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Article

Ochratoxin A and Aflatoxins: Fine-Tuning to the ELISA Test on Table Olives

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Abstract: This study is aimed to point out the possible application of the Enzyme-Linked ImmunoSorbent Assay (ELISA) test on table olives. Because olives, like many other foods, are not free from mycotoxin contamination, the main focus of this work was assessing the presence or absence of aflatoxins and ochratoxin A. The work was carried out in two phases. In the first phase, tests were carried out on commercial olives to assess the efficacy of the method and the kit (Veratox® 'Neogen' and AgraQuant® 'Romer Labs') by comparing them with the Liquid Chromatography coupled to a mass-mass spectrometer (LC-MS/MS); in order to understand if there were any interferences in antigen-antibody recognition; subsequently, mycotoxins were inoculated on the same samples with a known quantity, in order to assess the concentrations obtained and compare them to the expected ones. The second step involved modifying the method, so to improve the recovery of inoculated mycotoxins in the samples under study. Afterwards we evaluated the presence of mycotoxins in table olives supplied in the project. The results showed the effectiveness of the ELISA test, especially in the mycotoxin recovery phase. Indeed, after the modification of the mycotoxin extraction method, recoveries increased from 55% to over 70% for aflatoxins, and from 40% to over 55% for ochratoxin A. When analysing the samples provided by the above-mentioned company, the results showed some false positives, in particular in relation to ochratoxin A 's analysis, mainly related to the first sample submission. Taking this into account, the ELISA test can be considered as an initial screening method applicable as a control programme for the evaluation of mycotoxins in olives which helps by saving time and costs. It should always be kept in mind that, if positive values should emerge, the LC-MS/MS analysis should be carried out.

Keywords: table olives; ochratoxin A; aflatoxins; ELISA; LC-MS/MS

1. Introduction

In the Mediterranean regions, the olive (*Olea europaea* L.) and its derivatives are of strategic importance in terms of production, consumption and trade. In fact, almost the entire world production of olive oil is concentrated in this area, with Italy, Spain and Greece being the largest producers of the total output, of about 80%. Therefore, proper preservation and sanitisation of these products, especially in the pre-storage phases, is the right preventive approach to prevent mycotoxins from irreparably contaminating the raw material.

In view of the global situation on climate change, mycotoxin contamination is becoming more and more present in food, so it is crucial to ensure that food becomes increasingly safer [1,2].

Mycotoxins in foodstuffs constitute a major health and hygiene problem for both humans and animals, so they must be prevented since as of now there is no effective method to remove this type of contamination at later stages. Mycotoxins are rather stable by-products of fungal metabolism and

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therefore resistant to decontamination treatments. Because of this, once produced, they tend to remain in the substrate.

Table olives constitute a type of raw material which could hypothetically be contaminated by a wide range of fungal species, including potentially toxigenic ones belonging to the genera *Aspergillus* (e.g.: *A. flavus* and *A. parasiticus*, producers of aflatoxins and *A. ochraceus*, producer of ochratoxin A) and *Penicillium* (e.g.: *P. verrucosum*, producer of ochratoxin A) [3–7] (Figure 1), i.e. capable of producing toxic compounds such as mycotoxins at one or more stages of the production cycle.

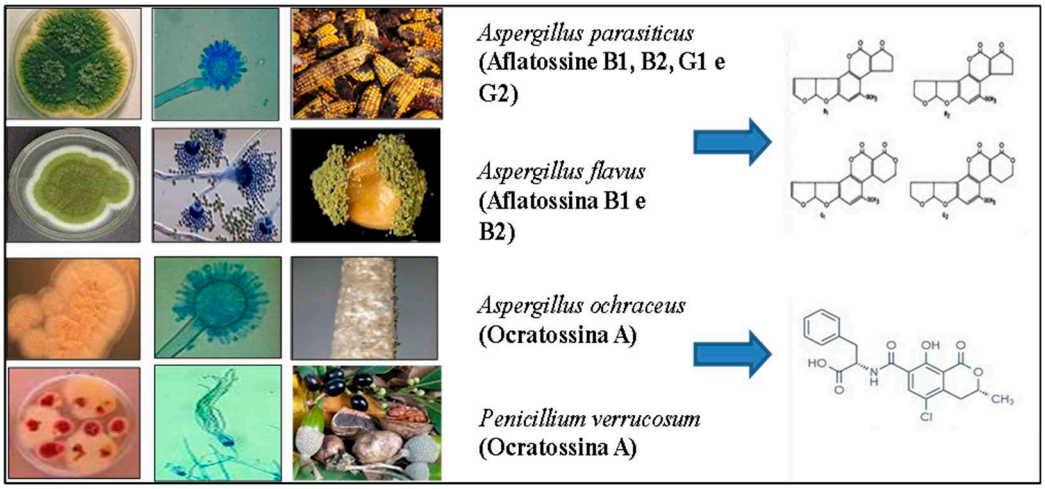

Figure 1. Aflatoxins and ochratoxin A, and the main toxigenic producing fungi.

To improve the health and hygiene quality of the final product and protect consumers, it is essential to optimise the steps of the process in order to safeguard against the risk of exposure to toxic metabolites through the consumption of contaminated table olives.

Aflatoxins B1, B2, G1 and G2, secondary fungal metabolites highly toxic to animals and humans due to their carcinogenicity, genotoxicity and mutagenicity characteristics, have been classified by the International Agency for Research on Cancer (IARC) as 'carcinogenic to humans' (Group 1); while ochratoxin A, which is markedly nephrotoxic, has been classified as 'possibly carcinogenic to humans' (Group 2B) [8].

Aflatoxins (AFB, AFG) are very stable organic compounds and therefore particularly resistant to degradation, as well as physical and chemical detoxification systems when in foodstuffs. Their toxic action on animal organisms happens mainly due to the interaction of these molecules with the nucleic acids of cells, impairing protein synthesis and the regulation of gene expression. For these reasons, these types of mycotoxins, in particular aflatoxin B1, show mutagenic and teratogenic characteristics and are considered the most potent carcinogens of natural origin [9].

Several studies have examined the presence of aflatoxins in fresh olives assessments. Using the HPLC under rather adverse storage conditions (rain, humidity and high temperatures) and the simultaneous presence of the toxins, which tends to increase the risks of toxicity, underlines the importance of having a good control over the storage of olives after harvest [10–12]. Other recent studies have led to the use of innovative techniques that allow simultaneous purification and enrichment in a single sample extraction step [13].

The production methods of table olives also play a key role in preserving the product, and if brine is used, also its composition [14–16]. Some studies have adopted the use of the electronic nose to determine changes in brines caused by microorganisms due to its ability to pick up volatile substances, as an alternative to other destructive techniques, [17].

However, it has been shown in the literature that yeasts and lactic acid bacteria, responsible for the fermentation process, constitute the dominant microflora; and their number, associated with metabolic activity, causes unfavourable conditions for the development of pathogenic microflora (aflatoxins and ochratoxins) which, in fact, is always absent [18–20].

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Other studies examined the growth and aflatoxin production of five strains of *Aspergillus flavus* and two strains of *Aspergillus parasiticus* isolated from: natural black olives in brine (for table consumption), whole fresh black olives, damaged fresh black olives and fresh black olive paste. All aflatoxigenic strains inoculated on damaged fresh black olives and fresh black olive paste grew extensively, while on fresh whole black olives they grew weakly. The strong aflatoxigenic strains produced low levels of aflatoxins, while the weak strains did not produce detectable amounts of the mycotoxin [7].

The ochratoxin group includes nine mycotoxins; of these, ochratoxin A (OTA) is the best-characterised molecule with the highest toxicity. These molecules are soluble in polar organic solvents and in weak basic solutions. They can be produced on many types of foodstuffs (including olives, both before and after harvesting) as a result of contamination by potentially producing fungi. The highest levels permitted of each type of mycotoxin in food are regulated by European regulations [21,22]. For the consumer, 'labelled' olive oils offer more guarantees, such as olive oil quality indices. Instead, 'unlabelled' oils offer no guarantees because the declared “extra virgin olive oils” have not been supported by any analyses. Furthermore, olive oil quality claims alone, without any kind of label, do not provide guarantees to the consumer about potential AFB1 and OTA contamination [23].

In the product 'table olives', the occurrence of high microbiological loads and the growth of pathogenic and potentially mycotoxin-producing microorganisms, suggested the need for a rapid technique for the identification of the hygienic quality of the product itself (although the methods of choice for the identification and determination of mycotoxin concentrations are generally of the chromatographic type - HPLC, GC, etc.). However, *Enzyme Linked ImmunoSorbent Assay* (ELISA) methods can also be used in the preliminary phase of screening of contaminated samples and then it’s possible to proceed with confirmatory chromatographic methods.

2. Materials and Methods

2.1. Table Olives Samples

The experimental design consisted mainly of two phases. In the first phase, tests were carried out on three commercial olive samples to verify the conditions suitable for the purpose of this study.

In the second phase, samples from the Ficacci Olives company located in Lazio region (Central Italy) were used to confirm what was observed in the commercial samples and analyses were carried out on these to verify the contamination of the two mycotoxins.

The tests were conducted on the following samples (4 shipments every six months for 3 batches) and then stored at 4°C.

1st shipment

- Nocellara del Belice (Castel Vetrano method) batch no. (335/20-402/20-409/20)
- Nocellara Etnea (Natural method) batch no. (217/20-233/20-259/20)
- Bella di Cerignola (Sivigliano method) batch no. (A-B-C without identification)
- Itrana Bianca (Natural method) batch no. (492/19-499/19-502/19)

2nd shipment

- Nocellara del Belice (Castel Vetrano method) batch no. (335/20-402/20-409/20)
- Nocellara Etnea (Natural Method) batch no. (217/20-233/20-259/20)
- Bella di Cerignola (Sivigliano method) batch no. (049/20-074/20-161/20)
- Itrana Bianca (Natural method) batch no. (492/19-499/19-502/19)

3rd shipment

- Nocellara del Belice (Castel Vetrano method) batch no. (408/21-422/21-474/21)
- Corservolea Nera (Californian method 1st drying) batch no. (024/22-047/22-056/22)
- Bella di Cerignola (Sivigliano method) batch no. (033/21-326/21-341/21)
- Hojiblanca Nera (Californian pitted method) batch no. (002/22-182/22-498/21)

4th shipment

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- Nocellara del Belice (Castel Vetrano method) batch no. (408/21-422/21-474/21)
- Hojiblanca Nera (Californian pitted method) batch no. (002/22-182/22-498/22)
- Bella di Cerignola (Sivigliano method) batch no. (033/21-326/21-341/21)
- GR 2177 (Californian pitted method) batch no. (one lot)

Specifically, the processing method which was used, called debittering, differs depending on whether the olives are green or black.

For green olives, the methods used were:

- The Sivigliano method, which eliminates the bitterness of raw olives by using sodium solution. This treatment takes between eight and fifteen hours from start to finish depending on the temperature, the size of the olives, and their degree of ripeness. This process is repeatedly followed by washing to remove the soda.
- The Castelvetrano method, also known as the 'soda method', which proceeds through the following stages: sorting of the olives, grading, sodium-saline solution, softening and fermentation, which are essential for final consumption.
- The Natural method proceeds through the following stages: sorting of the olives, grading, possibly crushing and stoning, brine, fermentation, grading, packaging, and possibly pasteurisation, which are essential for final consumption.

While for black olives, the method used was:

- The Californian method proceeds through the following stages: sorting of the olives, grading, brine, treatment with soda, air oxidation, washing, treatment with ferrous salts, brine, eventual pasteurisation, grading, packaging, and sterilisation, which are essential for final consumption.

2.2. Obtaining Olive Paste

The olive paste was obtained using two instruments: Ultra Turrax (IKA mod. T18 digital) and a commercial coffee grinder. Because the results were the same for both instruments, for a matter of speed in obtaining the olive paste and for ease-of cleaning, the choice of instrument fell on the second option. After homogenisation, the sample was divided into two falcons: one for analysis using the ELISA method; the other was sent to the Palermo Customs Office, which performed the analyses on the two mycotoxins using the official LC-MS/MS method.

2.3. Solvents Used and Olive Pasta Drying

The following organic solvents were used for the analyses: dichloromethane - CAS 75-09-2 (analytical grade RPE or ACS, Honeywell) and methanol - CAS 67-56-1 (≥ 99.8%, AnalaR NORMAPUR® ACS, VWR); deionised water was obtained by continuous deionisation equipment (Water Purification System Zeener Power I, Human Corporation, Seoul, Korea). Methanol and dichloromethane (15 ml for both) were used as mycotoxin extracting solvents in the olive paste. The better option seemed to be the one to use dichloromethane because it was found to have greater extractive power (data shown in results). The drying phase of the samples was carried out with the Büchi Rotavapor (mod. T-114).

2.4. Study of the Preparatory Phase

Sampling was carried out on the basis of representative criteria, and all milled samples were stored at 2–8 °C until the analysis [24]. During the analysis, in the first phase, 3 g of sample were taken, and 15 ml of 70% methanol were added (as stated in the ELISA kits), then the sample was vigorously shaken for 3 minutes, followed by centrifugation for 15 minutes at 15 degrees (Allegra 25R by Beckman Coulter) at 3500 RCF. The supernatant was filtered through Whatman No.1.

8 ml of the filtrate was then pre-dispensed and placed in a test tube and then dried with Rotavapor.

Once the samples were dried, they were resuspended in 3 mL of 70% methanol, they were then vortexed, centrifuged and then 2 mL of supernatant were transferred to a test tube.

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Subsequently, the pH was measured and if necessary an adjustment was made in the range between 6 and 7 (as reported in the methods of the ELISA kits).

2.5. Adjustments to the Preparatory Phase

During the first preparatory phase, 70% methanol was found to be ineffective in the extraction of mycotoxins, particularly for ochratoxins (data shown in results). Therefore, during the analysis, 3 g of sample were taken, and 15 ml of dichloromethane absolute were added, because they resulted in having a greater extraction power, this reagent was chosen. Once the sample is dry, it was resuspended in 3 mL absolute methanol, vortexed, centrifuged and then 2 mL of supernatant were transferred to a test tube, after which 850 µl of deionised water was added to restore the methanol concentration to 70%. Subsequently, a 2 ml aliquot was taken, and the pH was measured and if necessary an adjustment was made in the range between 6 and 7 (as reported in the methods of the ELISA kits).

2.6. ELISA Method

Aflatoxins and ochratoxin A content was assessed using two types (Veratox® 'Neogen' and AgraQuant® 'Romer Labs' kits following the manufacturer's instructions. The following direct-competitive ELISA kits were used: Veratox® for Aflatoxin (Neogen, product 8030), AgraQuant® Total Aflatoxin 4/40 ELISA kit (Romer Labs, article number 100002098/100002099), Veratox® for Ochratoxin (Neogen, product 8610), AgraQuant® Ochratoxin 2/40 ELISA kit (Romer Labs, article number 100002102/100002103).

Standard solutions (STDs) were used for the inoculation of mycotoxins into the olive paste samples: AFLATOXIN B1 (BIOPURE®) 2 ug/ml x 1 ml (ref. no. 10003652), Mix 5 AFLATOXINS (BIOPURE®) 0.25 ug/ml x 1 ml (ref. no. 10003657) and OCHRATOXIN A 10 ug/ml (BIOPURE®) x 1 ml (ref. no. 10003658).

2.7. LC-MS/MS Method

This method allowed us to identify, quantify and confirm ochratoxin A, aflatoxins B1, B2, G1 and G2, by Liquid Chromatography coupled to a mass-mass spectrometer (LC-MS/MS) . The method was applied on the olive paste matrix.

The method involved the extraction of mycotoxins from the homogenised matrix with a mixture of methanol:water (80:20 v/v).

The extract was purified with immunoaffinity columns specific for Aflatoxins (B1, B2, G1 and G2) and for Ochratoxin A. The purified extract was added with the labelled analogues of each mycotoxin used as internal standards.

The extract was analysed by (LC-MS/MS), a technique which simultaneously provides a confirmatory analysis and a quantitative determination. Quantification was performed by linear calibration.

Sample preparation was made by using about 5 g of olive paste and weighed into a 50 mL conical tube, then a sample was extracted with 30 mL of a mixture of methanol:water (80:20 v/v). The mixture was stirred and vigorously mixed on a multi-tube vortexer for 2 minutes, and sonicated for thirty minutes in an ultrasonic bath, followed by centrifugation at 5000 rpm for 15 minutes. The supernatant was collected and purified by immunoaffinity columns specific for aflatoxins and ochratoxin A. Specifically, for aflatoxin B1, B2, G1 and G2 4 ml of supernatant were taken and diluted to 25 ml with a phosphate buffer solution (Phosphate Buffer saline pH 7.4), then afterwards they were purified with the immunoaffinity column. After being washed with water (20 ml), the aflatoxins were eluted with 3 ml of methanol. Then, 0.5 ml of eluate was added to 0.5 ml of solution containing the internal standards as labelled for each aflatoxin (B1, B2, G1 and G2). As for ochratoxin A, however, 5 ml of supernatant were taken and diluted to 50 ml with a phosphate buffer solution (Phosphate Buffer saline pH 7.4) and then purified with the immunoaffinity column. Next, a wash was performed with 20 ml PBS and 10 ml water, Ochratoxin A was eluted with 3 ml of a mixture of acetic acid and

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methanol (2:98 v/v). 0.5 ml of eluate was then taken and added to 0.5 ml of solution containing the labelled internal standard for Ochratoxin A.

The instrument used to perform the analyses was a Thermo UHPLC Ultimate 3000 equipped with an autosampler and a Thermo MS/MS Q Exactive Focus detector.

Analytes were detected by injecting 10 µl into the liquid chromatograph coupled to the high-resolution mass spectrometer and acquiring the signal in Full-Scan and parallel on monitoring (PRM) modes.

In Full Scan, the instrument allowed us to visualise the signal of selected molecular adduct ions (H+) of mycotoxins by knowing their exact mass (m/z).

In PRM mode, the instrument isolated and fragmented the mycotoxin precursor ions, and the fragments were selected to be quantified.

Separation was done with Thermo UHPLC model Ultimate 3000 consisting of the binary pump, autosampler and column heater set at 25 ^{0}C.

The column used was a Thermo Scientific Accucore RP-MS 100 x 2, 1mm, 2.6 µm.

The mobile phase was divided as follows:

Phase A, composed of Water with 5 mM ammonium formate, 0.1% formic acid.

Phase B, composed of methanol with 5 mM ammonium formate, 0.1% formic acid.

The gradient:

Started with 5% B, maintained for 1 minute and used a linear gradient up to 95% B for 4.5 minutes, maintained for 3.5 minutes, then decreased to the original 5% B and equilibrated for another minute for a total run time of 13 minutes with an LC-MS/MS flow rate of 0.350 mL/min.

For calibration, six concentration levels were prepared in methanol.

Aflatoxin B1 and G1 at concentrations between 0.1-2.0 ng/mL (equivalent to 0.9-18 ng/g). Aflatoxin B2 and G2 at concentrations between 0.025-0.5 ng/mL (equivalent to 0.2-4.5 ng/g). Ochratoxin A at concentrations between 0.05-2.5 ng/mL (equivalent to 0.4-18 ng/g). Labelled analogues of each mycotoxin, used as internal standards, were added to each calibration level at a concentration of 1 ng/mL.

For each mycotoxin, the software performed regression analysis by working out a linear calibration function that reported the ratio of Area STD/Area Internal standard in function of the concentrations. These linear functions were used to quantify the concentration of each mycotoxin in the olive paste sample. All information and specifications were given in the supplementary material in Table 1.

2.8. Statistical Analysis

Statistical processing of the data was carried out with R Core Team software [25], with the packages 'agricolae', 'car', and 'emmeans' for the development of ANOVA analyses, the preparation of graphs [26] and the Tukey's post-hoc test [27–29].

3. Results

3.1. Determination of Mycotoxin Concentration

In the preliminary phase, sample preparation was optimised for the subsequent extraction phase according to the methods of the kits. Tests were carried out on three commercial samples, which demonstrated the use of olive paste was more effective, in relation to the smaller particle size obtained, favouring greater representation of individual subsamples for subsequent analyses. As far as the determination of ochratoxin A is concerned, the results obtained on the 'blanks' in the preliminary tests did not reveal interferences in antigen-antibody recognition for both kits used; the absorbance levels obtained were well below the limit of quantification of the methods (Veratox®: LOQ= 2 µg/kg; AgraQuant®: LOQ= 2 µg/kg). To confirm the lack of interference for the specific antibody, fortification tests were performed directly on the extracts obtained, according to the methods.

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Unfortunately, the results of the recovery tests on the above-mentioned samples, after being fortified with known amounts of ochratoxin A, did not give satisfactory results; in fact, on the fortified extracts, the values obtained gave a recovery of ≤ 40% for both kits.

The poor extraction capacity of mycotoxin was attributed to the ineffectiveness, for this type of matrix, of the solvent mixture used in the two kits (methanol 70%). Therefore, a revision of the method was carried out, both in the extraction procedures and in the replacement of the organic solvent mixture for the treatment of the samples, once combined with the mixture. Considering the high vegetable fat content of the 'olive' matrix, it was deemed appropriate to use solvents with a greater extractive force than diluted methyl alcohol (70% *v/v* as reported in the methods), which instead is evidently sufficient for other types of matrices with different characteristics from olives. Dichloromethane was then chosen as the main extractant. Preliminary tests were then carried out to recover ochratoxin A according to the modified method, using the same commercial samples. The results were encouraging, indeed previously the recoveries after using methanol 70% were at around 40%, while with dichloromethane the recoveries increased to a percentage between 55% and 75%. Concerning the determination of the total number of aflatoxins, the results obtained on the same unfortified samples (blanks) showed, for both kits used, that the olive matrix does not interfere in antigen-antibody recognition, resulting in absorbance levels well below the quantification limit of the methods (Veratox®: LOQ= 5 µg/kg; AgraQuant®: LOQ= 4 µg/kg).

To confirm the lack of interferents for the specific antibody, fortification tests were carried out on the extracts obtained according to the methods and the results confirmed again a good match between the concentrations obtained and those expected.

When evaluating the results, the different specificity characteristics of the antibodies used in the two different kits were considered; in particular, concerning the Veratox® kit, the reaction specificity is greater towards aflatoxins B1 and B2, while regarding AgraQuant® the percentage of specificity towards the four aflatoxins is as follows AFB1 = 100%, AFB2 = 65%, AFG1 = 70% and AFG2 = 42%.

Recovery tests on samples of green olive paste, fortified with known quantities of total aflatoxins (20 µg/kg of each of the 4 AFB and AFG or individually B1) and carried out according to the methods indicated in the two kits used, demonstrated sufficient efficacy of the solvent mixture (methyl alcohol at 70% v/v in water) used in the extraction of mycotoxins with values of around 55% on total aflatoxins (no. 3 samples), while for aflatoxin B1 they reached 76% (no. 1 sample). However, when applying the same OTA extraction conditions (new method), a recovery check was also carried out for aflatoxin B1, for concentrations between 6 and 10 µg/kg. The results showed that, in the case of AgraQuant®, the new extraction conditions improved the recovery of AFB1 in comparison with the method (MetOH 70%) to over 90% (no. 6 samples). A good recovery was also obtained in the case of Veratox® on the same samples and for the same concentration range (approx. 70%), which confirms the extraction validity of the modified method (Dichloromethane).

Another aspect evaluated concerned the optimisation of the sample preparation method, which involved the comparison of two types of sample treatment once combined with the extraction solvent. Olive paste samples fortified with known amounts of ochratoxin A and prepared in parallel were subjected to both high-speed homogenisation for 1 minute and vortexing (30 Hrz) for 3 minutes. The results reported at concentrations in the range of 20 to 100 µg/kg (n =4) demonstrated the non-significance ($p < 0.05$) of the differences in final mycotoxin recovery.

Once all the tests had been performed, the new method was applied to the samples provided by the Ficacci company to assess the contamination levels of aflatoxins and ochratoxin A. In parallel, the same analyses were also carried out in LC-MS/MS.

The data analysed with the chromatographic method (LC-MS/MS) showed that all samples had levels below the LOQ, while false positives were obtained with the ELISA method.

Specifically, for ochratoxin A the results showed several false positives, for both kits, especially in the first and fourth shipments, while all negatives were confirmed (Table 1).

Table 1. Average Ochratoxin A content in the batch processing of the olive pasta samples belonging to the four shipping groups (μg/kg), ± SD (standard deviation), $n = 4$.

Cultivar	ELISA method								LC-MS/MS analysis
	I shipment		II shipment		III shipment		IV shipment		
	Veratox®	Agraquant®	Veratox®	Agraquant®	Veratox®	Agraquant®	Veratox®	Agraquant®	
Nocellara del Belice	<LOQ	3.41	1.82±0.08	1.67±0.08	<LOQ	<LOQ	2.04±0.32	1.54±0.55	<LOQ
Castelvetrano method	2.15±0.35	<LOQ	<LOQ	<LOQ	<LOQ	1.29±0.35	2.42±0.72	1.79±0.46	<LOQ
	2.43±0.10	<LOQ	<LOQ	<LOQ	<LOQ	2.06±0.38	3.00±0.37	<LOQ	<LOQ
Nocellara Etnea	3.16±0.90	5.53±0.98	2.41±0.15	<LOQ	-	-	-	-	<LOQ
Natural method	2.18±0.99	5.59±0.52	1.73±0.16	<LOQ	-	-	-	-	<LOQ
	2.96±0.13	4.88±0.82	2.40±0.92	<LOQ	-	-	-	-	<LOQ
Bella di Cerignola	1.85±0.59	6.21±0.77	<LOQ	3.98±0.18	<LOQ	<LOQ	2.07±0.30	<LOQ	<LOQ
Sivigliano method	2.05±0.79	5.16±0.59	2.05±0.79	<LOQ	<LOQ	<LOQ	1.82±0.26	1.64±0.47	<LOQ
	1.67±0.19	3.95±1.00	1.67±0.19	2.80±0.23	1.91±0.07	<LOQ	2.11±0.45	1.90±0.33	<LOQ
Itrana Bianca	<LOQ	3.00±0.19	<LOQ	3.00±0.19	-	-	-	-	<LOQ
Natural method	<LOQ	2.96±0.41	<LOQ	2.96±0.41	-	-	-	-	<LOQ
	<LOQ	<LOQ	<LOQ	<LOQ	-	-	-	-	<LOQ
Conservolea Nera	-	-	-	-	1.62±0.24	<LOQ	-	-	<LOQ
Californiano I° drying method	-	-	-	-	<LOQ	1.66±0.28	-	-	<LOQ
	-	-	-	-	2.53±0.52	<LOQ	-	-	<LOQ
Hojiblanca Nera	-	-	-	-	1.99±0.53	<LOQ	1.90±0.48	<LOQ	<LOQ
Pitted	-	-	-	-	4.57±0.12	<LOQ	2.85±0.44	<LOQ	<LOQ
	-	-	-	-	3.86±0.30	<LOQ	1.70±0.28	<LOQ	<LOQ
GR 2177	-	-	-	-	-	-	<LOQ	<LOQ	<LOQ
Californiano pitted method	-	-	-	-	-	-	<LOQ	<LOQ	<LOQ
	-	-	-	-	-	-	<LOQ	<LOQ	<LOQ

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Therefore, after noticing these false positives, they were statistically processed in order to understand the reason for these results. It was found that the Nocellara Etnea variety (3.47 μg/kg) is significantly different from the other cultivars (Supplementary Table 2).

Whereas, regarding the processing methods for table olives, the natural method (3.30 μg/kg) showed to be statistically different, with a consistent intermediate case of the Californian pitted method (3.10 μg/kg) (Supplementary Table 3).

Among the kits used, it was found that there were significant differences between one another. (Supplementary Table 4).

Concerning aflatoxins content, the results of the LC-MS/MS analysis showed that the samples under study were below the LOQ, while the ELISA test gave some false positives for this mycotoxin, which was significantly lower than those found for ochratoxin A (Table 2). False positives emerged mainly with the AgraQuant® kit, around 25%, while with the Veratox® kit they were below 3%.

Also in this case, after finding these false positives, we wanted to understand the reason for them, so we processed the results statistically.

Significant differences were found between the group of Itrana Bianca (3.77 μg/kg) and Hojiblanca Nera (3.61 μg/kg) with an intermediate group consisting of Conservolea Nera (3.09 μg/kg), Nocellara del Belice (3.02 μg/kg) and Bella di Cerignola (3.01), compared to the statistically lower values of Nocellara Etnea (2.77 μg/kg) (Supplementary Table 5). Among the transformation methods, only the Californian pitted method (3.61 μg/kg) showed a statistically significant difference compared to the others (Supplementary Table 6). The kits did not show statistically different values (Supplementary Table 7).

Having observed the differences between all the cultivars and methods, we wanted to focus on the only two cultivars present in all 4 shipments (Bella di Cerignola and Nocellara del Belice).

When looking at results regarding aflatoxins specifically, the ANOVA analysis did not reveal any statistically significant differences in the two years over which they were observed and between the two cultivars (Figures 2 and 3). In contrast, for ochratoxin A, there is a statistically significant difference (*p-value <0.001*) between the averages in the two years (Figure 4), but not between the two cultivars (Figure 5).

Taken individually, the cultivars showed a different trend and distribution for both aflatoxins and ochratoxin A. Specifically, the Bella di Cerignola cultivar had a higher concentration of aflatoxins between the minimum value of 2.29 μg/kg and the maximum value of 4 μg/kg (Supplementary Figure 1), while the Nocellara del Belice cultivar had a more homogenous concentration for the year 2020 and became more uneven in the year 2021 (Supplementary Figure 2). Regarding the concentration of ochratoxin A, the Bella di Cerignola cultivar had a more homogeneous distribution for the year 2021 (Supplementary Figure 3) in contrast to the Nocellara del Belice cultivar which has a different distribution for both years (Supplementary Figure 4).

Table 2. Average content of Aflatoxins in the batch processing of the olive pasta samples belonging to the four shipping groups (µg/kg), ± SD (standard deviation), *n* = 4.

Cultivar	ELISA method								LC-MS/MS analysis
	I shipment		II shipment		III shipment		IV shipment		
	Veratox®	Agraquant®	Veratox®	Agraquant®	Veratox®	Agraquant®	Veratox®	Agraquant®	
Nocellara del Belice	<LOQ	2.78±0.23	<LOQ	<LOQ	<LOQ	2.88±0.63	<LOQ	<LOQ	<LOQ
Castelvetrano method	<LOQ	2.74±0.31	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Nocellara Etnea	<LOQ	2.49±0.28	<LOQ	2.49±0.28	-	-	-	-	<LOQ
Natural method	3.74±0.05	<LOQ	<LOQ	<LOQ	-	-	-	-	<LOQ
	<LOQ	2.29±0.08	<LOQ	<LOQ	-	-	-	-	<LOQ
Bella di Cerignola	<LOQ	<LOQ	<LOQ	2.93±0.80	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Sivigliano method	<LOQ	<LOQ	<LOQ	3.42±0.32	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	<LOQ	<LOQ	<LOQ	3.00±0.56	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
Itrana Bianca	<LOQ	3.86±0.41	<LOQ	<LOQ	-	-	-	-	<LOQ
Natural method	<LOQ	<LOQ	<LOQ	<LOQ	-	-	-	-	<LOQ
	<LOQ	<LOQ	<LOQ	<LOQ	-	-	-	-	<LOQ
Conservolea Nera	-	-	-	-	<LOQ	<LOQ	-	-	<LOQ
Californiano I° drying method	-	-	-	-	<LOQ	<LOQ	-	-	<LOQ
	-	-	-	-	<LOQ	<LOQ	-	-	<LOQ
Hojiblanca Nera	-	-	-	-	<LOQ	4.00±0.39	<LOQ	<LOQ	<LOQ
Pitted	-	-	-	-	<LOQ	<LOQ	<LOQ	<LOQ	<LOQ
	-	-	-	-	<LOQ	3.44±0.69	<LOQ	<LOQ	<LOQ
GR 2177	-	-	-	-	-	-	<LOQ	<LOQ	<LOQ
Californiano pitted method	-	-	-	-	-	-	<LOQ	<LOQ	<LOQ
	-	-	-	-	-	-	<LOQ	<LOQ	<LOQ

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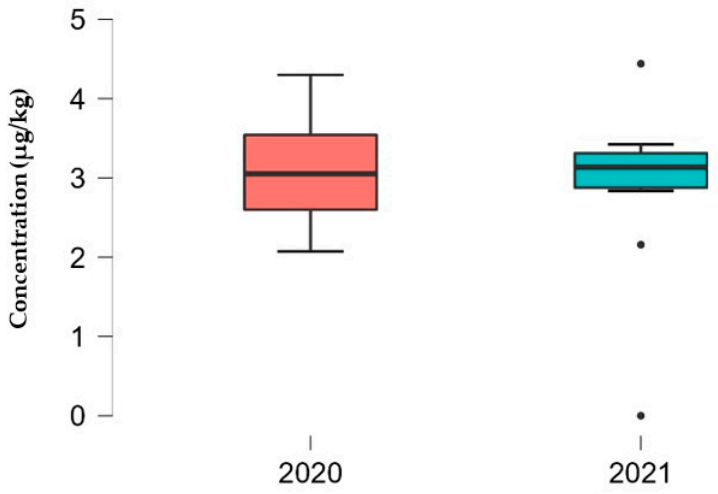

Figure 2. Aflatoxins concentration in the two crop years present in all 4 shipments.

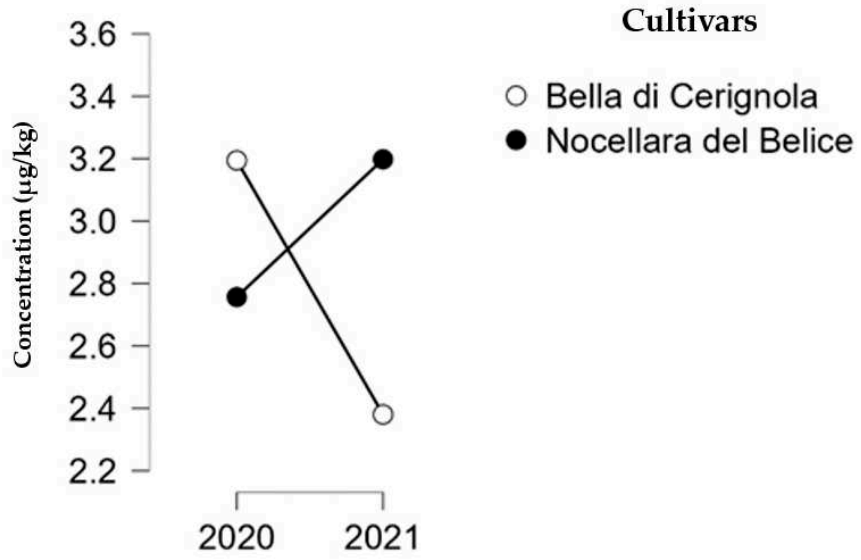

Figure 3. Aflatoxins concentration in the two cultivars present in all 4 shipments.

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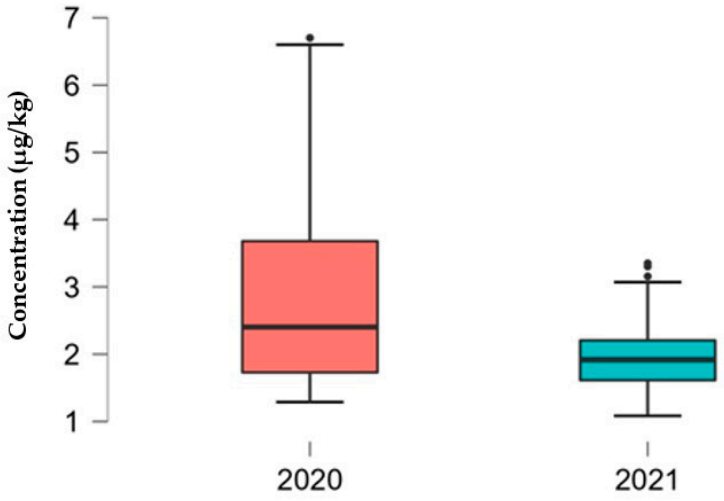

Figure 4. Ochratoxin A concentration in the two crop years present in all 4 shipments.

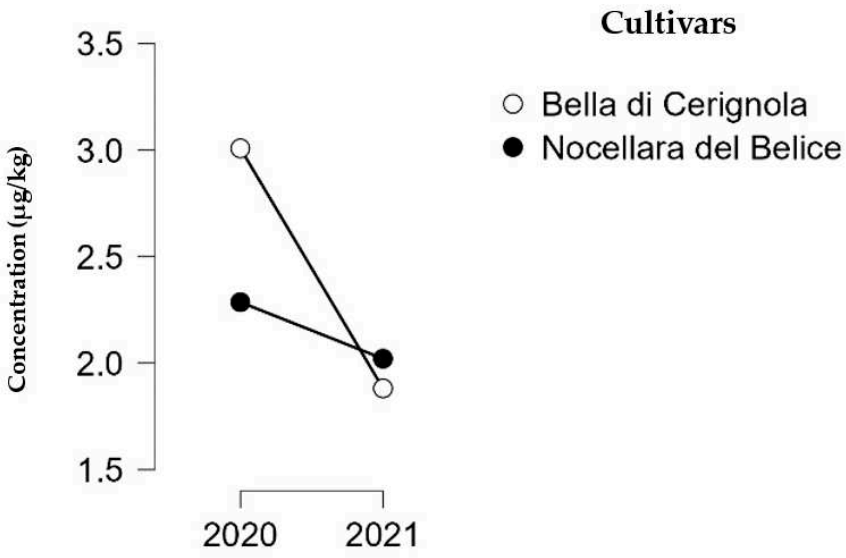

Figure 5. Ochratoxin A concentration in the two cultivars present in all 4 shipments.

4. Discussion

This study aimed to address the issue of organic contamination in table olives. In the present study, ELISA kits were used as a tool to monitor the hygienic levels of the olives.

In particular, the modification of the method of extraction of mycotoxins with the use of dichloromethane on inoculated table olive samples, allowed satisfactory results to be obtained, mainly for ochratoxin A. In fact, the results went from 40% to about 55%. This method has been also effective in the recovery of aflatoxins, despite good results when using 70% methanol.

Mycotoxin recovery tests, using the new method, were also carried out on project samples and the results were comparable with those obtained from commercial olives.

Afterwards, the samples were analysed, and the results showed the presence of some false positives, mainly for ochratoxin A (Table 1). These results will have to be food for thought to understand what were the problems that caused them. Moreover, as already mentioned for the ochratoxin A, the first and the fourth shipment, for both kits, were those with a greater presence of false positives. This was not found in aflatoxins.

In particular, the aflatoxins had a much lower percentage of false positives (Table 2), which suggests that the kits have a greater affinity with mycotoxin, resulting in less interference.

It must be said that the results of the false positives found by the analyses are rather low, close to the limit of quantification (LOQ), therefore the margin of error could increase.

In this regard, it would be useful to perform further tests on more samples by varying the concentration of inoculated mycotoxins.

Overall, the results for both types of mycotoxins underline the importance of keeping an eye on initial levels of contamination of table olives to ensure the safety of the consumer.

5. Conclusions

Regarding the technical and practical aspects of the present research, the use of the ELISA test undoubtedly responds to the new need to reduce production costs and response times, hence the need to have objective and comparable reading criteria. In fact, its performance, in terms of high diagnostic sensitivity, makes it particularly suitable as a screening test to be used during control programmes; especially in the early stages of evaluation, where a negative result would avoid the need to recourse to deeper and costly analysis using the LC-MS/MS method.

Therefore, by applying this method, it is possible to have an initial assessment of the presence or absence of mycotoxin contamination in olives and their derivatives.

The analyses carried out on olive paste indicated that the two ELISA kits (Veratox® and AgraQuant®), used for the assessment of the concentration of the two mycotoxins (aflatoxins and ochratoxin A), are not completely comparable, but are able to detect and quantify the mycotoxins in the various samples provided. Considering the complexity of the food matrix studied, correct sampling was essential to overcome the uncertainty of the divergent results obtained from the kits.

Our data showed that some variability within the kits could be expected, given the antibody-antigen affinity, but it also confirmed the reliability of the tests on negative values.

The importance of having a revised method for sample processing using dichloromethane as the main extractable agent, lies in obtaining the best recovery in percentage, in the inoculated matrix, of ochratoxins and aflatoxins.

In conclusion, the ELISA method can be used as an initial screening method, to assess the presence or absence of mycotoxins; only if the analysed samples prove positive, a more specific analysis with the determination of mycotoxins by LC-MS/MS is deemed necessary.

Supplementary Materials: The following supporting information can be downloaded at the website of this paper posted on Preprints.org.

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Data Availability Statement: All data are contained within the article.

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