Pressurized Fluid Extraction for Quantitative Recovery of Aflatoxins B1 and B2 from Pistachio

Hassan S. Ghaziaskar* , Ali Sheibani

Department of Chemistry, Isfahan University of Technology
Isfahan, I.R. Iran, 84156-83111. ghazi@cc.iut.ac.ir

Abstract

A pressurized fluid extraction method for the extraction of aflatoxins B1 and B2 from contaminated pistachio samples was developed using a modified supercritical fluid extractor. The parameters of temperature, pressure, and flow rate of the solvent were optimized for the extraction process. The pressure variation had slight effect on the aflatoxins extraction yield. Methanol-water (80:20) solution as the extraction solvent at flow rate of 0.5 mL/min and temperatures of higher than 80^{o}C were obtained for efficient extraction of aflatoxins B1 and B2 from spiked or naturally contaminated pistachio samples. Purification and separation of the extracted aflatoxins were also performed by the method approved by the Association of Official Analytical Chemists (AOAC). The developed method offered almost the same repeatability, while the extraction yield or recovery of the analytes was about 20% higher corresponding to those obtained with the AOAC method.

Keywords: Aflatoxins B1 and B2; Pressurized Fluid Extraction; Accelerated Solvent Extraction; Aflatoxin analysis; Pistachio

1. Introduction

Aflatoxins are a group of highly oxygenated heterocyclic compounds which are produced as secondary metabolites by the food spoilage fungi, particularly *Aspergillus Flavus* and *Aspergillus Parasiticus* growing on a variety of food products, such as nuts, grains, dried fruits, and spices [1-2]. They are highly toxic and carcinogenic compounds that cause disease in livestock and human. Among these, aflatoxin B1 (AFB1) is one of the most toxic and most carcinogenic [3, 4].

Generally, the analysis of aflatoxins consists of two major steps: (a) the extraction of aflatoxins from the sample matrix and (b) quantitative determination of extracted aflatoxins by different methods. Extraction is an important step in the determination of aflatoxins in different samples. Liquid-solid extraction is one of the basic procedures for aflatoxins analysis in agricultural products. In this procedure, extraction is achieved based on the solubility of aflatoxins in different organic solvents. The solvent ideally should extract the analyte quantitatively with as little additional compounds as possible in order to avoid interferences in the final determination step [5].

Methods based on thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC) with various detection systems are used for aflatoxins determination step [6, 7]. As an official method for aflatoxins analysis of pistachio samples recommended by the Association of the Official Analytical Chemists (AOAC) classical liquid-solid and liquid-liquid extractions are used for the extraction of the aflatoxins and clean up of the extract, respectively and TLC/HPLC is used for separation and quantitation of the extracted aflatoxins [8].

Pressurized fluid extraction (PFE), with trade name of accelerated solvent extraction (ASE), uses solvents at elevated pressures and temperatures to achieve complete extraction of analytes from solid and semi-solid samples with lower solvent volumes and shorter extraction times. The PFE method has been applied for the quantitative extraction of different analytes from sample matrices such as soils, sediments, and also for the analysis of food, biological, and environmental samples [9, 10].

In this work, we have developed a PFE method for the quantitative extraction of aflatoxins from pistachio samples at high temperature and pressure using a modified SFE apparatus. This method compared with the conventional extraction methods has significant advantage for the extraction of aflatoxins that is higher recovery. The effect of parameters such as temperature, pressure, flow rate,

and the type of the extraction solvent on the PFE yield of the aflatoxins was investigated. The accuracy and the precision of the method were established by the analysis of spiked and naturally contaminated samples. Validation of the developed method was accomplished by HPLC analysis of different similar matrix.

2. Materials and Methods

2.1 Materials

Pistachio (Pistachia Vera L) was obtained from Rafsanjan area of Iran. The pistachio kernels were ground passed through mesh 16 sieve, to facilitate mass transfer of analytes from the matrix during the extraction process. It was kept within a sealed bag in a refrigerator until it was used. The extraction kinetic is mainly controlled by the kernel particle size, moreover, the sieving is an important step to obtain reproducible extraction yield.

2.2. Chemicals

Methanol, chloroform, acetone, n-hexane, acetonitril, phosphate-buffered saline (PBS), and potassium bromide supplied by Merck Co. Aflatoxin standards (AFB1 and AFB2) were purchased from Sigma Chemical Co. Stock standard solutions were prepared in concentrations of 8-10 (μg/mL) by dissolving the solid standards in benzene-acetonitril (98:2). The concentrations of aflatoxin standard solutions were checked by UV-Vis absorption spectrophotometer as explained in the AOAC method [11]. Working standard solutions of AFB1 and AFB2 were prepared daily by dilution of stock standard solutions. Aflatoxin standard solutions was kept in a refrigerator at temperature lower than 5^{o}C till was used.

2.3. Apparatus

The SFE apparatus which is described in detail elsewhere [12] was modified to accomplish PFE. The TLC-scanner 3 and the UV-lamp (at 254 and 366 nm) were from Camag (Switzerland). The pre-coated silica gel 60 TLC aluminum plates were purchased from Merck Co. A HPLC system (waters, model 1525, USA) with a multi-wavelength fluorescence detector (waters model 2475) and a Kobra cell for the post-column derivatization was used. UV-Vis spectrophotometer (Model V-570) was from Jasco Co. An analytical balance (Sartorius, model LP 1200s, Germany) was used for the gravimetric determinations of the PFE yield (ng of aflatoxins/g of sample) based on the sample dry weight. The conditioned immunoaffinity column (IAC AflaTest®, Vicam, USA) for cleaning up the sample before aflatoxin analysis was used.

2.4. Preparation of the pistachio samples for PFE

The ground pistachio kernels were mixed with crushed Pyrex glass (that is passed through a mesh 40 sieve and retained by a mesh 60 sieve) to increase the contact surface between the extraction solvent and the sample. Sodium chloride was added (as much as 5% w/w of the sample), as recommended in the official procedures [8]. The salt probably increases the ionic strength of the solvent and the extraction selectivity and yield of the aflatoxins in the liquid-solid extraction process. The fat content of the pistachio nuts is more than 50% [13], therefore, it was defatted with solvents such as n-hexane during the extraction process of aflatoxins. Usually, n-hexane can be either added to the extraction solvent or to the samples into the extraction cell. Since the extraction solvent and n-hexane are immiscible and cannot be pumped together. Therefore, n-hexane was added to the samples into the extraction cell before the PFE was performed.

2 5. Type of solvent

The choice of the extraction solvent depends on the chemical properties of the matrix and the toxin. Often, mixtures of some solvents with water have proved to be very effective for the aflatoxin extraction [14]. The solubility of many toxins in water is low but aqueous solvents can better penetrate into hydrophilic tissues that result in an increase in the PFE extraction yield. The most commonly used solvents for aflatoxins extraction are mixtures of chloroform-water [15], methanol-water, [16] or acetonitril-water [17]. To avoid chlorinated solvents and to use less hazardous solvents, the solutions

of 80% and 55% methanol-water were used as the extraction solvent. The solution of 55% methanol-water led to a lower extraction yield [18]. Therefore, a solution of 80% methanol-water was used throughout the work.

2.6. The PFE procedure

The pistachio samples (~7 g) were loaded into the 10 mL stainless steel high pressure cell. Cotton wool was packed at the exit end of the cell to prevent transfer of solid samples to the tubing and clogging of the system. The extraction program consisted of filling the extraction cell with methanol-water (80:20) solvent within 5 min (static time) followed by running the extraction solvent over the sample at different flow rates, temperatures, and pressures. The mixture of extraction solvent and n-hexane were collected at the system exit within two phases. The fat of the pistachio samples was extracted by n-hexane (upper phase) and it is separated and discarded. Aflatoxins were extracted by the methanol-water (80:20) solvent (lower phase), and was purified with chloroform (clean up step), separated on the TLC plate (as recommended by the AOAC method) [8], detected and quantified using Camag TLC scanner 3 with Cats software for fluorescence measurement (section 2.8). Extraction yield were calculated as ng of Aflatoxins extracted per g of sample (i.e. ppb).

2.7. The AOAC extraction procedure

The ground sample pistachio (50 g) was blended with 2.5 g NaCl, 200 mL methanol (80% in water) and 100 mL of n-hexane for 5 min. 100 mL of the slurry solution was then centrifuged to remove the fatty phase, i.e. the upper phase. 25 mL of the lower phase, after filtration, was shaken for 1 min with 25 mL of chloroform in a separatory funnel. Finally, the chloroform phase (the lower phase) was separated and evaporated at the temperature of less than 50^{o}C.

2.8. Determination of aflatoxins by TLC method

The extracted aflatoxins were redissolved in 200 µL mixture of benzene-acetonitril (98:2) and then 5 µL of the solution was spotted on the TLC plate. The working standards were also spotted on the same plate and the aflatoxins were separated by TLC method using chloroform-acetone (90:10) as the developing solvent. The developed plate was scanned using the TLC scanner equipped with a mercury lamp at the wavelength of 366 nm as a source for fluorescence measurement. Determination of aflatoxins level in the samples was performed by comparing the area of the chromatographic peak of the samples and the standard solution of aflatoxins.

2.9. *Extraction and clean up procedure for HPLC analysis*

A 50 g of ground pistachio sample was blended with 2.5 g NaCl, and 200 mL solution of 80% methanol in water for 5 min and then the mixture was filtered on Whatman filter paper. The sample extract was diluted with 25 mL of distilled water and 5 mL PBS with pH=7.4. Finally, 20 mL of it was cleaned up on the immunoaffinity column activated with 20 mL of PBS by gentle syringe pressure at a flow rate of 1 mL/min and then the column was washed with 20 mL of deionized water. The column was dried by blowing air through it for 2 to 3 s with a syringe. The aflatoxins were slowly eluted from the column with 2 mL of HPLC grade methanol and then was diluted with 2 mL of deionized water [19, 20].

2.10. Determination of aflatoxins by HPLC method

Aflatoxins were isocratically separated using an HPLC (Waters model 1525), with an ODS column (250 × 4.00 mm), a fluorescence detector and 50 µL of sample injection. The mobile phase was 60% deionized water, 20% acetonitrile, and 20% methanol with the addition of 350 µL of 4 M HNO_3 and 120 mg of KBr at a flow rate of 1 mL/min. The fluorescence detector was set at the excitation wavelength of 362 nm and emission wavelength of 450 nm. Quantification of each toxin was performed by measuring their peak areas and comparing them with their relevant standard calibration curve [21].

3. Results and Discussion

3.1. The effect of extraction temperature

The temperature of the extraction is one of the important parameters that affect the extraction yield. Increasing the temperature of the extraction increased the extraction yield of aflatoxins, as shown in Figure 1. Based on the thermodynamic principles, increasing the temperature can lower the viscosity of organic solvents and also the strong aflatoxin-matrix interactions, resulting in higher penetration of organic solvent into the matrix that lead to a higher extraction yield. Furthermore, an increase in temperature could increase the solvating capacity of the extraction solvent.

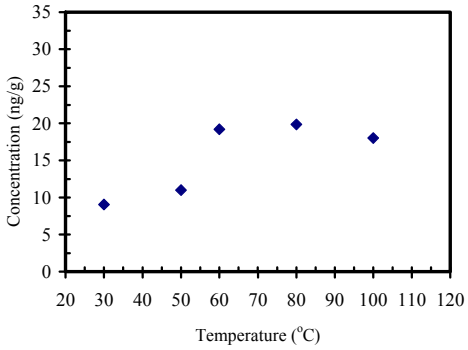

Figure 1. The extracted total aflatoxins (ng/g) versus temperature at the pressure of 50 bar and the flow rate of 1 mL/min.

3.2. The effect of flow rate

The extraction solvent was passed through the samples at different flow rates. As shown in Figure 2, increasing the flow rate of the extraction solvent had significant adverse effect on the extraction yield of aflatoxins. The PFE yield of aflatoxins was higher at the lower flow rates. Although the extraction time increased at the lower flow rates, but higher extraction yields have been achieved because of the enhanced contact time of solvent and the samples. At the lower flow rate, the consumption of the extraction solvent may also be reduced.

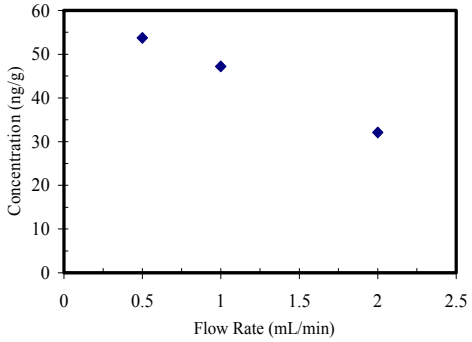

Figure 2. The extracted total aflatoxins (ng/g) versus the flow rate of the extraction solvent at the pressure of 50 bar and the temperature of 60°C.

3.3. The effect of pressure

The use of high pressure allows solvent to remain liquefied above its boiling point and also increases the solvent penetration into the sample matrix and as a result the extraction yield improves. The change in the PFE extraction pressure had a little effect on the extraction yield over the range of 10-100 bar. This is in agreement with what has been observed for the PFE of pesticides from soil [22]. In order to keep the extraction solvent as liquid at temperatures higher than the boiling point (61^{o}C for methanol) and also to achieve high yield, pressure of 50 bar was applied.

3.4. Comparison of the AOAC and the PFE-TLC method

Many natural samples contaminated with aflatoxins have been analyzed by the AOAC [8] and the PFE-TLC method. The PFE-TLC method presented is applicable for the quantitative determination of aflatoxins in the pistachio samples. As mentioned previously, these two methods are different in the extraction step but the clean up procedure, separation, and quantification steps are similar. As shown in Table 1, the analysis of several samples by the developed method showed that the PFE extraction yield is superior in comparison with the AOAC method. For most of the results from Table 1, t_{cal} is larger than t_{cri} at the 95% confidence level at 4 degrees of freedom. The t test showed a significant difference between the results of two methods. This difference could be related to the higher extraction temperature and efficient contact of the extraction solvent with the sample.

Analysis of naturally contaminated pistachio samples (n=5) was performed and the relative standard deviation (RSD) was calculated as the repeatability of the PFE method. The RSD is comparable with what has been achieved by the AOAC method, respectively 13.5 and 12%.

Recovery measurements were carried out by spiking non-contaminated samples with 15 (ng/g) AFB1 standard samples. The recovery of the developed PFE-TLC method was superior in (about 20%) comparison with the AOAC method (~80%).

Supercritical fluid extraction (SFE) technique had a poor recovery (i.e. 77.3% for AFB1) [23] in comparison with the developed PFE method. Moreover, the variables of temperature and pressure are less important in the PFE method in comparison with a SFE due to difficulties in maintaining supercritical conditions that is not so critical in the PFE [24].

In order to validate the PFE-TLC method, peanut samples as other similar matrix were also analyzed, and compared with that obtained by the AOAC method (Table 2). Furthermore, the aflatoxins of pistachio samples were extracted by the PFE method, purified with immunoaffinity column and quantified by HPLC method (section 2.9 and 2.10). The results of two methods (PFE-HPLC and HPLC) are listed in Table 2. According to the results of this Table, the accuracy of the method is acceptable.

Table 1
Comparison of the AOAC and the PFE-TLC methods in terms of total aflatoxins B1 and B2 (ng/g ± SD[a]) extracted from pistachio samples and relative increase in the extraction yield.

Pistachio Samples	Method		Increase in yield (%)
	AOAC	PFE-TLC	
1	N.D[b]	2.3 (±0.3)	-
2	8.3 (±1.0)	14.0 (±1.9)	68.7
3	13.1 (±1.5)	21.3 (±2.7)	62.6
4	14.7 (±1.7)	19.0 (±2.8)	29.2
5	15.7 (±2.0)	19.2 (±2.8)	22.3
6	73.0 (±7.3)	96.4 (±8.0)	32.0

a) SD = Standard deviation; b) Not detected.

Table 2
Comparison of the AOAC and the PFE-TLC methods in terms of total aflatoxins B1 and B2 (ng/g) extracted from peanut samples and the HPLC analysis of pistachio samples for validation of the developed method.

Type of Sample	Method	
Peanut	AOAC	PFE-TLC
1	21.0	39.6
2	18.0	31.6
3	3.7	5.1
Pistachio	HPLC	PFE-HPLC
1	22.6	30.4
2	11.1	17.6

4. Conclusion

A PFE-TLC method was developed for the extraction of aflatoxins in pistachio samples. The developed PFE method has a 20% higher extraction yield or recovery for aflatoxins in comparison with the AOAC method using similar extraction solvent. The accuracy of the developed method was validated by the HPLC analysis and use of peanut samples as other similar matrix. Increasing temperature and lowering flow rate enhanced the extraction yield. The temperature of 80^{o}C, flow rate of 0.5 mL/min were found as optimum PFE parameters. Acceptable repeatability (RSD = 13.5%) was achieved by the developed method that was comparable to the AOAC method.

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