoxins under UV light and kept under protected conditions(16).

The olive oil sample (approximately 200 ml) was purchased from the major vegetable oil producing in Tehran.

2.5. Method of analysis

We poured 5.0 g olive oil in four tubes, three tube spikes with AF at 0.5,2 and 6 ng/g and one tube as blank. We used the intermediate standard as the spiking solution for recovery studies. We waited one hour to toxin linked to samples; and then added 1.0 g NaCl and 25 ml methanol 55%, then shook the tubes at 400 rpm for 1min (Centrifuge at 5000 mm/s^2(g value) for 10 min).We discarded oil layer and passed the aqueous through filter paper, separated 15 ml and mixed with 30 ml water. Then passed through glass microfiber filter paper and collected 30 ml filtrated aqueous.

We removed top cap from afla-test column and connected to syringe reservoir of manifold. Then transferred 30 ml filtrate in to the syringe and passed filtrate through column by gravity force, then added 10 ml methanol 10% as washing solution. We dried the column by passing air, placed 2ml vial in to the manifold under column. Then eluted the column with 0.6ml HPLC grade methanol, after passing the last drop, waited 1min,eluted with additional 0.6ml HPLC grade methanol, dried the column by passing air and then added 0.8ml water to vial, closed the vial cap tightly and vortex. We put the vial in cool and dark place until injection to HPLC.

2.6. HPLC analysis and calculation

120µl AF working standard and test solution were injected to HPLC column. Peaks were isolated respectively; AFG_2, AFG_1, AGB_2 and AFB_1.Derivativedevice AFB_1 and AFG_1 to AFB_{2a} and AFG_{2a}shows enhanced fluorescence compare with AFB_1 and AFG_1(17).

Concentration of each aflatoxin in olive oil sample was determined by standard curve and following formulae; standard curve for each AF was drawn with working standard solutions (Figure 3).

AF (ng/g) = [(R – I)/S] / D
R = Sample peak area
I = Intercept
S = Slop
D = Dilutionfactor

Recovery % = [AF (ng/g) / C] × 100
C = spiking solution at threelevels 0.5, 2 and 6ng/g

3. Results

Figure 1 has shown the chromatogram of AF working standard solution (AF 1.25ng/ml) and Figure 2shown the chromatogram of an olive oil sample, spiked with AF (2ng/ml), it is evident that the peak of aflatoxin G_2, G_1, B_2 and $B_1$are well separated. As a result, the selectivity of the method is considered satisfactory.

RSD_R are the relative standard deviation calculated from results generated under repeatability and reproducibility conditions [(S_R / Recovery) ×100] and were compared to the reference value, required in Decision L70/31, 2006 (European Union). RSD_R was compared to the values derived from Horwitz Equation and RSD_r was calculated as 0.66 times precision RSD_R.

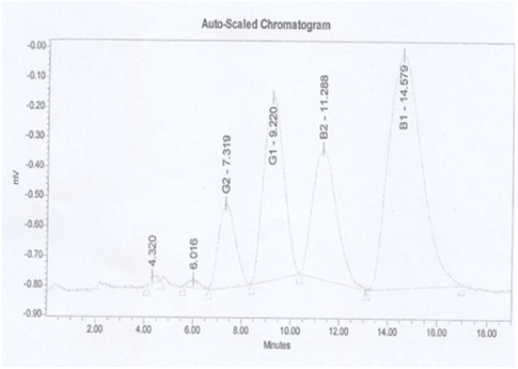

Figure 1. Liquid chromatogram of AFs working standard solution (AF 1.25 ng/ml)

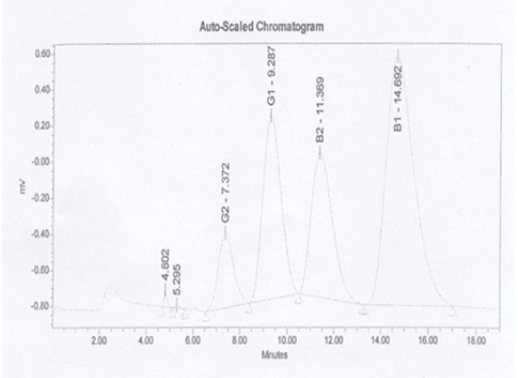

Figure 2. Liquid chromatogram of olive oil sample, spiked with AFs (0.5ng/ml)

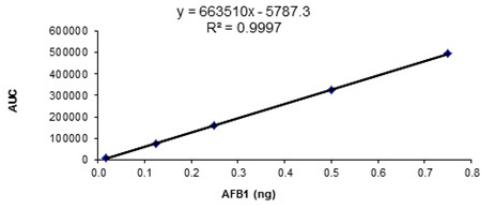

Figure 3. AFB_1 calibration curve

Table 1. Concentration of working standard solution, ng/ml

Working standard solution	AFB_1	AFB_2	AFG_1	AFG_2	AF_S
1	0.18	0.045	0.18	0.045	0.45
2	1.25	0.312	1.25	0.312	3.124
3	2.5	0.625	2.5	0.625	6.25
4	3.75	0.937	3.75	0.937	9.374
5	5	1.25	5	1.25	12.5
6	7.5	1.875	7.5	1.875	18.75
R^2	0.999	0.999	0.998	0.999	
slope	663510x-5787.3	1E+06x-4192.3	312859x-4541.7	470645x-676.8	

Table 2. LOQ and LOD of AF_S

Aflatoxins	LOQ	LOD
AFB_1	0.5	0.16
AFB_2	0.12	0.04
AFG_1	0.5	0.16
AFG_2	0.12	0.04

LOD: Limits of detection, LOQ: limit of quantification

In this study recovery of added AFS were more than 92%, 85% and 84% at the spiking levels of 0.5ng/g, 2 ng/g and 6 ng/g, respectively. Limits of quantification (LOQ) and limits of detection (LOD) were according to Table 2. RSD_r and RSD_R were according to Table 3. The obtained values are below the reference values.

4. Discussion

Mycotoxin analysis in developed countries has in recent years become almost invariably based on HPLC, because it offers good sensitivity and precision, coupled with ease of automation.

Therefore, liquid chromatography is the technique of choice for AF separation (5,13-15). A number of toxins have natural fluorescence, for example; aflatoxin, ochratoxin and citrinin can be detected in HPLC- FLD. One reason for the use of HPLC is the very low limit of detection (LOD); this method improves the sensitivity of aflatoxin measurements. Low detection limits of aflatoxin in foods and animal and human tissues are important for calculate health risk assessment (18).

Analytical methods based on immune-affinity column clean-up and liquid chromatography represents the most recent and advanced tool for the determination of mycotoxins.

Le Tutour et al (1982) determined AFB1 in olive oil utilizing TLC and their detection limit was 4 mg/kg(19). Miller et al (1985), Marjerus et al(2009) and Ferracane et al (2007) used HPLC for determining levels of AFB1 in oils, detection limits were 5 mg/kg and 0.36 mg/kg, respectively (9, 20-22). Ferracane et al. (2007) and Lei Bao et al (2010) used the immunoaffinity column for the clean-up and detection limits for AFB_1were 56 Pg/g and 0.1ng/g (21,23,24). In this study, immunoaffinity column was used and detection limit obtained for AFB_1 and AFG_1, 0.5ng/g and for AFB_2 and AFG_2 was 0.125 ng/g.

The developed method to better optimize of all the chromatographic factors, had to involve the best way for sample preparation during extraction process with acetonitrile /toluene. There were also solvent mixtures that were tested to obtain a high and stable extraction for the full group of analytes that are present in a very complex biological mixture.

Table 3. Average recoveries, RSD_r and RSD_R of AF_S spiked to olive oil

Spiking solution, ng/g		Recovery,%	RSD_r,% (n=3)	RSD_R,% (n=9)
0.5	AFB_1	92.26 ± 0.07	8.71	15.4
	AFB_2	100.33 ± 0.01	12.15	13.37
	AFG_1	109± 0.04	5.91	7.68
	AFG_2	94.66± 0.01	5.68	8.21
2	AFB_1	87.19±0.23	6.38	10.85
	AFB_2	88.04± 0.04	9.59	11.21
	AFG_1	88.64 ± 0.23	7.33	11. 5
	AFG_2	85.99± 0.04	8.97	9.58
6	AFB_1	87.65 ± 0.72	3.83	12.81
	AFB_2	86.77± 0.11	3.81	8.78
	AFG_1	86.38± 0.77	3.54	14.91
	AFG_2	84.54± 0.15	3.89	12.07

RSD: Relative standard deviation

According to the values obtained during the proposed extraction procedure for the selected aflatoxin, acetonitrile /toluene had engaging acceptable and sustainable and recovery (25).

HPLC method validation showed that the simple modular system capable of solving complex simultaneous measurement of aflatoxin, and quantitative restrictions, although a simple fluorescence detector was used for multiple channels.

5. Conclusion

The validated method showed that a simple HPLC liquid chromatography as modular system is capable to handle such a complex simultaneous determination of aflatoxin, and the quantification limits, although a simple fluorescence detector for multiple channels was used. The procedure for method optimization included the liquid-liquid extract ion process and is settled longer to allow more samples to be analyzed and to check the status of the column, after the washing stage for each injection.

In the present study the detection limits were 0.16 ng/g for AFB_1, AFG_1 and 0.05 ng/g for AFB_2, AFG_2.

The parameters obtained in the validation procedure show that the procedure described and validated is suitable for the determination of aflatoxinvB_1,B_2 ,G_1 and G_2 in traditional olive oil. The LOQ obtained by chromatographic parameter optimization, for each of the mycotoxins were 0.5ng/g and the LOD obtained by chromatographic parameter optimization, for each of the mycotoxins were 0.16 ng/g in traditional olive oil.

Conflict of Interests

Authors have no conflict of interest.

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