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# Occurrence of Aflatoxins in Tigernuts and Their Beverages Commercialized in Spain

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A method based on matrix solid phase dispersion extraction was applied to determine aflatoxins $B_1$, $B_2$, $G_1$, and $G_2$ from tigernuts and tigernut beverages. Recoveries of each aflatoxin from tigernut (spiked at 10 $\mu$g/kg level) and from tigernut beverages (spiked at 10 $\mu$g/L level) ranged from 72.3 to 82.1% and from 74.0 to 86.3%, respectively. The limits of quantification ranged from 0.21 to 1.49 $\mu$g/kg (for tigernuts) and from 0.13 to 0.57 $\mu$g/L (for tigernut beverages) studied using liquid chromatography with fluorescence detection. The proposed extraction method followed by liquid chromatography−fluorescence detection determination was applied to 37 and 25 samples of tigernuts and tigernut beverages, respectively, 3 positives being found in each category.

**KEYWORDS: Aflatoxins; tigernuts; tigernut beverages; MSPD; fluorescence detection; liquid chromatography**

## INTRODUCTION

*Cyperus esculentus* is a species of sedge native to warm temperate to subtropical regions of the northern hemisphere. There are several varieties such as *esculentus* from the Mediterranean region east to India, *hermannii*, *leptostachyus*, and *macrostachyus* from the United States, and *sativus* from Asia. All of these varieties are able to grow also in Ghana, Nigeria, Burkina Faso, and Mali. It is an annual or perennial plant, growing to 90 cm tall, with solitary stems growing from a tigernut tuber. This tuber is widely eaten, as dried and mixed with roasted groundnut, soaked in water and unprepared, in West and Central Africa (*1*). Tigernut was found to be a good substitute for cereal grains to preparate Kunnu (*2*), a nonalcoholic beverage used as a thirst quencher, as a refreshment, and also as a weaning drink for infants. This nut was reported to be rich in sucrose (17.4−20%), fat (25.5%), protein (8%), and mineral contents. In Spain, tubers are used to make a refreshing, nonalcoholic beverage of dairy appearance, called "horchata", originated in Alboraya, a town in northern Valencia (*3*, *4*).

The production of horchata requires the careful selection of the best tigernuts, and it is summarized in four steps. First, the process begins with the washing of the tigernuts, which are soaked for about 8 h; during this time, they are shaken intermittently, and the water is periodically renewed. They are subsequently washed for a second time in the same way. The second step is the grinding; it is done in a grinder with a uniform flow of water, approximately 3 L/kg of tigernuts. The third step is the pressing, where the mass obtained is left to soak, more water is added, approximately 2 L/kg, and then it is pressed and sifted. Finally, the making of horchata is completed by dissolving the desired proportion of sugar, in general, between 100 and 120 g/L of extract (*5*).

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Since 1992, the European Union and national and regional administrations have been promoting the protection of agro-food products under different quality labels. Regulation 2081 (*6*) launched labels such as the Protected Origin Designation, which applied to this tuber in the Spanish context is equivalent to the Origin Designation of Valencia's tigernuts.

The processing of the tigernut is critical due to the presence of physical, chemical, and biological contaminants, such as stones (*7*), antinutrient contents (*8*), bacteria such as *Listeria monocytogenes* (*9*) and *Enterobacter aerogenes* (*10*), and fungi including *Dematophora necatrix* (*11*), *Fusarium sporotrichioides*, *Fusarium moniliforme* (*12*), and *Alternaria alternata*, *Aspergillus flavus*, *Aspergillus niger*, *Penicillium citrinum*, and *Rhizopus arrhizus* (*13*). With regard to the fungus contaminants, *Fusarium* spp. and *A. flavus* are the most dangerous because they possess the ability to produce trichothecenes and fumonisins (*12*) and aflatoxins (*13*), respectively, in tigernuts. The control of the tuber important, because other studies have detected the presence of aflatoxins in tigernut-based drinks (*14*).

The objective of this study was to develop a rapid and cheap extraction method based on the use for the first time of $C_{18}$ solid phase to extract and purify aflatoxins in tigernuts and their beverages, the determination of which is carried out by liquid chromatography−fluorescence detection (LC-FLD). Finally, the proposed method was applied to 62 real samples commercialized in Spain.

## MATERIALS AND METHODS

**Samples and Sampling.** Sampling was carried out according to mandated procedures (*15*). Samples of tigernuts were bought from supermarkets, street vendors, and candy shops that sell nuts. Tigernut beverages were obtained from different supermarkets, street vendors, juice bars, and ice cream parlors and were stored at 4 °C until they were analyzed. A total of 37 tigernut and 25 tigernut beverage (including sterilized, concentrated, and pasteurized) samples were investigated.



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**Table 1.** Validation of Aflatoxin Determination by LC-FLD Analysis of the Studied Samples

| | linearity | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| aflatoxin | range | $R^2$ | repeatability RSDr (%) | reproducibility $RSD_R$ (%) | recovery ± RSD (%) | LOD[a] | LOQ[b] | SSE[c] (%) |
| | | | | **Tigernuts** | | | | |
| $AFB_1$ | 2−50 ng/g | 0.998 | 7.9 | 12.8 | 74.1 ± 6.3 | 0.17 ng/g | 0.58 ng/g | 83 |
| $AFB_2$ | 2−50 ng/g | 0.994 | 7.1 | 12.6 | 72.3 ± 6.5 | 0.06 ng/g | 0.21 ng/g | 72 |
| $AFG_1$ | 2−50 ng/g | 0.999 | 8.1 | 12.7 | 77.4 ± 7.9 | 0.45 ng/g | 1.49 ng/g | 69 |
| $AFG_2$ | 2−50 ng/g | 0.997 | 8.9 | 12.9 | 82.1 ± 7.1 | 0.14 ng/g | 0.46 ng/g | 71 |
| | | | | **Tigernut Beverages** | | | | |
| $AFB_1$ | 0.6−50 ng/mL | 0.997 | 7.1 | 10.9 | 76.3 ± 4.1 | 0.05 ng/mL | 0.17 ng/mL | 49 |
| $AFB_2$ | 0.6−50 ng/mL | 0.995 | 6.8 | 10.5 | 74.0 ± 5.0 | 0.04 ng/mL | 0.13 ng/mL | 53 |
| $AFG_1$ | 0.6−50 ng/mL | 0.997 | 7.5 | 11.4 | 79.0 ± 6.7 | 0.17 ng/mL | 0.57 ng/mL | 59 |
| $AFG_2$ | 0.6−50 ng/mL | 0.991 | 8.0 | 11.5 | 86.3 ± 6.9 | 0.07 ng/mL | 0.25 ng/mL | 47 |

[a] Limit of detection. [b] Limit of quantification. [c] Signal suppression/enhancement.

**Chemical and Reagents.** All employed solvents were of HPLC grade (Merck, Darmstadt, Germany), and HPLC grade water was obtained by filtration of distilled water through a Milli-Q system (Millipore, Bedford, MA). Solvents and water were degassed for 20 min using a Branson 5200 (Branson Ultrasonic Corp., Danbury, CT) ultrasonic bath, and $C_{18}$ bonded silica (50 $\mu$m) used as solid phase was from Análisis Vínicos (Tomelloso, Spain). Pure aflatoxins $B_1$, $B_2$, $G_1$, and $G_2$ (≥98%) were purchased from Sigma Chemical Co. (St. Louis, MO), each stock standard solution being prepared in acetonitrile with concentrations of 500 $\mu$g/mL, wrapped in aluminum foil, and stored at −20 °C for < 3 months. Working solutions were diluted in acetonitrile and stored at 4 °C in darkness for a maximum of 4 days (*16*).

**Aflatoxin Extraction.** An aliquot (1 g or 1 mL) of the sample was placed into a mortar, and 2 g of the $C_{18}$ sorbent was added; they were blended using a pestle to obtain a homogeneous mixture. The mixture was introduced into a Pyrex glass tube of a centrifuge, 10 mL of hexane was added, and the content was thoroughly mixed for 1 min. Then the tubes were placed into a centrifuge at 4500 rpm (4173*g*) for 15 min. The extract was discarded, and the solid was dried with a stream of $N_2$ to eliminate hexane completely. After that, 10 mL of acetonitrile was added and centrifuged for a second time at 4500 rpm for 15 min. The extract was evaporated to dryness with a gentle stream of $N_2$ at 45 °C. Then the extract was redissolved with 100 $\mu$L of trifluoroacetic acid for 5 min, re-evaporated to dryness with $N_2$ at 45 °C, and finally reconstituted in 1 mL of acetonitrile/water (25:75 v/v) for LC-FLD.

**Liquid Chromatography−Fluorescence Detection Analysis.** A Shimadzu (Kyoto, Japan) SCL-GA system LC equipped with two LC-GA pumps, a Rheodyne model 7125 injector (20 $\mu$L loop), and an SRF-535 fluorescence detector was used. The column used was a 150 mm × 4.6 mm i.d., 5 $\mu$m, Kromasil SC-18 (Scharlau, Barcelona, Spain) with a 4 mm × 4 mm i.d. guard column of the same material, with a mobile phase consisting of a mixture of acetonitrile/water (25:75 v/v) at a flow rate of 0.7 mL/min. Detection of aflatoxins was carried out using 365 and 435 nm as wavelengths for excitation and emission, respectively (*16*).

## RESULTS AND DISCUSSION

**Method Performace.** Validation of the analytical methods was based on several parameters, including recovery percentage, linearity, limits of detection (LOD) and quantification (LOQ), sensitivity, and repeatability.

The range of studied linearity for each mycotoxin is shown in **Table 1**. The response linearity was obtained in triplicate with five concentrations in tigernuts (2, 5, 10, 20, and 50 $\mu$g/kg) and in tigernut beverages (0.6, 2, 7, 15, and 50 $\mu$g/L). The correlation coefficients obtained for each compound were acceptable ($r^2 > 0.99$).

For the study of the repeatability and reproducibility, five series of five extractions of a tigernut and tigernut beverage spiked at 50 $\mu$g/kg and 10 $\mu$g/L, respectively, with the studied mycotoxins were analyzed. The corresponding relative standard deviations

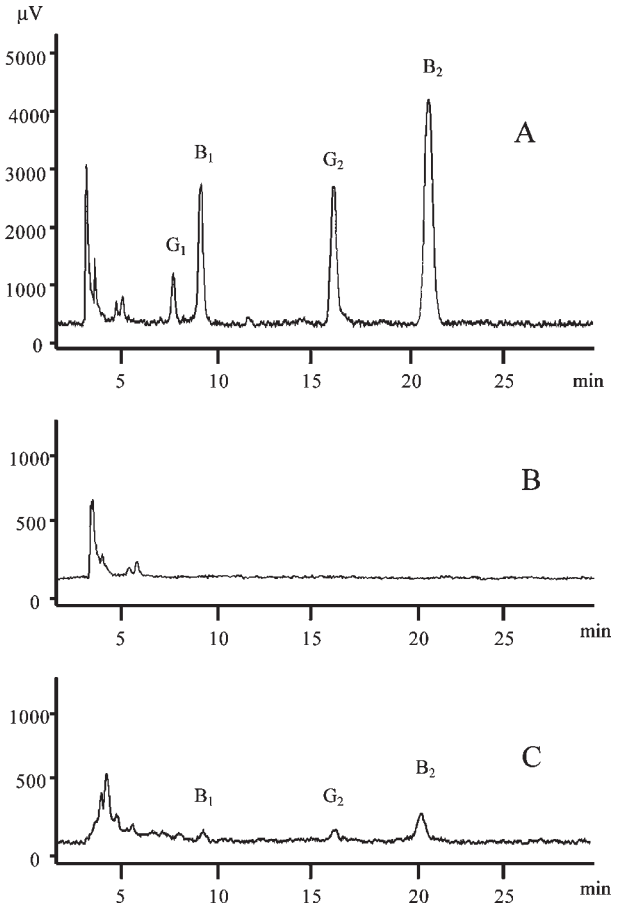


**Figure 1.** LC-FLD chromatograms obtained after proposed procedure: (**A**) tigernut beverage matrices fortified with 10 ng/mL for each aflatoxin analyzed; (**B**) blank of tigernut beverage; (**C**) tigernut beverage containing 1.2, 1.4, and 2.0 ng/mL of aflatoxin $B_1$, aflatoxin $G_2$, and aflatoxin $B_2$, respectively.

(RSDs) were calculated and are shown in **Table 1**. The RSDs obtained for intraday and interday variations ($n$ = 5) ranged from 6.8 to 8.9% and from 10.5 to 12.9%, respectively, for both matrices. Recoveries obtained with this procedure for tigernut (spiked at two levels, 10 and 50 $\mu$g/kg) and tigernut beverage (spiked at two levels, 10 and 50 $\mu$g/L) ranged from 72.3 to 82.1% and from 74.0 to 86.3%, respectively; such recoveries, repeatability, and reproducibility were considered to be valid for analyzing studied mycotoxins in both matrices (*15*) for the official

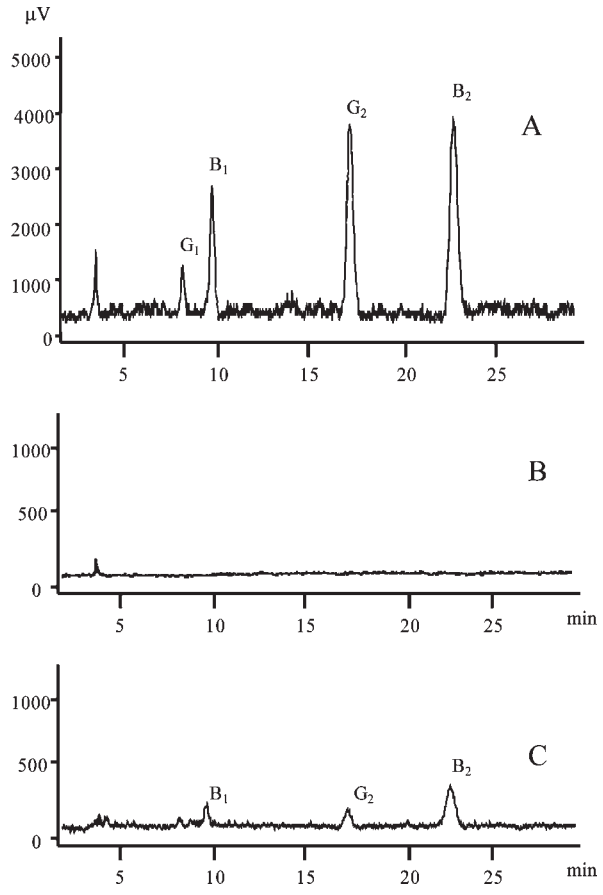


**Figure 2.** LC-FLD chromatograms obtained after proposed procedure: (**A**) tigernut matrices fortified with 10 ng/g for each aflatoxin analyzed; (**B**) blank of tigernut; (**C**) tigernut containing 2.0, 2.7, and 3.5 ng/g of aflatoxin $B_1$, aflatoxin $G_2$, and aflatoxin $B_2$, respectively.

control of mycotoxins including aflatoxins. For the estimation of the linearity and matrix effects, raw extracts of sample spots without visible fungal infections were fortified using a multianalyte standard on a range of studied concentration levels, diluted, and analyzed, and the corresponding peak areas were compared to a standard prepared and diluted in neat solvent. To differentiate between extraction efficiency and matrix-induced signal suppression/enhancement, the slope ratios of the linear calibration functions were calculated, and the signal suppression/enhancement (SSE) due to matrix effects was determined. The results calculated are indicated in **Table 1**. Panels **A** and **B** of **Figure 1** show the LC-FLD chromatograms, obtained following the proposed procedure, for a tigernut beverage fortified and nonfortified, respectively. Panels **A** and **B** of **Figure 2** show the LC-FLD chromatograms, obtained following the proposed procedure, for a tigernut fortified and nonfortified, respectively.

The LODs and LOQs of the studied mycotoxins were experimentally calculated by considering signal values 1:3 and 1:10 times, respectively, that of the background noise given by the software, on the basis of five independent determinations. LOD values ranged from 0.06 to 0.45 $\mu$g/kg for tigernut and from 0.04 to 0.17 $\mu$g/L for tigernut beverage. LOQ values ranged from 0.21 to 1.49 $\mu$g/kg for tigernut and from 0.13 to 0.57 $\mu$g/L for tigernut beverage.

**Application to Real Samples.** The proposed extraction method followed by LC-FLD determination was applied to 25 and 37 samples of tigernuts and tigernut beverages, respectively, and positive samples are shown in **Table 2**. Maximum levels of total aflatoxins and $AFB_1$ are laid down in European legislation (*17*) as

**Table 2.** Aflatoxin Concentrations for Tigernuts (Nanograms per Gram) and Tigernut Beverages (Nanograms per Milliliter) from 3 of 37 and 3 of 25 Positive Samples, Respectively

| sample | $AFB_1$ | $AFB_2$ | $AFG_1$ | $AFG_2$ | total aflatoxins |
|---|---|---|---|---|---|
| tigernut 1 | 2.0 | 3.5 | | 2.7 | 8.2 |
| tigernut 2 | 0.7 | 3.1 | 3.8 | 1.9 | 9.5 |
| tigernut 3 | 4.5 | 2.2 | | 1.6 | 8.3 |
| tigernut beverage 1 | 1.2 | 2.0 | | 1.4 | 4.6 |
| tigernut beverage 2 | 3.1 | 1.3 | | 1.3 | 5.7 |
| tigernut beverage 3 | 2.8 | 2.2 | | 1.4 | 6.4 |

4 and 2 $\mu$g/kg, respectively, present in groundnuts, nuts, dried fruits, cereals, and processed products therefrom intended for direct human consumption or as an ingredient in foodstuffs. Recently, the Codex Alimentarius, in 2009 (*18*), set a maximum level of 10 $\mu$g/kg total aflatoxins in ready-to-eat almonds, hazelnuts, and pistachios at a level higher than that currently in force in the European Union (EU) for total aflatoxins. Currently, the European Commission and Member States are discussing the alignment of EU legislation for these nuts with the Codex Alimentarius decision. In our study, one of three and two of three positive tigernuts and tigernut beverages, respectively, contained $AFB_1$ at levels higher than the EU legislation. Positive samples of tigernuts and their beverages were of unknown origin because they had not been identified with the Protected Origin Designation from Valencia, which guarantees their quality. **Figures 1C** and **2C** show the chromatograms obtained for a positive sample found from a tigernut beverage and tigernut, respectively. The presence of mycotoxin/aflatoxin contamination has been reported in tigernut matrix, the quantitity of aflatoxin **B1** ranging from 10 to 120 $\mu$g/kg (*13*). The concentrations of trichothecenes and fumonisins depend on the variety of tigernut used as substrate, the African sample of imported tigernut being more susceptible to the production of these mycotoxins (*12*). Twenty-two tigernut beverage samples from Spanish and Belgian supermarkets were analyzed, and only one of them presented $AFB_1$, at a 0.06 $\mu$g/L concentration (*14*). Bankole et al. (*13*) detected $AFB_1$ ranging from 10 to 120 $\mu$g/kg in 27 of 77 marketed tigernut sanples in Nigeria.

In conclusion, the developed method is faster, cheaper, and easier than the conventionally used immunoaffinity columns. It could be applied for the establishment of monitoring programs for aflatoxins in these types of matrices.

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