

## ORIGINAL ARTICLE

**OTA-producing fungi isolated from stored cocoa beans**S. Amézqueta<sup>1</sup>, E. González-Peñas<sup>1</sup>, C. Dachoupan<sup>2</sup>, M. Murillo-Arbizu<sup>3</sup>, A. López de Cerain<sup>3</sup> and J.P. Guiraud<sup>2</sup><sup>1</sup> Organic and Pharmaceutical Chemistry Department, Faculty of Pharmacy, University of Navarra, Pamplona, Spain<sup>2</sup> Qualisud cc023, University of Montpellier 2, Montpellier Cedex, France<sup>3</sup> Department of Nutrition and Food Sciences, Physiology and Toxicology, Faculty of Pharmacy, University of Navarra, Pamplona, Spain**Keywords**

cocoa, fungi, mycotoxins, ochratoxin A.

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**Abstract****Aims:** The aim of this study was to identify fungal populations in unroasted cocoa beans stored in Spain in order to evaluate the ochratoxin A (OTA)-production ability of certain *Aspergillus* isolates.**Methods and Results:** Twenty batches of cocoa beans from different origins and with different OTA content were selected for this study. Three *Aspergillus carbonarius* and 13 *Aspergillus niger* aggregate strains isolated from these cocoa bean samples were selected to evaluate their OTA synthesis ability, being the only *A. carbonarius* isolates which are OTA producers [ $<$ limit of detection (LOD) = 3520  $\mu\text{g kg}^{-1}$  culture medium; LOD = 6  $\mu\text{g kg}^{-1}$  culture medium].**Conclusions:** No correspondence was found between the OTA levels in cocoa beans and the presence of OTA-producing fungi. Nonetheless, some samples contained *A. carbonarius* with a high OTA-producing ability and, consequently, specific fungal controls should be set up during storage to avoid this toxin.**Significance and Impact of the Study:** Toxigenic fungi in cocoa beans are not well understood. This study attempted to identify these fungi and evaluate their OTA-producing ability.**Introduction**

Ochratoxin A (OTA) is a secondary fungal metabolite present in a wide variety of foodstuffs. Several mould species have been identified as possible producers of OTA, and *Aspergillus ochraceus* and *Penicillium verrucosum* were the first to demonstrate an ability to synthesize this mycotoxin. The most relevant OTA-producing species are *P. verrucosum*, *A. ochraceus*, *Aspergillus niger* aggregate and *Aspergillus carbonarius*, owing to their prevalence in foodstuffs and the number of isolates able to produce OTA.

Most of the studies on the ochratoxigenic capability of *P. verrucosum*, *A. ochraceus* and *Aspergillus* section Nigri strains have been carried out on isolates from grapes, coffee and cereals, but little scientific work has been published on cocoa. Monjouenpou *et al.* (2008) isolated some *A. niger* and *A. carbonarius* strains from cocoa beans which were able to synthesize the mycotoxin. *Aspergillus carbonarius* strains showed the greatest OTA-producing capability (up to 2772  $\mu\text{g kg}^{-1}$  in a rice medium).

In order to reduce OTA contamination, the identification of toxigenic species, together with fungal control and other preventive measures, need to be established. To determine the OTA levels produced by potential toxigenic species, adequate validated methods of analysis are needed. Moreover, validated methods are essential for obtaining reliable data so as to avoid additional work and to aid in correct decision-making (Ermer 2001). The aims of this study were to identify fungal population in unroasted cocoa beans stored in Spain and to evaluate the toxigenic potential of certain isolated fungi using validated methods.

**Materials and methods****Materials**

Ochratoxin A was purchased from Sigma (St. Louis, MO, USA). A stock standard solution of 100 mg l<sup>-1</sup> was prepared by dissolving 1 mg of OTA in 10 ml of methanol and stored at -20°C. The concentration of OTA was

determined by UV at 333 nm (MW: 403.8;  $\epsilon$  (molar extinction coefficient) = 5500 mol l<sup>-1</sup> cm<sup>-1</sup>) (Bacha *et al.* 1988). Ochrates immunoaffinity columns (IAC) were obtained from Vicam Inc. (Watertown, MA, USA).

Phosphate-buffered saline (PBS) was prepared by adding sodium chloride (8 g), monobasic potassium phosphate (0.20 g), di-basic sodium phosphate (1.2 g) and potassium chloride (0.20 g) to 0.9 l of water. The pH of the dissolution was adjusted to 7.0. Finally, two drops of Tween-20 were added and the volume adjusted to 1 l.

Potato dextrose agar (PDA) was prepared by diluting 19.5 g of PDA for *in vitro* use from Merck (Darmstadt, Germany) in 1 l of distilled water. 0.1% peptone was prepared by diluting 0.09 g of peptone from Biolife (Milan, Italy) in 90 ml of distilled water. 0.01% Tween-80 was prepared by diluting 8.5 g of sodium chloride from Merck (Darmstadt, Germany) and 0.5 ml of Tween-80 (Sigma) in 1 l of distilled water. All of these solutions were autoclaved.

## Methods

### Samples

Twenty samples (about 1 kg each) from different origins (15 from Ivory Coast: samples 1–15, and five from Nigeria: samples 16–20) of dried unroasted cocoa beans were collected from a Spanish cocoa factory. The cocoa beans had been stored in bags for a 6- to 12-month period in environmentally controlled warehouses. Sample humidities were under 7.5% in all cases.

### OTA extraction from cocoa bean samples, purification and HPLC analysis

The method used for OTA extraction from cocoa bean samples and its validation are described in Amézqueta *et al.* (2004). In short, OTA is extracted from 25 g of milled cocoa with aqueous 3% NaHCO<sub>3</sub> : methanol (3 : 7 v/v). After centrifugation and filtering, the extract is diluted with PBS, passed through an immunoaffinity column and the OTA eluted with methanol. The eluate is evaporated to dryness and the residue is re-dissolved in the mobile phase.

For HPLC analysis, an Agilent Technologies 1100 liquid chromatographic system equipped with a fluorescence detector (model G1321A; Agilent Technologies) was used. The sample compounds were separated on a 5  $\mu$ m (25  $\times$  0.4 cm) Tracer Extrasil ODS2 column with a Tracer Extrasil ODS-2 pre-column, both from Teknokroma (Barcelona, Spain). The injection volume was 100  $\mu$ l, the flow rate 1.5 ml min<sup>-1</sup> and the mobile phase 29 : 29 : 42 (v/v/v) methanol–acetonitrile–sodium acetate (5 mmol l<sup>-1</sup> acidified to pH 2.2 with phosphoric acid). Chromato-

graphy was performed at 40°C and the fluorescence conditions were:  $\lambda_{\text{Ex}}$  = 225 nm,  $\lambda_{\text{Em}}$  = 461 nm. The OTA had a  $t_{\text{R}}$  of approximately 5 min.

### Fungal isolation and identification

To isolate the moulds, a 30-g subsample of cocoa beans was stirred for 5 min at room temperature in 90-ml 0.1% peptone water (w/v), and 0.3 ml were spread-plated onto PDA. In a previous work in our laboratory, results obtained on DG18 for cocoa beans were in general qualitatively and quantitatively equal or inferior to those obtained on a PDA medium. Petri dishes were incubated at 25°C for 5–7 days. Growing moulds were then subcultured on PDA for identification purposes. They were identified according to the identification key for common food-borne fungi (Samson *et al.* 1995). *Aspergillus carbonarius* and *A. niger* aggregate were identified by comparison with reference standards. These strains belong to our laboratory's fungal collection (references: 05/01 for sample 3, 05/02 for sample 4 and 05/03 for sample 8).

### Inoculation and incubation to evaluate toxigenic potential

Inoculation and incubation were done by following the methods of Suárez-Quiroz *et al.* (2004b): *Aspergillus* isolates were grown on PDA medium, pH 3.5 at 25°C,  $a_{\text{w}}$  0.85, 0.90, 0.95 and 0.99 for 7, 14 and 24 days. Conidia were collected by scraping in a 0.01% Tween-80 solution. Five microlitres of a 10<sup>6</sup> conidia per ml suspension were deposited in the centre of a Petri dish containing PDA and incubated at 28°C.

### OTA extraction from culture medium, purification and HPLC analysis

Three agar plugs of 5-mm diameter were removed from the central area of the colony and introduced into a small vial. OTA was extracted with 2.5 ml methanol : formic acid (25 : 1), for 15 min in an ultrasound bath.

Extracts were passed through filter paper and 600  $\mu$ l was evaporated to dryness. In the case of the *A. niger* aggregate, the residue was redissolved in 200  $\mu$ l of the mobile phase. For *A. carbonarius*, 50  $\mu$ l was evaporated and the residue was re-dissolved in 3 ml of the mobile phase.

In the case of *A. carbonarius*, the HPLC method was that used for OTA determination in cocoa beans. However, another method was developed for *A. niger* aggregate owing to the presence of substances which interfered with the OTA peak in chromatograms. The new mobile phase portions were of 23 : 23 : 54. The OTA had a  $t_{\text{R}}$  of approx. 5 min for the *A. carbonarius* method and a  $t_{\text{R}}$  of approx. 16 min for the *A. niger* aggregate one.

## Method validation

Method validation for OTA determination in culture medium was based on the following criteria: selectivity, linearity, limit of detection (LOD) and limit of quantification (LOQ), precision (within- and between-day variability) and recovery.

In the assessment of linearity, three calibration curves were plotted in the ranges  $11 \times 10^2$  to  $11 \times 10^3$  and  $11 \times 10^3$  to  $11 \times 10^4 \mu\text{g kg}^{-1}$  for *A. carbonarius* strains and  $8\text{--}80 \mu\text{g kg}^{-1}$  for the *A. niger* aggregate. Three replicates of six calibration standards were analysed for each range.

The LOD was calculated using the following relation:

$$LOD = \frac{Y_{bl} + (K \times S_{bl})}{b}$$

where  $Y_{bl}$  is the area of the blank, and  $b$  is the slope of a curve made representing the area for each concentration level *vs.* the concentration after analysing three samples of spiked PDA containing mould isolates at each of the following levels: 12, 20 and  $32 \mu\text{g kg}^{-1}$ .  $K$  is a factor of 3.  $S_{bl}$ , the standard deviation of the blank, is the intercept of the curve obtained representing the standard deviation for each concentration level *vs.* the concentration.

The LOQ was calculated as the lowest concentration for which acceptable data of recovery and precision are obtained.

Within- and between-day precision and recovery were studied at 22, 260 and  $1680 \mu\text{g kg}^{-1}$  OTA levels, spiking PDA containing mould isolates with adequate volumes of methanolic OTA solutions. The repeatability and reproducibility of this process was tested by carrying out the complete sample process of the recovery experiment in triplicate for each OTA level, on 1 day and on the three different days, respectively.

## Results

### Distribution of OTA in naturally contaminated samples

Table 1 shows the OTA levels found in the 20 cocoa bean samples analysed. A total of 60% of the cocoa bean samples were contaminated with a level  $>LOD$ , 10% over  $2 \mu\text{g kg}^{-1}$ . The mean and median values were 0.821 and  $0.108 \mu\text{g kg}^{-1}$ , respectively, and the OTA levels were within an interval below an LOD of  $6.9 \mu\text{g kg}^{-1}$ .

### Microbiological analysis and isolate identification

Although yeasts were the major fungi present in the cocoa bean samples, moulds were also isolated. These moulds

**Table 1** Ochratoxin A (OTA) levels in cocoa bean samples

Sample	Origin	OTA content ( $\mu\text{g kg}^{-1}$ )	Sample	Origin	OTA content ( $\mu\text{g kg}^{-1}$ )
1	Ivory Coast	0.165	11	Ivory Coast	<LOD
2	Ivory Coast	0.052	12	Ivory Coast	<LOD
3	Ivory Coast	0.80	13	Ivory Coast	<LOD
4	Ivory Coast	1.11	14	Ivory Coast	1.52
5	Ivory Coast	0.049	15	Ivory Coast	6.9
6	Ivory Coast	0.248	16	Nigeria	<LOD
7	Ivory Coast	0.95	17	Nigeria	<LOD
8	Ivory Coast	2.83	18	Nigeria	<LOD
9	Ivory Coast	0.93	19	Nigeria	<LOD
10	Ivory Coast	0.89	20	Nigeria	<LOD

LOD, limit of detection.

belonged mainly to the species *Rhizopus stolonifer* (Ehrenb.) Lind. (27%), *A. niger* aggregate (17%), *Aspergillus flavus* Link (31%) and *Penicillium citrinum* Thom (13%). Other species, such as *A. carbonarius* were found to a minor extent.

Species selected for the OTA-producing study were *A. carbonarius* and *A. niger* aggregate. *Aspergillus carbonarius* was isolated only from three of the 20 cocoa samples under study, being a minor component in fungal flora. In the preliminary study, no other fungi than *Aspergillus* section Nigri was able to synthesize OTA (data not shown).

### Method validation

Cocoa bean method recovery, LOQ and LOD were 88.9% (RSD, relative standard deviation = 4.0%), 0.04 and  $0.1 \mu\text{g kg}^{-1}$ , respectively (Amézqueta *et al.* 2004).

In the case of the culture medium method, no interferences with the OTA peak were observed during the HPLC quantification. When OTA was added to the positive samples, an increase in OTA peak area was observed.

The assays exhibited linearity between the peak area ( $y$ ) and the OTA concentration ( $x$ ) in all of the intervals assayed. Moreover, an analysis of the three calibration samples for each interval and over the 3 days showed adequate values of precision and accuracy (RSD and RE, relative error <10%, respectively).

The LOD and LOQ were 6 and  $12 \mu\text{g kg}^{-1}$ , respectively. At the LOQ level, good precision ( $n = 3$ ) (6.8%) and recovery (72%) values were achieved.

Recovery percentages for the within- and between-day recovery experiments were similar (66.1% and 63.9%, respectively) and the RSD obtained were below 5% (2.63% and 2.49%, respectively). Results have been corrected with the recovery value (63.9%).

**Table 2** Ochratoxin A (OTA) levels ( $\mu\text{g kg}^{-1}$ ) produced by *Aspergillus carbonarius* isolates after 5, 10, 15 and 20 days of incubation

Batch	$a_w$	OTA ( $\mu\text{g kg}^{-1}$ culture medium)		
		7 days	14 days	24 days
05/01	0.850	<LOD	<LOD	<LOD
	0.900	<LOD	<LOD	<LOD
	0.950	<LOD	<LOD	<LOD
	0.990	247	640	482
05/02	0.850	<LOD	9.14	<LOD
	0.900	57.9	130	108
	0.950	146	199	196
	0.990	3520	2030	1300
05/03	0.850	44.5	82.0	16.8
	0.900	134	86.4	61.4
	0.950	262	213	152
	0.990	2620	1630	1400

LOD, limit of detection.

### OTA-producing potential of the *Aspergillus* species

From all the strains isolated in the microbiological analysis, 13 *A. niger* aggregates (nine isolated from samples containing OTA levels over LOQ, one isolated from a sample containing OTA in the range LOQ–LOD, and three isolated from samples under LOD) and three *A. carbonarius* (isolated from samples 3, 4 and 8, all containing OTA levels over LOQ) strains were evaluated for OTA production ability on PDA culture. Table 2 shows the OTA levels produced by the three *A. carbonarius* isolates after 7, 14 and 24 days of culture.

*Aspergillus carbonarius* isolates produced the mycotoxin, production reaching optimum level after 14 days in one case and after 7 days in the other two cases. None of the *A. niger* aggregate isolates synthesized OTA, not even after 38 days of culture.

### Discussion

Few studies have been carried out on the incidence of OTA in cocoa beans. Miraglia and Brera (2002) reported a study in which none of their 96 cocoa bean samples had OTA levels exceeding  $2 \mu\text{g kg}^{-1}$  (the maximum OTA level found was  $1.8 \mu\text{g kg}^{-1}$ ). CAOBISCO/ECA/FCC (2003), Bonvehí (2004) and Amézqueta *et al.* (2004) found that 14% of 1220 samples, 5% of 21 samples and 13% of 46 samples were above  $2 \mu\text{g kg}^{-1}$ , respectively. These data are in line with those obtained in the present study and reveal that cocoa is not exempt from OTA contamination.

Regarding the microbiological analysis, *Rhizopus stolonifer* was essentially found in samples containing low OTA levels. Varga *et al.* (2005) have recently demonstrated that certain *R. stolonifer* strains are able to degrade OTA and, perhaps, the presence of large quantities of

*R. stolonifer* in some cocoa samples could be related to the low OTA levels found.

In order to analyse the OTA levels produced by potential toxigenic species, adequate validated methods of analysis are needed. In this work, a validation study covering all the OTA range expected was carried out before analysis.

The OTA production profile from the *A. carbonarius* isolates coincides with that of Sage *et al.* (2002), Bellí *et al.* (2005), Leong *et al.* (2006), and Astoreca *et al.* (2007). Despite OTA being a stable metabolite, the mycotoxin content decreased with incubation time. Other studies (Bellí *et al.* 2004; Esteban *et al.* 2006; Astoreca *et al.* 2007; Romero *et al.* 2007) have also noted this phenomenon. It is possible that OTA production could have stopped owing to the limited amounts of nutrients in the synthetic medium (Bellí *et al.* 2004).

None of the *A. niger* aggregate isolates considered for the OTA-production study synthesized OTA. Other authors (Taniwaki *et al.* 1999, 2003; Suárez-Quiroz *et al.* 2004a; Illic *et al.* 2007; Leong *et al.* 2007) have reported that 1–9% of *Aspergillus niger* strains isolated from coffee beans produce the toxin. This difference could be attributed to a natural selection in the strain or to adverse environmental conditions.

The results are similar to those obtained from strains isolated from coffee beans (Taniwaki *et al.* 1999, 2003; Joosten *et al.* 2001; Leong *et al.* 2007). Coffee and cocoa are cultured in tropical regions and, although differences in nutrients exist, the rest of the fungal growing parameters remain quite similar.

*Aspergillus carbonarius*, the only mould found that was able to produce OTA, was of negligible content in cocoa beans. There was no correlation between the OTA content and the OTA producers isolated from stored cocoa beans. Pardo *et al.* (2004) suggested that this might indicate that OTA is not a result of storage, but is more likely linked to adverse conditions during the harvesting, drying and transportation stages. At some point in these stages, the moulds would have developed, produced OTA and disappeared following another change in the environmental conditions. As OTA is a stable metabolite, it would have remained unaltered. Other OTA sources could be field-contaminated materials, such as infected or spoiled pods. The use of different preventive practices in stages previous to exporting are of great importance in minimizing the final OTA content in cocoa beans, and consequently, in cocoa derivatives. More specifically, it seems that drying is the most critical process in coffee processing (Joosten *et al.* 2001), and by extrapolation, in cocoa beans.

Nevertheless, significant amounts of OTA can be produced in only 7 days by *A. carbonarius* in stored cocoa

beans if certain environmental conditions are met. As a result, storage conditions must always be maintained under strict control.

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