Preliminary data on the presence of mycotoxins (ochratoxin A, citrinin and aflatoxin B1) in black table olives "Greek style" of Moroccan origin

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Many mould strains, in particular *Aspergillus* and/or *Penicillium*, are able to develop on olive and produce ochratoxin A (OTA) and/or citrinin (CIT) and/or aflatoxin B (AFB) after harvest, during drying and storage of olives. The development of fungi on olives is responsible for the reduction of nutritional quality of olive because they can disturb the synthesis of the fatty acids. OTA, CIT and AFB are particularly dangerous for health, inducing cancer of urinary tracts or liver carcinoma. In this study, ten olive samples bought at retailer and at supermarket in Morocco were analyzed for their OTA, CIT and AFB contents. These three mycotoxins were extracted simultaneously by a method based on solvent partition validated in-house, then separated by HPLC coupled to a fluorescence detector. All olive samples contain OTA ranging from LOQ to 1.02 μg/kg. Respectively, 50 and 25% from retailer and supermarket samples were contaminated by more than 0.65 μg/kg. In addition, 80% of olive samples contained CIT above LOD, and 100% of olive tested contained AFB above 0.5 μg/kg. As simultaneous presence of these toxins increases toxic risks, it is thus essential to have a good control of the conservation of olives after harvest.

Keywords: Aflatoxin B1 / Black olive / Citrinin / Mycotoxin / Ochratoxin A

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1 Introduction

Mycotoxins are toxic metabolites produced by some species of mould genera in the field as *Aspergillus*, *Penicillium* and *Fusarium*, which invade crops and may grow on food during storage under favorable conditions of temperature and humidity. Among the 400 known mycotoxins, ochratoxin A (OTA), citrinin (CIT) and aflatoxin B (AFB) receive now more attention. These three mycotoxins are produced by fungi belonging to *Aspergillus* genera (*Aspergilli ochraceus*, *carbonarius*, *niger*, *parasiticus, flavus*) and *Penicillium* genera (*Penicillium verrucosum*, *griseofulum*, *citrinum* and *expansum*) [1, 2]. OTA and CIT have been detected in wheat, oat and barley [3–5]. Moreover, OTA has also been detected in beverages such as wine, juices, beer, coffee and dried fruits [6, 7] and CIT has been detected in cereals [8]. These two mycotoxins are very stable during food processing (Bhat, R. V., Vasanthi, S., in: Third Joint FAO/WHO/UNEP, 1999, *International Conference on Mycotoxins, Tunis, Tunisia. MYC-CONF/99/49)*. AFB contaminated mainly peanuts, maize, cottonseed, but also spices, and tree nuts [9, 10].

OTA and CIT seem to be implicated in the Balkan endemic nephropathy in some Balkan areas [8, 11, 12]. The International Agency of Research on Cancer (IARC) has classified OTA as a possible human carcinogen (Group 2B) and AFB as a human carcinogen (group 1) [13]. CIT is nephrotoxic [14, 15] and genotoxic [16] and enhances OTA renal toxicity in pig [14, 17], the incidence of renal cell tumors in male DDD mice [18], and kidney adenoma in male F344 rats [19], although CIT alone was not carcinogenic.

Several studies have reported that olives could be a substrate for the growth of moulds [20–23]. Among all kinds of

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Abbreviations: ACN, acetonitrile; **AFB**, aflatoxin B; **CIT**, citrinin; **OTA**, ochratoxin A

olives, the black one, "Greek style", is the highest contaminated by toxinogenic molds [24–26].

Many studies described the contamination of Greek style black olives by aflatoxin B1 [20, 22, 24, 27]. However, up to now, few studies analyzed contamination of virgin olive oils and Greek style black olives by OTA [28]. No information exists about contamination of olive by CIT.

The aim of this study was to evaluate the simultaneous presence of OTA, CIT and AFB in black olive Greek style of Moroccan origin collected from retail and supermarket. The extraction procedure of these three mycotoxins was accomplished simultaneously by a method using partition developed in our laboratory and then quantified by HPLC [29, 30]. The advantage of this method is the simultaneous extraction of the three toxins and avoids the problem of interferences with immunoaffinity column clean up as seen for other foods [29, 31]. Moreover, the aim was also to develop a method less expensive than method using immunoaffinity column, suitable in developing countries.

2 Materials and methods

2.1 Sample collection

Ten olive samples of Moroccan origin were purchased from retailer (six samples) and supermarket (four samples) in Morocco as would be done by a consumer.

2.2 Chemicals

All reagents (potassium chloride, sodium hydrogen carbonate, sulfuric acid, phosphoric acid, sodium hydrogen phosphate) were of Normapur grade. All solvents (methanol, chloroform, ACN, propanol-2-ol, n-hexane) were of HPLC grade from ICS (France). Deionized water was used for the preparation of all aqueous solutions and for HPLC. OTA, free from benzene, CIT, AFB1 and carboxypeptidase were from Sigma Chemicals (France).

2.3 Preparation of standard solutions

Standard solutions of OTA, CIT, and AFB1 were prepared by dissolving 10 mg of OTA or CIT or AFB in 1 mL of methanol. A series of working standards from 0.2 to 100 ng/mL of mycotoxin/mL were prepared by dilution. They were used to calibrate the LC detector response. The OTA stock solution was determined by measuring the absorbance at 333 nm and calculated by using the molar extinction coefficient e of 5500 $mol^{-1}cm^{-1}$. CIT stock solution was determined by measuring the absorbance at 321 nm and calculated by using the molar extinction e of 5490 $mol^{-1}cm^{-1}$. AFB stock solution was determined by measuring the absorbance at 360 nm and calculated by using the molar extinction e of 21 800 $mol^{-1}cm^{-1}$.

2.4 Extraction/purification method for simultaneous OTA, CIT and AFB extraction

2.4.1 Homogenization of samples

One hundred grams of each black olive sample, as purchased, were cooled for 2 h at –80°C, and then olives without stone were crushed to olive paste using a Waring Blendor at high speed for a short period to avoid heating of the sample.

2.4.2 Extraction

Eight mL of a 4% aqueous solution of potassium chloride acidified to pH 1.5 with undiluted sulfuric acid, were added to each 10-g aliquot of olive paste sample. The mixture was homogenized and extracted with 72 mL ACN on an orbital shaker for 20 min. The extract was then passed through a Whatman No. 4 paper in a porcelain filter under vacuum, collecting in a measuring cylinder.

2.4.3 Purification of the extract

The n-hexane (40 mL) was added to the filtrate and shaken for 1 min. After separation, the upper phase (n-hexane) was discarded. This defatting operation was repeated twice. To the lower phases, 20 mL deionized water and 40 mL chloroform were added. The mixture was shaken for 10 min. After separation, the lower phase (chloroform) was collected. The upper phase was re-extracted three times with 20 mL of chloroform using the above conditions. The four chloroform extracts were pooled, extracted with 20 mL sodium bicarbonate and the mixture shaken for 10 min. After separation, the aqueous phase (bicarbonate) was acidified to pH 1.5 with concentrated hydrochloric acid. The acidified aqueous phase was extracted three times with chloroform (40, 10, 10 mL). The pooled chloroform extracts were evaporated under vacuum using a rotator evaporator in a 40°C water bath at low speed. Methanol (1 mL) was added and the solution was sonicated and filtered through cartridges (Spartan 0.2 µm, Schleicher and Schuell, Germany) and finally evaporated to dryness under nitrogen. The pellet was re-suspended in 500 µL of methanol and stored at –20°C until analysis by HPLC.

2.5 HPLC analysis of OTA and CIT

HPLC analysis used a Gilson 811B dynamic chromatography pump coupled to a Spectra Physics 2000 fluorescence

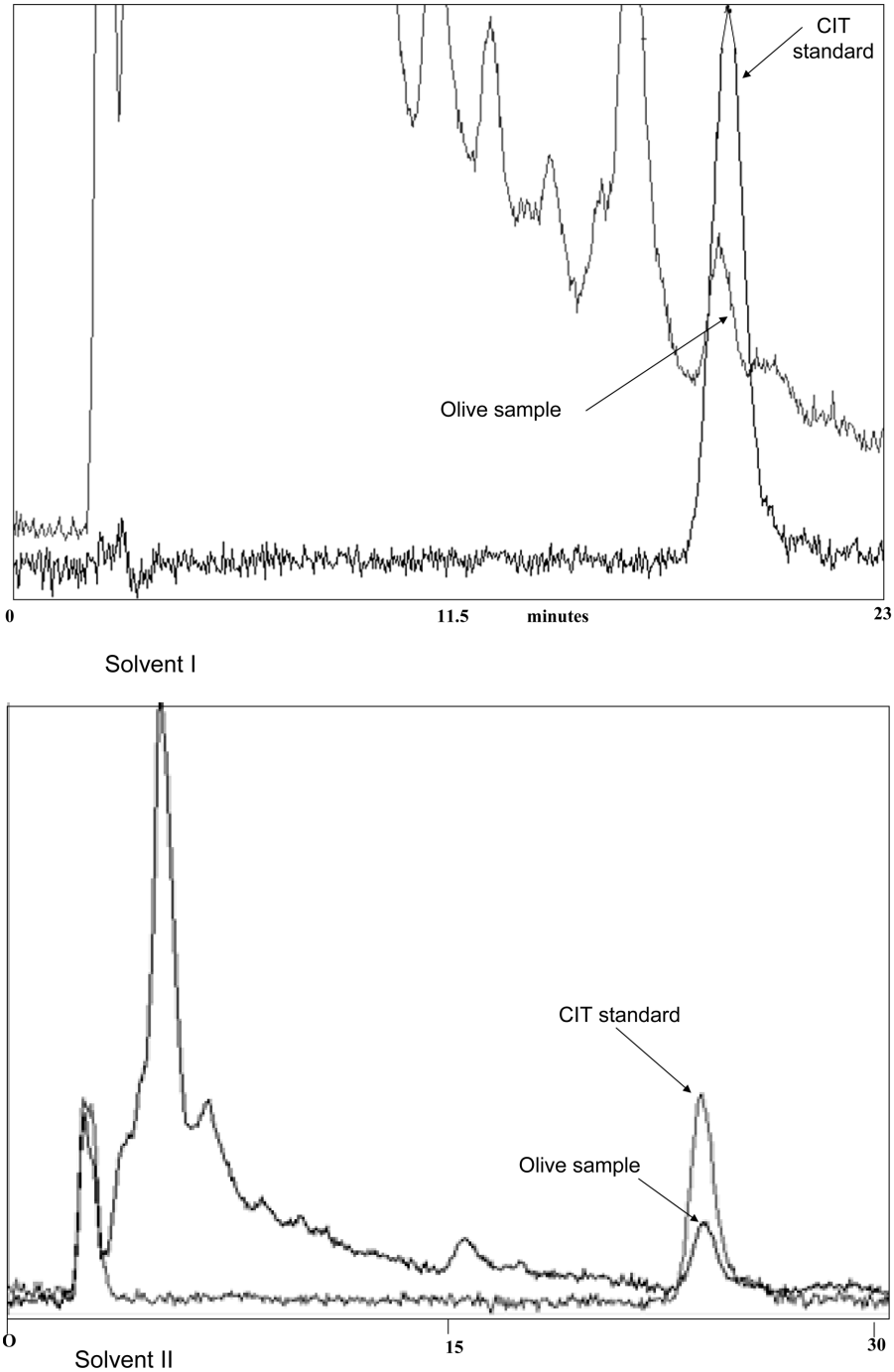

Figure 1. HPLC separation of CIT. Solvent I: H_3PO_4 (0.33 M)/ACN/propanol 2-ol (600/400/50). Solvent II: H_3PO_4 (0.33 M)/ACN/propanol 2-ol (700/300/50).

spectrophotometer. The analysis was performed on a C18 nucleosil column (4 μm C18, 0.46 × 25 cm), preceded by a C18 pre-column from ICS. The elution phase used was H_3PO_4 (0.33 M)/ACN/propanol 2-ol (600/400/50), flow rate was 0.7 mL/min, elution time of OTA and CIT was about 29 and 16 min, respectively.

The excitation and emission fluorescence of OTA, CIT and AFB were 335 and 465 nm; 331 and 500 nm; 365 and 440 nm, respectively. The chromatograms were analyzed by a Normasoft software provided by ICS (France).

2.6 Confirmation of the presence of OTA

The confirmation of the presence of OTA in olive samples was performed by the following technique. An aliquot taken from the purified extract of a sample where OTA was

detected by the HPLC analysis was dried. The pellet was dissolved in 975 µL of a buffer solution of 0.04 M Tris-HCl, 1 M NaCl, pH 7. 5. Then, 25 µL of carboxypeptidase (100 U/mL H_2O) was added and the mixture was incubated at room temperature overnight. The sample was analyzed by the same HPLC chromatographic conditions used above. The OTA peak disappeared whereas the peak of OT alpha appeared.

2.7 Confirmation of the presence of CIT

The confirmation of the presence of CIT in olive samples was performed using another elution phase in which the amount of H_3PO_4 was increased and ACN was decreased as follows H_3PO_4 (0.33 M)/ACN/propanol 2-ol (700/300/50). Under these conditions, CIT eluted at 25 min, and was separated from contaminating peaks (Fig. 1).

2.8 Confirmation of the presence of AFB1

Underivatized AFB1 were separated by HPLC and detected by spectrofluorimetry after post-column derivatization in the Kobra cell® where they are converted into their 9,10-dibromo derivatives.

3 Results and discussion

3.1 Method development for OTA, CIT and AFB analysis in black olive

3.1.1 General remarks

The analytical protocol for OTA; CIT and AFB determinations was characterized in-house by regarding the following criteria: linearity, accuracy, repeatability, and reproducibility, LOD and LOQ. As no reference material exists for OTA, CIT and AFB in black olive, accuracy was estimated by determining the OTA or CIT or AFB recovery factors on spiked olive samples with different concentrations of these three mycotoxins.

3.1.2 OTA recoveries

Four black olive samples (without stone) were spiked with 0.2, 0.6, 2 or 6 µg/kg and analyzed by the same operator on the same day with the same HPLC system. The average recoveries were 72 ± 5.3; 71 ± 2.8; 74.5 ± 2.1 and 72.3 ± 3.2%, respectively.

3.1.3 Repeatability test for OTA analysis

One black olive sample was spiked with 2 µg/kg and analyzed six times by the same operator on the same day with the same HPLC system. The average OTA recovery was 1.38 ± 0.23 µg/kg. In a naturally contaminated sample (sample N°1, Table 1), the average level from six analyses was 1.150 ± 0.31 µg/kg.

Table 1. OTA and CIT levels in black olive samples of Moroccan origin

Sample number	OTA (µg/kg)	AFB (µg/kg)	CIT (µg/kg)
1[a,b]	1.02	5[d]	< LOQ
2[a,b]	0.79	0.6[d]	0.45
3[a,b]	LOQ	1.7[d]	LOD
4[a]	< LOQ	NA[e]	LOD
5[a,b]	0.31	NA	LOD
6[a,b]	0.73	NA	LOQ
7[b,c]	< LOQ	NA	ND[e]
8[c]	< LOQ	NA	ND
9[b,c]	0.48	NA	< LOQ
10[b,c]	0.68	2.4[d]	0.52

a) Samples from retailer.
b) Confirmed by carboxypeptidase.
c) Samples from supermarket.
d) Confirmed by post-column derivatization.
e) ND: not detected, NA: not analyzed using Kobra cell.

3.1.4 Dose-response curve for OTA analysis

Four black olive samples were spiked with quantity of OTA equivalent to a contamination of 0.02 to 4 µg/kg and analyzed by HPLC. The coefficient of linearity (R^2) was 0.997. The LOD was 0.05 µg/kg and the LOQ was 0.2 µg/kg of olive without stone.

3.1.5 CIT recoveries

Four black olive samples were spiked with 0.5, 2 or 6 µg/kg and analyzed by the same operator on the same day with the same HPLC system. The average recoveries were 74 ± 5.2, 75.4 ± 4.1 and 77.2 ± 6.3%, respectively

3.1.6 Repeatability test for CIT analysis

One black olive sample was spiked with 0.5 and 2 µg/kg and analyzed six times by the same operator with the same HPLC system. The average CIT concentration was 0.48 ± 0.05 and 1.400 ± 0.3 µg/kg, respectively. In a naturally contaminated sample (sample N° 10, Table 1), the average level from six analyses was 0.5 ± 0.04 µg/kg.

3.1.7 Dose-response curve for CIT analysis

Four black olive samples were spiked with 0.2 to 10 µg/kg and measured by HPLC. The linearity coefficient (R^2) was 0.978. The LOD was 0.2 µg/kg and the LOQ was 0.5 µg/kg of olive without stone.

3.1.8 Recovery test of OTA and CIT simultaneously

Four black olive samples were spiked with each of the two toxins at 0.5 or 2 μg/kg on the same day and analyzed by the same operator on the same day with the same HPLC system. The average recovery for OTA and CIT were 75 ± 3.5 and 74.3 ± 3.3%, respectively when olive sample was spiked with 0.5 μg/kg and 79.2 ± 2.8 and 77.3 ± 4.7%, when olive sample was spiked with 2 μg/kg.

3.1.9 Aflatoxin recovery

The method showed a recovery factor of 94.5 ± 6.5% with a LOD of 0.1 μg/kg and the LOQ was 0.5 μg/kg. The linearity coefficient (R^2) was 0.997.

3.2 Results of the sample analysis

The overall results, uncorrected for recoveries are presented in Table 1.

Seven of ten black olive samples analyzed had OTA concentration above or equal to LOQ value (0.2 μg/kg). The most contaminated samples (OTA amount above 0.65 μg/kg) were bought at the retailer (3/6) and only one came from supermarket (1/4). This is probably due to the storage conditions, which are relatively better in supermarket compared to retailer. Moreover, 50% of olives samples were co-contaminated by CIT and OTA. The amount of CIT was close to the LOQ (0.5 μg/kg of olive without stone). Although the amount of CIT found in our sample is low, the data are in line of those of Heperkan *et al*. [23] who found 77% of olive sample collected in Turkey, contaminated by CIT. Presence of AFB has been confirmed in four olive samples and ranged between 0.5 and 5 μg/kg. In their surveys, Heperkan *et al*. [23] in Turkey as well as Ghitakou *et al*. [27] found AFB1 in black and green olives of Greek origin. Both reports show the simultaneous presence of OTA and AFB1, as in our study.

Considering the four highest OTA contamination samples and the mean daily consumption of 60 g of olive without stone per person, the OTA intake *via* consumption of the sample 1, 2, 6 and 10 would, respectively, be 60, 46, 42 and 40 ng/day. Based on the Joint expert committee on food and additives (JECFA), the provisional tolerable weekly intake (TWI) of OTA (based on nephrotoxicity in pig) is 100 ng/kg bodyweight/week [31] equivalent to 6 μg/week for a person weighing 60 kg. If we take into account the EU legislation based on carcinogenicity of OTA, the daily intake (TDI) is much lower and corresponds to 5 ng/kg bodyweight/day. This means a consumption of 300 ng/day for a person weighing 60 kg (JEFCA, WHO, Evaluation of certain mycotoxins, fifty-sixth report of the Joint FAO/WHO Expert Committee on Food on Food Additives, 906.). Consumption of these four olive samples represents, respectively, 20, 15, 14 and 13% of the TDI of OTA. Our results suggest that human exposure to OTA *via* contaminated black olive could contribute significantly to the total daily intake of OTA and consequently be a hazard to public health. In addition, the possibility of synergism or additive effects with other mycotoxins like AFB1 and CIT present in the same food commodities contaminated even at low levels must be taken into consideration [14–19] (Commission of the European Community, Scientific committee on Food Opinion on Ochratoxin A. CS/CNTM/MTC/14 Final, Annex II to Document XXI/2210/98,Brussels: CEC, 1998).

4 Concluding remarks

Because the occurrence of mycotoxins in black table olives is linked to the presence of toxinogenic moulds in olives, the efficient way to prevent contamination is to take good handling procedures during harvest and best storage conditions. Moreover, the obligatory regular controls are essential during these two steps to reduce the risk of the growth of mycotoxin-producing moulds and to safeguard the health of consumers.

However, additional studies on more olive samples are necessary to determine with precision the consumption impact of black olives on human health.

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