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Simultaneous Detection of Aflatoxin B_1 and Ochratoxin A in Olive Oil

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ABSTRACT

A screening method has been developed for simultaneous determination of aflatoxin B_1 and ochratoxin A in olive oil. The technique includes extraction of both mycotoxins with aqueous methanol, clean-up using lead acetate, partitioning in chloroform, and thin layer chromatography. The detection limits achieved are, respectively: 4 μg aflatoxin B_1 and 40 μg ochratoxin A/kg. 60 samples of crude farm olive oil were analyzed using this method: three of them contained traces of ochratoxin A while none was contaminated with aflatoxin B_1.

INTRODUCTION

The natural occurrence of aflatoxins in a wide range of fatty products has often been reported, but to our knowledge, comparatively few references dealing with mycotoxin contamination of olive oil can be found in the literature.

However, in 1962, Gracian et al. (1) identified *Aspergillus flavus* in olive fatty cakes; and in 1973, San Perez et al. (2) obtained aflatoxin on black olives artificially contaminated with either *A. flavus* or *A. parasiticus*.

More recently, olive oil samples originating from Greece (3) and Spain (4) have been reported to contain aflatoxins.

For our part, mycological surveys carried out in our laboratories on Moroccan olives, while not resulting in the isolation of *Aspergillus* from the *flavus* group, did result in the identification of *A. ochraceus* in many samples (5).

The toxigenesis of these strains was tested after cultivation on whole wheat, using two methods (6, 7) for extraction and analysis. None of the strains secreted measurable quantities of ochratoxin.

Nevertheless, we thought it interesting simultaneously to determine contamination levels for Moroccan crude farm olive oil with both ochratoxin A and aflatoxin B_1, since farmers sometimes store their olives for several weeks in conditions that promote the growth of molds. Furthermore it must be emphasized that refining which would remove aflatoxins from contaminated crude oil (8) is not applied to so-called "virgin" olive oil.

Therefore, this paper comprises two distinct parts: (a) the description of a method for simultaneous detection of aflatoxin B_1 and ochratoxin A in olive oil; and (b) an investigation of these two mycotoxins in crude farm olive oil samples collected from producers in the area of Beni-Mellal (250 km southeast of Rabat).

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EXPERIMENTAL PROCEDURES

Materials

Glass plates 20 × 20 cm; Quickfit applicator; dessicator; spotting template; microsyringe 10 μl; Desaga developing tank; longwave ultraviolet (UV) lamp (366 nm); Pleuger, chromatolux 2L.

All reagents are analytical grade chemicals. Solvents: acetonitrile, benzene, chloroform, ethyl acetate; formic acid 98-100%; hexane, toluene. Extraction solvent: methanol/water solution (6:4, v/v) containing 4% NaCl. 4% NaCl aqueous solution. Clean-up solution: 20% lead acetate aqueous solution, prepared from Pb $(CH_3COO)_2$, $3H_2O$. Drying agent: anhydrous sodium sulfate. Silica Gel for TLC: HR 60, Merck.

Mycotoxin standard solutions: aflatoxin B_1: determine concentration and purity, following basic methodology described by Rodricks and Stoloff (9); using a Pye Unicam UV-visible SP 1800 spectrophotometer (correction factor, CF = 0.997). Ochratoxin A: follow same method, using information given by Stoloff et al. (10).

Developing solvents: benzene/hexane (3+1) (11), toluene/ethyl acetate/formic acid (6+3+1) (11), ethyl acetate/formic acid (99+1), benzene-methanol/acetic acid (18+1+1) (7), chloroform/methanol (92+8).

Methods

Extraction and clean-up. Extraction procedure follows method of Toussaint et al. (3) with slight changes.

Pour a 50 g sample with 100 mL hexane into a 500 mL separating funnel. Extract first with three 100 mL portions of methanol/water extraction solvent (6:4; v/v, containing 4% NaCl), next with two 50 mL portions of aqueous 4% NaCl solution. Shake for 2 min each time.

Combine aqueous and aqueous/methanolic extracts in a separating funnel and wash twice with 50 mL hexane each time. Transfer aqueous/methanolic phase to a beaker, add 20 mL clean-up solution (20% acetate aqueous solution), and stir well. Filter the solution on Buchner funnel and wash the precipitate with 50 mL distilled water. Transfer the

developing station in Abu-Gharib near Baghdad. Seeds which were medium in size and light yellow in color, were stored for two months at 5 C before pressing. They were sorted by hand, ground by a laboratory grinding mill, and oil expressed by a laboratory press (Carver model C). The expressed oil was filtered twice through cotton and stored in amber bottles after flushing with nitrogen and kept frozen at −20 C for ca. 1 month until used.

Commercial brand oil (Bint oil) was supplied by the local General Company for Vegetable Oils (Iraq), in one-kg plastic bottles. This refined oil was composed of 50% sunflower oil and 50% cottonseed oil and had no added antioxidants. Samples of this oil were stored in amber glass bottles under nitrogen at −20 C for about one month until used.

The physicochemical and compositional characteristics of the two oils are reported elsewhere (16). They had identical saponification values (189); iodine values of 145 and 109; tocopherol contents (mg/100 g) of 67.0 and 70.3; copper contents (ppm) of 0.025 and 0.063; iron contents of 0.370 and 0.845; oleic acid contents (wt %) of 13.5 and 21.2; and linoleic acid contents of 76.2 and 66.6 for safflower and Bint oils, respectively.

Amino acids. Analytical grades L-amino acids used in this study were: methionine, proline, cysteine, arginine, valine, isoleucine, serine, histidine, hydroxyproline, glycine, threonine, alanine, aspartic acid, glutamic acid, tryptophan, leucine and tyrosine (supplied by Hopkins and Williams, UK), phenylalanine, glutamine, asparagine, histidine-HCl, arginine-HCl and glutamic acid-HCl (supplied by BDH-UK), lysine, cystine, cysteine-HCl and lysine-HCl (supplied by Riedel-Dehaen-AG, West Germany).

Methods

Addition of amino acids. The amino acids were added directly (as crystals) to oil samples or as 10% solution in redistilled water. However, tryptophan and glutamic acid were added as 8% solution in redistilled water, cystine was dissolved in 0.1 N NaOH and tryosine was added directly in the crystalline form.

Oil stability tests. The active oxygen method (AOM) employed a specially designed apparatus by (Sargent-Welch Scientific Co., USA) conforming to the AOCS specification (17). In this method, ca. 20 mL oil was placed in an aeration tube in a mineral oil bath (97.8 C), while a controlled flow of dry, filtered air was bubbled at 2.33 mL/sec through the sample. Oil samples were withdrawn periodically and examined for peroxide content (17). Results are reported in hours for samples to reach peroxide value of 100 meq/kg of fat.

Storage stability test was determined for the oil samples at 45 ± 1 C in a convection-type thermostatically controlled oven. Samples of 20 mL oil were stored in 100 mL test tubes with a loose cotton plug. At 5-day intervals, the samples were removed from storage, well mixed, and peroxide values (17) were determined in duplicate. Storage stability results are reported as days for samples to reach peroxide value of 100 meq/kg of fat.

Stability tests were carried out in duplicates and average values are reported. The effectiveness of amino acids are compared by their protection factors. The protection factor is defined as the stability value with additive divided by that of the control oil. Control experiments were carried out without any added material.

RESULTS AND DISCUSSION

Antioxidant Effect of Amino Acids

Amino acids added directly as solid material to Bint oil (Table I) showed negligible antioxidant activity. Cysteine-HCl was the only amino acid which showed a prooxidant effect.

The insolubility of amino acids in oil was apparently responsible for their lack of activity as antioxidants. When they were added to Bint oil as aqueous solutions at the same concentration (Table I), most had antioxidant activity. When the same amino acids were added in solution at the same level to safflower oil (Table I), they generally had lower antioxidant activities. Arginine-HCl had no antioxidant effect, while glutamic acid-HCl showed a prooxidant effect.

It is possible that the effect of aqueous solubilization is twofold. One, the amino acids are solubilized rather than dispersed, as when added solid. Therefore, a higher effective concentration results. Two, the metal prooxidants presumably enhancing the oxidation may partition into the water and be less available to the oil.

The present data regarding the relatively higher antioxidant activity of methionine and proline with safflower oil and lysine and arginine in Bint oil are in general accordance with many reports on other oils and fats (1-4, 8, 9, 15). However, it contradicts one report (7) on olive oil in

TABLE I

Antioxidant Effect of L-Amino Acids when Added as Aqueous[a] Solution on the Oxidation of Two Vegetable Oils under the Conditions of the Active Oxygen Method (AOM, hr at 97.8 C)[b]

Amino acids (0.02%)	Protection factors (AOM)	
	Safflower	Bint oil
Lysine	1.25	1.85
Arginine	1.25	1.85
Glutamic acid	1.04	1.72
Methionine	1.29	1.68
Hydroxyproline	1.09	1.60
Tryptophan	1.04	1.58
Proline	1.26	1.57
Leucine	1.03	1.53
Phenylalanine	1.12	1.51
Serine	1.12	1.51
Cystine[c]	1.12	1.51
Threonine	1.06	1.45
Isoleucine	1.15	1.41
Cysteine	1.25	1.36
Asparagine	1.09	1.32
Valine	1.13	1.30
Glycine	1.07	1.30
Aspartic acid	1.06	1.30
Alanine	1.07	1.24
Histidine	1.09	1.23
Lysine-HCl	1.03	1.19
Glutamine	1.09	1.17
Arginine-HCl	1.00	1.17
Tyrosine[d]	1.03	1.07
Histidine-HCl	1.01	1.07
Cysteine-HCl	1.06	0.51
Glutamic acid-HCl	0.91	0.36

[a]When added as solid, the protection factors were 1.0-1.05.

[b]Control stability values were 6.8 and 5.3 hours for safflower and Bint oils, respectively.

[c]Cystine was dissolved in NaOH before addition to oil.

[d]Tyrosine was added directly to oil.

TABLE II

Lowest Detectable Quantity (in ng) on TLC plate; Recovery (%); and Minimum Detectable Concentration (in μg/kg) for Aflatoxin B_1 and Ochratoxin A in Olive Oil

	Sensitivity on TLC plate (ng)		Recovery (%)		
	Solution of standard	Standard mycotoxin added to 6 μL extract spot	Quantity added to olive oil (μg/kg)	Recovery (%)	Lowest detectable concentration (μg/kg)
Aflatoxin B_1	0.4-0.5	0.6	20	90	4
Ochratoxin A	12	20	150	90-100	40

at low level, leads us to suppose that ochratoxin producing molds can grow on olives and secrete this mycotoxin. However, we have to verify whether or not olives are a good medium for ochratoxin production by toxicogenic *A. ochraceus* strains.

The recovery of ochratoxin in pressed oil and fatty cakes when processing contaminated olives should also be measured.

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The Antioxidant Activity of Amino Acids in Two Vegetable Oils

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ABSTRACT

Of 27 amino acids studied, most had some antioxidant activity when added in aqueous solution to either safflower oil or a mixture of sunflower and cottonseed oil (active oxygen and storage methods). Cysteine-HCl, glutamic acid-HCl (in the mixture), and glutamic acid-HCl (in safflower oil) behaved as prooxidants. When added as a solid, most amino acids were ineffective. The protection factors of these amino acids were less than 1.3 in safflower oil with methionine, proline, lysine and cysteine providing the highest activity. In the oil mixture (which had a higher metal content) lysine, arginine, glutamic acid, methionine, and hydroxyproline were antioxidant with protection factors of up to 1.85. Chelation of metals by amino acids was presumably responsible for the antioxidant activity. The increase in cysteine concentration up to 1% has more than doubled the protection factor in Bint oil (compared with the 0.01% level), whereas with some other amino acids the increase was either small or slight.

INTRODUCTION

Many contradictory results are found in the literature when comparing the relative effectiveness of amino acids as antioxidants in different oils and fats. For example, methionine was reported to have a good antioxidant effect in some fatty materials, rapeseed, olive and soy oils (1-4). It was also described as more effective than some other amino acids, including cystine or cysteine in some vegetable oils and lard (1, 3). In contrast, some reports found cystine as an effective antioxidant in soy, rice and corn oils as well as in lard and tallow (5, 6) and it was ranked as better than methionine in olive oil (7). However, other reports described cystine, proline and some other amino acids as ineffective in lards (8), with cysteine behaving as prooxidant in soy oil (5). Still, proline was found as an effective antioxidant in dry butter (9), in fresh sardine oil (10), and as proline nitroxide in menhaden oil (11), although it was not an effective metal deactivator in lard (12). Other amino acids were also investigated by many workers with different order of antioxidant effectiveness frequently reported (4, 6, 8, 12-15).

In view of this, it would not be possible to extend any findings in the literature in this respect to other oils. Therefore, it was the interest of the present work to investigate the antioxidant effects of 27 amino acids (including some derivatives) in two Iraqi vegetable oils.

MATERIALS AND METHODS

Materials

Oils. Safflower seeds (*Carthamus tinctorius* L.) of the "Gila" variety were supplied by the cotton and oilseed

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