



Original Article

Identification, purification and quantification of Toxins “Ochratoxin A” in Algerian grape juice

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ABSTRACT

This study aimed to investigate the mycotoxin of *Ochratoxin A* (OTA) which can contaminate grape in mediterranean basin. Its toxicity was subject of many animal studies and carcinogenic characters are well established today. So the European Committee has fixed the maximum in grape 2µg/L. Most methods used for mycologic identification is based mainly on the morphological characters. It was identified after its purification by the technique of culture monospore on the culture medias Potatoes Agar and Czapek Yeast extract Agar. The detection and quantification of this mycotoxin by uses combined techniques: immunoaffinity for the purification and the HPLC coupled with the fluorometric detector. This chromatographic system allowed us to estimate the contamination of Ochratoxin in the grape which is lower than 30 ng/L. The concentration of conidia at *Aspergillus* section Nigri was estimated at 3×10^6 conidia/mL. The value obtained is less than 30 ng / L and is lower than that fixed by the EC. So, we confirm that our sample does not present serious risks to the human health. We have also confirmed fungus belonging to *A. Niger* specie, which normally does not produce OTA.

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1. Introduction

Ochratoxins are a group of chemically related toxin fungal metabolites (mycotoxins). They are produced by a kind of moulds of the *Aspergillus* and *Penicillium* which are growing on a wide range of raw food commodities. They are considered as potent toxins and their presence in food is undesirable. The most important and most toxin ochratoxin found naturally in food is Ochratoxin A. Mycotoxins are a worldwide problem in food economy and public health. FAO (Food and

Agricultural Organization) estimated that about 25% of cereal crops worldwide are probably affected by mycotoxins. At the international level, guidelines and regulations have been enacted to restrict the marketing of food contaminated with their mycotoxins and the Joint Food and Agriculture Organization of the United Nations/ World Health Organization Expert Committee on the Food Additives have discussed the imposition of a maximum tolerable of 100 ng of OTA/kg of body

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weight by week. These mycotoxins can be presented in a wide variety of substrates such as grains, fruits and juices, dried fruit. It is also found in wine and grape juice, as well as in human blood and animal tissues [1-4]. The poisoning is through ingestion of contaminated foodstuffs. Chronic poisoning, however, occurs long-term results in fatty infiltration and is the cause of cancer or irreversible damage can be produced [5-7]. The highly toxigenic fungi associated with the fact that these fungal microorganisms grow on many foods around the world, which poses a problem of food hygiene and public health. This problem is even more worrying that in some countries such as central Africa or the diet is limited to the consumption of foods that are frequently contaminated by mold, because the geographical area under climate conditions is favorable to the development of this type of micro-organisms. Since then, several grape derived products have been found to be potentially contaminated. Ochratoxin contamination of grapes is caused mainly by black *Aspergilli*, especially *A. carbonarius*. The most important factors for the growth and mycotoxin production are oxygen, temperature, water activity, nature and condition of items substrat [3,8-10]. During the harvest, the use of clean farming equipment, mechanical damage prevention and overripe or fermented fruits discard are convenient practices. In the post-harvest, the storage is the most critical phase.

In our study, we chose as a substrate to study the grapes produced on the outskirts of Algiers, a region with a warm climate and optimal conditions for the development of mycotoxins. We are interested to the identification of toxigenic molds specific to grapes by optimizing the parameters of this culture of fungal flora followed by purification by immunoaffinity column, qualitative and quantitative analysis of OTA by HPLC [11, 12].

2. Materials and Methods

2.1. Material

2.1.1. Grape's sample

The sample of black raisin grapes to be analyzed

was collected in the area belonging to the Algerian Cinsault variety and *Vitis vinifera* species. The land area is 650 m² and the samples were taken at five homes, each one of about 1 kg.

2.1.2. List of kits used

- Columns of immuno-affinity "OchraPrep r-Biopharm, St Didier Au Mont D'Or, France" containing a gel of Sepharose to which monoclonal antibodies specific for OTA are covalently bonded.
- OTA witness "Sigma Aldrich".

2.2. Methods

2.2.1. Mycological analysis

2.2.1.1. Preparing dilutions of grape juice

25g of grapes are crushed and transferred to sterile saline solutions to obtain the dilutions of 10⁻¹, 10⁻² and 10⁻³.

2.2.1.2. Technical isolation of fungi

The inoculation of each dilution is performed on the surface of petri dishes with PDA medium flows at a rate of 0.1 mL and then incubated at 25 °C for 07 days. The first reading is from the 3rd day to avoid the invasion of fungi. The isolation of different strains of fungi is performed by sampling explantats culture of each strain using a cookie cutter (9mm), then subcultured on Petri dishes containing PDA and CYA media. Incubation is at 25°C for 15 days. Before the determination of OTA from grapes, we performed the extraction and purification on immunoaffinity column.

2.2.1.3. Purification of *Aspergillus* species by monoconidial culture

Monospore culture provides a pure culture from a single spore [13]. It takes two explantats sterile culture of fungi that are introduced in 10 mL of sterile distilled water. The suspension stirred manually for 10 min was filtered through sterile gauze to separate mycelial fragments conidia. The spore count was performed on Malassez cell after sedimentation on the central grid system. Count the number of spores located on the two diagonals and

rectangles are averaged. The figure obtained is the number of conidia contained in a rectangle in a volume of 0.01 mm^3 . Knowing that 10^{-2} mm^3 corresponds to 10^{-5} mL and the concentration is estimated number of spores /mL.

$X = \text{number of spores in a rectangle} \times \text{dilution factor} / 10^{-5}$. The average number X corresponds to the concentration of spores in the sample estimated a number of spores /mL. To obtain a monosporale culture (purity of the strain), we are plated aseptically 0.5 mL of fungal suspension adjusted to a concentration of 102 spores /mL on Petri dishes poured with water agar at 2%, then performs an incubation at 25°C for 24 hours. Germinated spores were subcultured on PDA medium and poured into Petri dishes. After 7 days of incubation at 25°C , our isolates were inoculated on CYA medium for identification.

2.2.2. Identification species of the genus *Aspergillus*

Species of *Aspergillus carbonarius*, *A. ochraceus* and *A. niger* are the most common contaminants of grapes. Their identification is based on morphological characteristics of *Aspergillus* heads. After taking a Scotch colony, we observe under a Scanning Electron Microscope (SEM) successively the different parts which accompany the *Aspergillus* heads.

2.2.3. Extraction and determination of OTA

2.2.3.1 Preparation of grape sample

- 125g sample of grapes is crushed using a mixer and filter.
- Centrifugation for 20 minutes at a speed of 4000 revolutions/minute.
- Adjust the pH of grape juice to 7.8 with KOH 30%.
- Dilute 10 mL of grape juice with 10 mL of phosphate buffered saline (PBS: Buffer solution of Na/ K, NaCl, glucose and distilled water).
- Adsorption and elution of OTA by immunoaffinity column.

2.2.3.2. Adsorption and elution of Ochratoxin A by immunoaffinity column

It is necessary for the extraction and purification of samples to use an immunoaffinity column [14]. The sample of grape previously prepared is chromatographed on immunoaffinity column, according to the following protocol:

- Balancing the immunoaffinity column with 2 mL of PBS.
- Add in column 5 mL of grape juice at a rate of approximately one drop every 2s.
- Washing the column with 5 mL of washing solution (2.5% NaCl + 0.5% Na HCO₃).
- Elute with 2 mL of pure methanol by drop wise with a flow rate of 1 drop per 2s.
- Evaporate the eluent at 50°C under nitrogen. Dissolve immediately in 250 μL of HPLC mobile phase and stored at 4°C until analysis by HPLC.

2.2.3.3. Determination of OTA

Most mycotoxins are commercially available with a purification greater than 98%, making these preparations suitable for the manufacture of standard solutions [15].

2.2.3.3.1 Preparation of standard solution of OTA.

Dissolve 1mg of OTA in the solvent mixture (toluene: glacial acetic acid, 99:1, v/ v) to obtain a solution containing approximately 20 to 30 μg /mL of OTA. To determine the exact concentration, the absorption spectrum was recorded of the solution between 300 and 370 nm in a quartz cuvette of 1 cm optical path using the solvent mixture as reference. Identify the absorption maximum and calculate the concentration of OTA in μg /mL using the following equation:

$$C = A_{\text{max}} \times M \times 100 / \epsilon \times \delta$$

A_{max} : Absorption determined at the maximum wavelength (333nm)

M: Molecular weight of OTA = 403.8g /mole

ϵ : Molar extinction coefficient of OTA in the solvent mixture, equal to $544 \text{ m}^2/\text{mol}$

δ = Optical path (1cm)

This solution is stable at -18°C for at least 4 years.

2.2.3.3.2 Preparation of standard solution of OTA at 2 μg /mL

Dilute the stock solution with the same previous solvent mixture to get a standard solution of OTA at a concentration of 2µg /mL. This solution can be stored at 4°C in the refrigerator. Stability must be tested regularly.

2.2.3.3.3. Analysis of sample's grape juice by HPLC

OTA is measured by HPLC reverse-phase isocratic fluorimetry (λ excitation = 333 nm, λ emission = 460 nm). The analytical column used was reverse phase C8-ODB (15 cm x 4.6 mm x 5 µm) while the mobile phase was acetonitrile: water: acetic acid 99/99/2 (v/v/v) at a rate of 1 mL /min. The injections were performed manually with a syringe of 100 µL and an injection volume was 100 µL. To assess the OTA concentration in the sample of grapes, a calibration curve was constructed using standard solutions of OTA (0.6; 2; 6; 20 and 60 ng/mL).

3. Results and discussion

3.1. Isolation and enumeration of the total mycoflora

Microbiological testing by inoculation of grape juice on selective culture media PDA, Sabouraud, CYA and Oxitetracline Glucose Agar (OGA)

allowed their detection and numerical evaluation using the technique of suspension-dilution. The analysis on the four culture media of total mycoflora revealed the presence of a strong yeast population and only two molds belonging to the type *Aspergillus* and *Penicillium*. *Aspergillus* type population lies between 4 and 10 Colony Forming Unit (CFU) only. However, yeasts are in uncountable number on the four culture substrates. This yeast charge could be explained by the high concentration of sugars in the grape juice.

The number of colonies of *Aspergillus* section *Nigri* isolated in 10^{-1} dilution increases in all culture media. We have also found once a discrete presence of *Penicillium* spp. of about 20 CFU /mL on PDA medium. In the dilutions 10^{-2} and 10^{-3} , we have observed a gradual decrease of the yeast population accompanied by disparition of *Aspergillus* flora on three culture media: OGA, Sabouraud and CYA. Contrari wise the PDA medium, section *Nigri* increased to 200 CFU/ mL in 10^{-2} dilution. This could be explained by a contamination on grapes by yeasts that cause a phenomenon of competition between these two fungi in the solution and the first dilution.

The results (Table 1) show that among the inoculated culture media, only the culture media PDA is selective for optimal development of the grape contaminant.

Table 1. Frequency of representatives of the total mycoflora of grapes, expressed as CFU /mL.

Dilutions	Solution	1/10	1/100	1/1000	Backgrounds			
Aspergillussection	08	0	160	0	0	OGA		
Nigri	Uncountable (nb > 300)	Uncountable (nb > 300)	0	0	0			
Penicillium spp. Yeast	300)	300)	65 x 1000	45 x 1000				
Aspergillus section	60	0	160	20	200	0	PDA	
Nigri Penicillium spp. Yeast	Uncountable (nb > 300)	Uncountable (nb > 300)	0	51 x 1000	0	35 x 1000		
Aspergillus section	04	0	80	0	0	0	0	Sabouraud
Nigr. Penicillium spp. Yeast	Uncountable (nb > 300)	Uncountable (nb > 300)	32 x 1000	25 x 1000				
Aspergillus section	10	0	100	0	0	0	0	CYA
Nigri Penicillium spp. Yeast	Uncountable (nb > 300)	Uncountable (nb > 300)	45 x 1000	55 x 1000				

3.2. Identification of the species *Aspergillus* section *Nigri*

The identification was based on macroscopic and microscopic characters described by [16]. We have

identified single species with black colonies belonging to section *Nigri* from the results obtained in the culture monospore of *Aspergillus* section *Nigri*. The calcul of the cell's concentration it's the

enumeration of the spores on Malassez hemacytometer evaluated at a concentration about 3×10^6 spores / mL. A serial dilution at 1/10 to achieve a concentration of 10^2 spores /mL.

3.3. Study of morphological characters of the *Aspergillus section Nigri*

Petri dishes inoculated with two drops of the fungal suspension concentration 102 spores /mL were incubated for 24h at 25° C. Germ tubes obtained, are planted on medium CYA to develop colonies of single species or monomicrobial. The identification of *Aspergillus* is done by analyzing the morphological criteria after macroscopic and microscopic observation.

3.3.1. Macroscopic examination

Culture on the medium CYA, colonies in early growth are white and become black anthrax after 48 to 72 h at 25 °C and the reverse remains colorless. After 5 days, the analysis of plates shows that the colonies are aerial platforms, granular edge with white fluffy consistency with a black color on the front and yellow on the back, the diameter varies between 71 and 74 mm (Figure 1 and 2).



Fig. 1 Macroscopic observation on the front of *Aspergillus* section *Nigri* on CYA medium after 7 days of incubation at 25°C.



Fig. 2 Macroscopic observation on the back of *Aspergillus* section *Nigri* on CYA medium after 7 days of incubation at 25°C.

Possibly with a magnifying glass, it is easy to distinguish easily carried by the heads *Aspergillus* conidiophores. We have also noticed the presence of long, thin rays of the back plates.

3.3.2. Microscopic examination by SEM

Aspergillus section *Nigri* was observed in the light microscope (x 40) on culture media CYA at 25°C for 7 days. On vegetative filaments, from a podal cell other filaments hyaline are smooth, large thick-walled wide, with an angle more or less straight and not partitioned, called conidiophores. These terminate in an *Aspergillus* head consisting of a globular form and radial around are arranged directly with two rows of sterigmata. The diameter of the vesicles is between 50 and 61 mm. Finally at the end of phialides conidia arise, unicellular spherical 4 to 4.75µm in diameter, are arranged in black unbranched chains. They are often globular, smooth or rough of echinulations (Figure 3 (A and B) and Table 2).



Fig. 3 (A). *Aspergillus section Nigri* observed by light microscope (x40), culture on CYA medium at 25 °C for 7 days

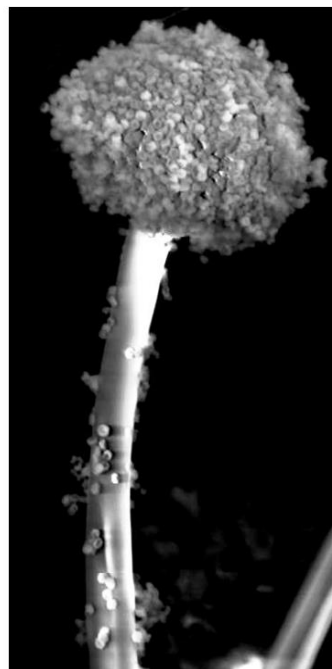


Fig. 3(B). Microscopic observation of *Aspergillus Gx 1200* at 25 °C for 7 days

Table 2. Description morpho-cultivation of *Aspergillus* spp. found on the grape. Culture on CYA, incubation at 25 °C for 7 days.

Charactéristic	Aspect	Color	Diameter
colony	Flat edge with fluffy white mycelial	black(front) pale yellow(verso)	71 –74mm
conidiophore	simple, smooth, large thick-walled	hyaline	
vésicule head <i>Aspergillus</i>	Globular, radial: metulae and phialides around vesicle	colorless to light brown	50 - 60,9 µm (x 40)
conidies conidies orphialospores	spherical, unicellular, rough, isolated echinulate or in small chains	black	4 - 4,75 µm (x 40)

After a comparative study, these mold contaminant grapes have no similarity to be confused with two species namely *Aspergillus ochratoxinogenic* A. *Ochraceus* and A. *carbonarius* described by [13],[14],[17]. In fact, all the morpho-cultural, macroscopic and microscopic, are considered sufficiently close to the third species of *Aspergillus ochratoxinogenic* that our isolate is included in the *Aspergillus section Nigri* strain reference type

described by the same authors mentioned above.

3.5. Determination of OTA by HPLC

The chromatogram of the sample reveals the appearance of three peaks. Only the third one is characterized by a retention time nearest standards (t_R) (Fig. 4), Thus confirming the presence of OTA in our sample (Fig.5).

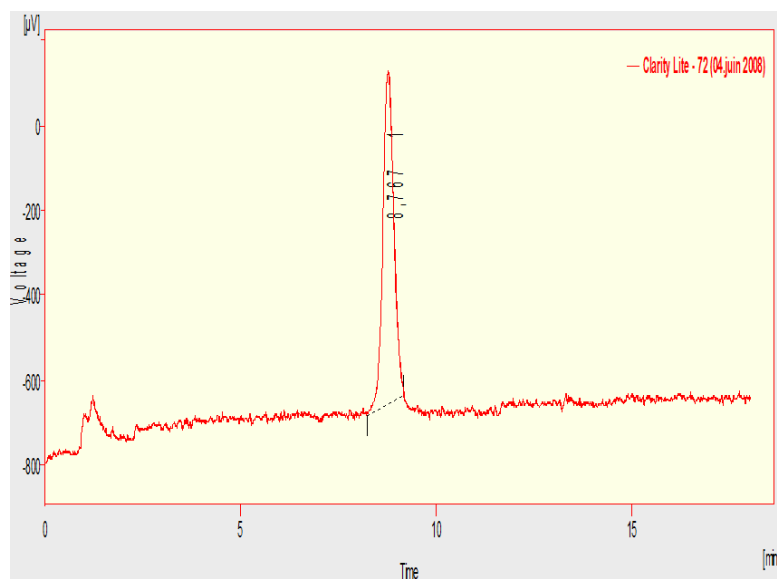


Fig. 4 Chromatogram of standard OTA at 6 ng/mL (TR : 8.767 mn ; A : 14925)

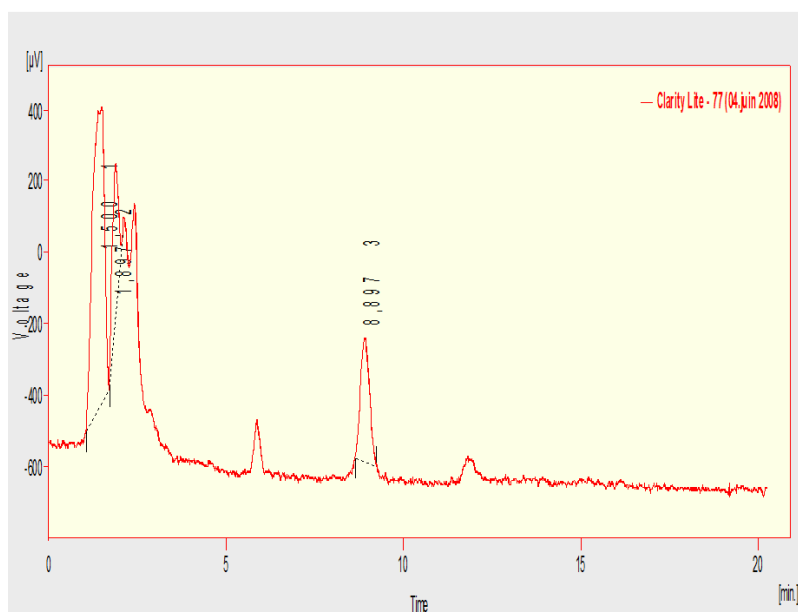


Figure 5. Chromatogram of sample (TR: 8.897 mn and A: 6.145)

The appearance of two peaks is due either to impurities in the sample that absorb at the same wavelength than OTA, or solvents used in previous stages and remained with the OTA after evaporation under nitrogen. The concentration of OTA in our sample is calculated according to the formula:

$$C_{OTA} \text{ (ng/mL)} = M_A \times 2/V_1 \times V_3/V_2$$

MA is the mass of OTA(ng) in the aliquot of the matrix injected on the column and determined from the calibration curve.

2: is the dilution factor,

V₁: is the volume of sample to analyze,

V₂ : is the volume of test solution injected into the column,

V₃ :is the solution used for redissolving the dry eluent.

The found amount is less than 30 ng / L and is lower than that fixed by the EC. So, we confirm that our sample does not present serious risks to the human health.

4. Conclusion

OTA is a mycotoxin may contaminate cereal and coffee and to a lesser extent the grape and wine. On grapes, fungi responsible for this contamination are *Aspergillus ochraceus*, *Aspergillus carbonarius* and *Aspergillus Niger*. Tests to isolate these *Aspergilli* spp. able to produce OTA were performed, filamentous fungi are found on the PDA and average 200 CFU /mL of grape juice.

Through the microbiological analysis on pigmentation, sporulation and mycelial aspect, we have demonstrated the presence of *Aspergilli* specie belonging to section *Nigri* in our sample of grapes.

The adsorption and elution of OTA and its analysis by HPLC revealed that the level of fungal contamination of vineyard Douéra (region based in 30 km of south Algiers) is slightly affected since the content of OTA revealed less than 30 ng / L in the grapes. This value is smaller than the limit imposed by the EC that 2µg / L (Regulation N°. 123/2005 of 26 January 2005). We have also confirmed fungus belonging to *A. Niger* specie, which normally does not produce OTA.

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Compliance with Ethical Standards

Conflict of Interest: The authors declare that they have no conflict of interest.

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