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**Determination of aflatoxin B1 in tiger nut based soft drinks**

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Additives/Contaminants:	Mycotoxins – aflatoxins
Food Types:	Beverages

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## Determination of aflatoxin B<sub>1</sub> in tiger nut based soft drinks

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### Abstract

An analytical method for the determination of Aflatoxin B<sub>1</sub> (AfB<sub>1</sub>) in a tiger nut based soft drinks named 'horchata' is described. The method is based on an immunoaffinity clean-up, followed by HPLC separation and fluorescence detection after electrochemical post-column derivatisation (PCD). The detection limit (S/N = 3) and the quantification limit (S/N = 10) were 0.02 µg/kg and 0.06 µg/kg respectively. The mean recovery at a level of 2 µg/L was 88% (n = 6) and the coefficient of variation (CV) 9%. The method was applied to conduct a small market survey for a beverage named 'horchata' that is frequently consumed by all kind of population in Southern Europe. A total of 22 samples from Spanish and Belgian supermarkets were analysed. As a result only one sample was found to contain AfB<sub>1</sub> at the LOQ of the method.

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### Keywords

Aflatoxin B<sub>1</sub>, tiger nuts, 'chufa', 'horchata', High-performance Liquid-Chromatography.

### INTRODUCTION

Tiger nut or 'chufa' is a plant that botanically belongs to the genus *Cyperus esculentus* L. There are two varieties with different applications: *Cyperus esculentus* L. var. *esculentus* and

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*Cyperus esculentus* L. var. *sativus*. The second one is cultivated for human consumption and the rhizomes are used to obtain the tiger nuts. (Fact Sheet HS-583)

In Europe, the cultivation of tiger nuts is widely spread through the eastern part of Spain (Region of Valencia). Tiger nuts are also grown in Central Africa and in the southern part of United States (Florida).

The Spanish annual consumption of tiger nuts is about 5-7 million of kg/year, and around 3 million kg were imported during 2004. Exporting countries were Nigeria, Niger, Burkina Faso, Benin, Mali and Ghana. The main destination of the tiger nuts in Spain is the production of 'horchata' (40-55 millions litres/year), a soft drink consumed by all kind of population including children (with special importance due to their low body weight).

'Horchata' is either available as liquid, hailstorm or in congealed consistency. It is considered mainly as a refreshing drink, but is also as nutritive due to its caloric contents. After collection of the tiger nuts, they are washed and sorted in order to separate the product from soil, insect- or otherwise damaged tiger nuts that can be present at the collection. This is an important step for minimizing possible aflatoxin contamination present in the raw material and it is nowadays performed with the help of industrial machines. In the next step the tiger nuts are dried to reduce the water content from 50 to 11 %, this is the last step in the processing and it should be performed carefully since mycotoxin producing micro organisms can still start to grow on not sufficiently dried tiger nuts.

For the preparation of 1 L of 'horchata' approx. 200 g of tiger nuts and 150 g of sugar are used. This product must be distinguished from another different type of 'horchata', that is based on rice and vanilla, is made in Central and South America. (Mosquera et al. 1996).

Tiger nuts are also used for the production of oil for human consumption and they are an important animal feed ingredient in the southern United States (Kelley and Fredricksin 1991)

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In 1996 Bankole and Esegbe recognized tiger nut as one of the commodities susceptible to aflatoxin contamination and detected aflatoxins in 35% of tiger nut samples collected from Nigeria with concentrations ranging from 10 to 120 µg/kg. Adebajo (1993) also reported the presence of aflatoxin in tiger nut at toxicological unsafe levels. Aflatoxins are considered one of the most significant mycotoxins, they are natural toxins produced by fungi affecting several food and feed commodities. They are classified as carcinogen to humans by the International Agency for Research on Cancer (IARC 1993; JECFA 1996). In Europe, regulations on aflatoxins exist for various products but not for tiger nuts or products thereof.

EU maximum levels for AFB<sub>1</sub> and total aflatoxins are 2 µg/kg and 4 µg/kg, respectively for most food products (Van Egmond 2004). Generally in the EU, imported food products from third countries are controlled as they enter the Community and in cases of consumer health risks, EU Member States can exchange information on contamination cases in the so called "Rapid Alert System for Food and Feed" (RASFF) ([http://europa.eu.int/comm/food/food/rapidalert/index\\_en.htm](http://europa.eu.int/comm/food/food/rapidalert/index_en.htm)). In 2002, three notifications were made about contamination levels of aflatoxin in EU imported tiger nuts. And in April 2004, one notification was made about tiger nuts with a contamination level of 300 µg/kg AFB<sub>1</sub> that were imported from Mali ([http://europa.eu.int/comm/food/food/rapidalert/reports/week15\\_en.pdf](http://europa.eu.int/comm/food/food/rapidalert/reports/week15_en.pdf)).

To our knowledge no method has been reported for the analysis of AFB<sub>1</sub> in 'horchata'. The here-described method has been developed on the basis of a previously described one by Stroka et al (2000). A small survey on commercially available 'horchata' from Spanish and Belgian supermarkets was conducted after method development.

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## MATERIALS AND METHOD

### *Samples*

The survey was carried out on 'horchata', from supermarkets in Spain and Belgium (imported product from Spain) with production dates from 2004. The method has been tested for 8 brands and different batches of some of these brands with a total of 22 samples.

### *Reagents*

**Solvents and reagents.** Solvents used were complying with grade ACS-ISO. Acetonitrile and methanol were HPLC grade from Merck (Darmstadt, Germany). Immunoaffinity columns type EASI-EXTRACT™ aflatoxins were from Rhone-rBiopharm (Glasgow, Scotland).

**Water.** Purified water was delivered by the Millipore water purification device Milli-Q Gradient A10 ( Bedford, USA).

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**Phosphate-buffered saline (PBS).** PBS was prepared by adding 0.2 g KCl, 0.2 g KH<sub>2</sub>PO<sub>4</sub>, 1.16 g anhydrous Na<sub>2</sub>HPO<sub>4</sub>, and 8.0 g NaCl in 1.0 L water. The pH was adjusted to 7.4 with NaOH c=(0.1 mol/L).

**Standards.** AFB<sub>1</sub> standard solution with a concentration of 10.0 µg/mL dissolved in chloroform was obtained from the Dutch National Institute for Public Health and the Environment, RIVM (Bilthoven, The Netherlands). From this, a dilution with a final concentration of 0.10 µg/mL solution was used for calibration standards purposes. Portions of 125, 250, 500, 750 and 1000 µL from this last standard were evaporated in 250 mL

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volumetric flasks, afterwards re-dissolved with 75 mL of methanol and filled up with water.

The following concentrations were achieved: 0.05, 0.10, 0.20, 0.30, 0.40 in ng/mL.

For recovery experiments, 5 mL of a 0.40 µg/mL AfB<sub>1</sub> standard solution was used to fortify 1 L of blank matrix the day prior to analysis, resulting in a level of fortification of 2 µg/L.

#### Apparatus.

**HPLC system.** The HPLC equipment consisted of a combination of Gilson HPLC pump type 309 with a solid phase extraction unit ASPEC XL (Milano, Italy), with a 100 µL injection loop. The fluorescence detector was from Waters type 474 (Milano, Italy). The PCD with KOBRA cell was from Rhone -rBiopharm (Glasgow, Scotland). The column used was a Supelcosil LC-18, 25 cm x 4.6 mm, 5 µm.

#### Sample preparation and clean-up.

25 mL of PBS and 25 mL of the 'horchata' were mixed and filtered over glass micro fibre filter (Whatman GF/A), 25 mL of this filtrate were passed through an immunoaffinity column (IAC) at a flow rate of approximately 3 mL/min. The IAC was washed twice with 10 mL of water at the same speed. For elution of AfB<sub>1</sub>, 0.5 mL methanol was applied by gravity on the IAC. The eluate was collected in a volumetric flask of 5.0 mL. After 1 min, a second portion of 0.5 mL methanol was applied. This step was repeated once more after another minute and air was pressed through the IAC to collect most of the applied methanol.

The volumetric flask, containing the combined portions of IAC eluate was filled up to approx. 4.5 mL, shaken and filled up further with water to the mark and shaken again. The clear extracts were directly injected into the HPLC system.

#### **HPLC equipment and reverse phase conditions**

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Deleted: **Extraction procedure**

1 The detector was set at 360 nm (excitation wavelength) and 420 nm (emission wavelength).  
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 3 The mobile phase was water/acetonitrile/methanol [62 + 22 + 16 (v+v+v)] with 120 mg of  
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 5 KBr and 350 µL of HNO<sub>3</sub> (4 mol/L) per litre of mobile phase. The flow rate was at 1 mL/min.  
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 8 Injection volume was 100 µL.  
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## 10 RESULTS AND DISCUSSION

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 16 It could be shown, that the clean-up procedure by immunoaffinity chromatography after  
 17 dilution of the sample with PBS and glass micro fibre filtration is fast, simple and reliable  
 18 despite a solid residue content of the product of a required minimum of 12 % and a fat content  
 19 of around 2 % in 'horchata' drinks. Chromatograms were free of interferences in the region of  
 20 the AFB<sub>1</sub> peak (see Figure 1).  
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 32 The mean recovery of the method was 88 % (n = 6). These recovery experiments were  
 33 performed using blank samples spiked at a level of 2 µg/L of AFB<sub>1</sub> in the product. This shows  
 34 that the method procedure is suitable to purify and concentrate the analyte by immunoaffinity  
 35 from the matrix and that the matrix has no significant effect on the recovery during clean up.  
 36 Additional recovery experiments at lower levels were not carried out as results from  
 37 collaborative studies and own experience showed that recovery figures for the here used  
 38 methodology (immunoaffinity clean-up with HPLC-fluorescence detection) and this type of  
 39 analyte (aflatoxins) are rather stable in terms of fortification levels, while being more  
 40 influenced by the matrix used for analysis (Arranz et al. 2005, Dragacci et al. 2001 and Stroka  
 41 et al. 2000, 2001, 2003).  
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The resulting relative standard deviation was 9 % under repeatability conditions. On the basis of the assumption that the internal reproducibility is linked by a factor of 3/2 with the internal repeatability, the estimate for the internal reproducibility is 14%.

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Based on a “fitness for purpose approach” which is characterised by the determination of an acceptable precision parameter that is derived from the function given below, the calculated reproducibility of 14% from this study was compared with the limit calculated from the function below. This approach has recently been implemented in EU legislation on mycotoxin method performance requirements (Commission Directive No. 38/2005) and can be used for those cases where no fully validated method precision (full collaborative trial) is available.

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$$U_f = \sqrt{(LOD/2)^2 + (\alpha \times C)^2}$$

In this formula  $U_f$  is the maximum acceptable standard uncertainty in  $\mu\text{g/kg}$ , LOD is the Limit of Detection expressed in  $\mu\text{g/kg}$ ,  $\alpha$  is a factor (0.1 – 0.2) that depends on the concentration of interest (C in  $\mu\text{g/kg}$ ). For concentration below 50  $\mu\text{g/kg}$ ,  $\alpha$  is 0.2. The resulting maximum acceptable standard uncertainty for a target level of 2  $\mu\text{g/L}$  (assuming a liquid density of 1 g/mL for ‘horchata’) and a LOD of 0.02  $\mu\text{g/L}$  is 0.40  $\mu\text{g/L}$ . Expressed as relative standard deviation this figure is 20 %.

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As the internal reproducibility is mostly accepted as standard uncertainty, the calculated figure of 14% shows that the method can be considered as “fit for purpose” according to this scheme.

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In none of the tested 22 sample materials AfB<sub>1</sub> could be quantified. Only in one case the presence of AfB<sub>1</sub> could be detected at the level of the estimated LOQ (Figure 1).

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CONCLUSION

1  
2 A rather simple and fast method for the determination of AfB<sub>1</sub> in 'horchata' was developed  
3  
4 and validated under single laboratory conditions. It could be demonstrated that the described  
5  
6 method shows good performance characteristics when compared with minimum requirement  
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8 data calculated from a "fitness for purpose" function that is currently discussed for use in EU  
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10 legislation on mycotoxin methodology in official food control. The described method  
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12 therefore is a good candidate for such use.

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14 Based on this small survey, no indication was found for a consumer risk from the 'tiger nut'  
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16 based soft drink as sold on the Spanish and Belgian market during the period 2004. However,  
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18 a survey with more samples would be necessary to obtain a full picture, which was partially  
19  
20 difficult as there is a limited amount of producers in Spain.

### 21 22 23 24 Acknowledgements

25  
26  
27 The authors are grateful to the RIVM in Bilthoven (The Netherlands) for providing us with  
28  
29 the aflatoxin standard and to the Regulating Council of Denomination of Origin "Chufa de  
30  
31 Valencia" (CRDO) in Valencia (Spain) for providing us with some technical information  
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33 about the 'horchata' consumption in Spain.  
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24 Tiger nuts are also used for the production of oil for human consumption and they are an  
25 important animal feed ingredient in the southern United States (Kelley and Fredricksin  
26 1991)

27  
28 In Spain, the quality of the tiger nuts must fulfil the specifications of the agriculture  
29 quality policy of the European Union and the name of the end product "Chufa de  
30 Valencia" is a product defined according to the conditions of a Protected Designation of  
31 Origin ((ECC) No. 2081/91)

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33 The quality of tiger nuts depends on the terrain used for cultivation. Therefore, it has  
34 been reported that also the quality and flavour of horchata is influenced by the production  
35 area (Mosquera et al. 1996)

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49 The end product

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17 also mouldy and bitten by insects tiger nuts should be eliminated.

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34 should be wash and leave to soak during at least 12 hours, afterwards they should get  
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39 wash again and mixed with

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43 , next is to blend this mixture and to sieve it

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49 The process ends with the addition of 1 L of water, the beverage should be served cold.

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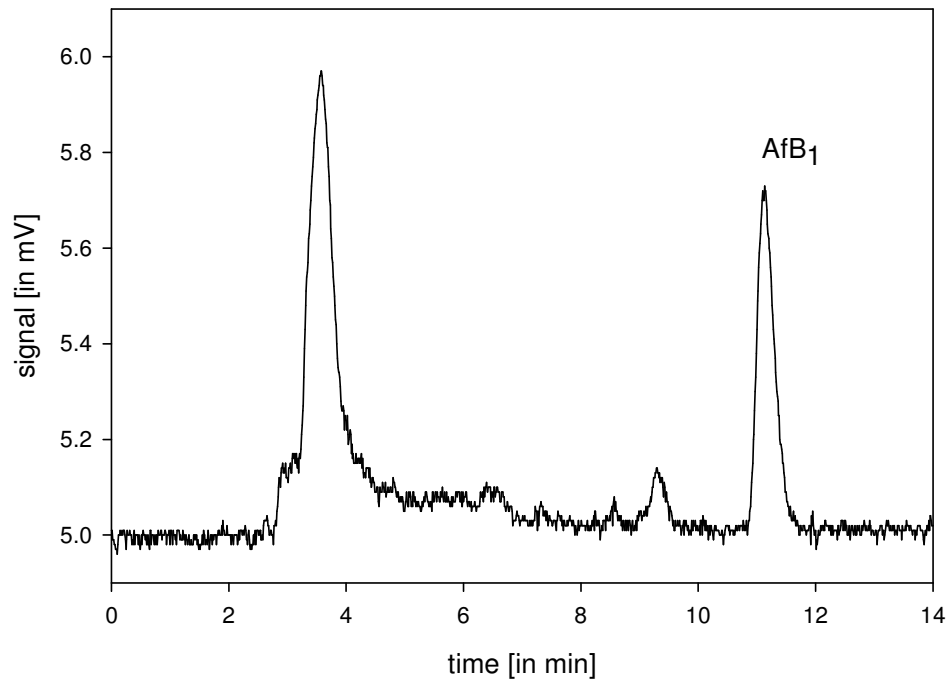
4 The determination is performed by HPLC with fluorescent detection and the need of  
5  
6 transforming AfB<sub>1</sub> in a nonquenchable derivative is solved by PCD involving  
7  
8 electrochemical bromination (Kobra cell) (Papadopoulou-Bouraoui et al.  
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12 **Page 9: [20] Deleted** **strokjo** **10/6/2005 12:03:00 PM**

13 Commission Regulation (ECC) No. 178/2002 of 28 January 2002 laying down the  
14  
15 general principles and requirements of food law, establishing the European Food  
16  
17 Safety Authority and laying down procedures in matters of food safety. Available  
18  
19 from: [Regulation EC/178/2002](http://ec.europa.eu/food/food/food_safety/legislation/regulation_178_2002_en.htm). Via the INTERNET. Accessed 2005 June 07.  
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Figure 1:



Chromatogram from a horchata sample containing aflatoxin B<sub>1</sub> at the estimated LOQ (0.06 µg/L).