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Discrimination and Quantification of Aflatoxins in *Pistachia vera* Seeds Using FTIR-DRIFT Spectroscopy after their Treatment by Greek Medicinal and Aromatic Plants Extracts

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Abstract: A method for detection and quantitative determination of aflatoxins (AFs) produced by *Aspergillus flavus* in *Pistachia vera* seeds (pistachio) using Fourier transform infrared spectroscopy, coupled with Diffuse Reflectance Infrared Fourier Transform Spectroscopy (FTIR-DRIFT) is examined in this study. Greek medicinal and aromatic plant extracts with established bioactivity against *A. flavus* AFs production were used as treatments on pistachio seeds. Classes were analyzed using discriminate analysis algorithm, and distance values were calculated in Mahalanobis distance units. Strong absorptions were detected in the spectral ranges between 1,750 and 1,500 cm^{-1}, which were different in contaminated and not contaminated samples. The spectra regions that gave matching values less than 50.00 in absolute difference for AFs in pistachio paste samples were 1,705-1,575 cm^{-1} and 1,350-900 cm^{-1}. Also, the high performance of FTIR as analytical tool for quantitative estimation of AFs was emerged by the Partial Least Square (PLS) regression model that found to fit perfectly the actual concentration values (R^2 = 99.99%) in combination with low levels of errors of calibration, validation and prediction along with a minimum and low-cost sample preparation. The developed methodology presents a great potential of a valuable and reliable technique for composition determination, differentiation and evaluation of AFs in food products. We also aimed to reduce sample preparation in the methodology described and to include pistachio and plant extract matrix in classification demonstrating the suitability of FTIR DRIFT spectroscopic methods for direct AFs detection and quantification in biorefineries and industry.

***Keywords*:** FTIR, aflatoxins, pistachio, medicinal and aromatic plants, *Aspergillus flavus*

1. Introduction

A substantial portion (> 25%) of the world's agricultural commodities is known to be contaminated with diverse mycotoxins due to fungal colonization. *Aspergillus* strains that producing aflatoxins (AFs) are one of the most significant from economic and public health perspectives due to their role in food spoilage (Benett, 2010). Of 18 different types of AFs identified to date, the more common types are aflatoxin B1 (most predominant and potent), B2, G1, G2, M_1, and M_2. The AFs have been directly associated with high toxicological, carginogenic and mutagenic properties [2]. The International Agency for Research on Cancer (IARC) conducted evaluation of several chemicals of aflatoxins' carcinogenic potential and classified them as the most potent natural, known human carcinogens [3]. Thus, food and feed grains and oil seeds contaminated with AFs can be extremely toxic and carcinogenic causing esophagus and liver cancers in humans and animals. In view of toxic and carcinogenic effects of aflatoxin contaminated foods, US department of Agriculture (USDA) and Food and Drug Administration (FDA) set for foods the tolerance limit of 20 ppb. European Commission (EC Commission Regulation 165/2010) established much lower permissible levels of AFs. Aflatoxin contaminated grains and oil seeds are also devaluated in the markets, resulting in substantial economic losses for farmers, animal producers, grain handlers, and food and feed processors.

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Pistacia vera is an economically important crop in Greece and worldwide. According to FAOSTAT (The Food and Agriculture Organization Corporate Statistical Database) the international pistachio production has been increased at about 146% from 1994-2014 due to the growing demand [4]. Between producing countries, Iran is the largest producer and Greece ranked at the 6th position internationally and at the 1st in European Union. According to ICAP Group data (Business Services Groups in South Eastern Europe), edible pistachios are the 2nd exporting product from dry fruits of Greece after almonds [5]. Due to the great economic importance of food safety several research attempts have been widely conducted on analytical methods for the detection and quantification of AFs. These techniques, which are available in laboratory and non-laboratory locations, include high-performance liquid chromatography (HPLC) [6], liquid chromatography-mass spectrometry (LC–MS) [7], thin layer chromatography (TLC) [7, 8], enzyme-linked immuno-sorbent assay (ELISA) [9] and fluorometric assays with immunoaffinity column clean-up [10]. All the above-mentioned techniques are very reliable, very sensitive but require accredited laboratories with sophisticated instrumentation and trained skilled technicians. Moreover, they need extensive sample pretreatment, expensive apparatus and consumables with the exception of ELISA method that has found application outside regulatory testing, but with ongoing issues regarding cross-reactivities and the need to handle solvents and testing solutions. The ELISA technique except the disadvantage of its high cost in commercial kits, requires multiple washing steps, which may at times proved to be not only laborious but also time consuming, matrix dependent with limited application. A review about ELISA and similar assay-based methods was published recently [11].

As an alternative to the current detection methods, biosensors and complicated nanostructures entered recently the field [12-16]. Biosensors offer a rapid, specific, real-time monitoring, adaptable to remote sensing. However, at the same time there are still some limitations in their use because of their tend to be cross-reactive and insensitive. Also, one of the main problem is reproducibility while more complicated structures are often used for multifunctional signal transduction and amplification to achieve high sensitivity [11, 17].

Therefore, the search for simple, label-free, and more rapid and sensitive tools that are based on immune-biosensor format, appears to offer for the near future, versatile, portable, sensitive, and accurate field use devices for AFs detection. Many studies have demonstrated the potential use of Fourier transform infrared spectroscopy, coupled with attenuated total reflectance IR spectroscopy-based for the detection of AFs in oil seeds and mold infestation on crops and a summary of state-of-the-art was recently provided [18]. Specifically, FTIR is a powerful analytical tool for mycotoxin analyses, especially when coupled with chromatographic methods. Several studies have shown that FTIR-based methods can be successfully applied in the food industry, in association with detection of substances that affect the quality of food products or that are employed for adulteration [19]. Several studies have shown that FTIR-based methods can be used for the detection of AFs in many food products. Nevertheless, there is no published research that could address AFs detection and their direct quantification not interfering pistachio matrix or fungal contamination, that would enable it suitable for this purpose.

In this study, we examine the potential of FTIR-DRIFT method as a rapid and direct method for detection and quantification of AFs in pistachio contaminated samples compared to ELISA, as well as an evaluation tool for the biological activity of Greek medicinal and aromatic plants extracts on AFs production by *A.flavus* on pistachio seeds.

2. Materials and methods

2.1 Plant material and extract preparation

All plant extracts were prepared by dry plant material as described in [20] without any modification. TrHf treatment corresponds to *Hyssopus officinalis* extract (hyssop), TrMf *to Melissa officinalis* (lemon balm), TrSv to *Salvia officinalis* (sage), TrDt to *Origanum dictamnus* (dittany), TrOr to *Origanum vulgare* (oregano) and TrSf to *Crocus sativus* (saffron). Additionally, a TrRs 8.6 mg/mL rosmarinic acid (RA) treatment solution was prepared as RA is the common and characteristic phenolic compound of all the Lamiacae family plants, and 8.6mg/mL is the approximate concentration of RA in TrMf according to [21], in an attempt to interpret the bioactivity of the previous extracts against a specific compounds. Prior to application on pistachio seeds, aliquots of the plant extracts were further filtered through a sterile and endotoxin free 0.2μm PES filter media (Whatman Puradisk 25mm, country) in order to reduce the risk of interference by microorganisms. Test solutions were prepared by 5mL of each plant extract in which 45mL of double distilled water ddH_2O and 0.01% of the surfactant tween 20 were added.

2.2 Determination of total phenolic content

The total phenolic content of plant extracts was determined using Folin-Ciocalteu assay (Singleton et al., 1999), with some modifications. Thus 100μL of sample tested, 500μL of Folin-Ciocalteu reagent and 6mL of deionized water

were transferred in a 10mL flask and shaked thoroughly. After 3min, 1.5mL of 20% Na_2CO_3 was added in the flask which was filled with water until 10mL. The absorbance was measured at 725nm after two hours standing. Calibration curve was plotted by using Caffeic acid as standard (0-500mg/L) and the results were expressed as mg caffeic acid per mL. All measurements were performed in triplicate. Caffeic acid standard was supplied by from Sigma-Aldrich Corporation.

2.3 High Performance liquid chromatography (HPLC)

HPLC analysis was conducted for all the plant extracts prepared using an Agilent model 1100 (Agilent Corporation, MA, USA) system equipped with a diode array detector. A reverse phase column Supelco (Discovery HS C18), length 250mm, internal diameter 4.6mm with material porosity 5μm was used. The HPLC system is controlled by Agilent Chemstation software. Absorptions spectras were obtained at 260, 280 and 330nm for all samples and standard solutions.

The flow rate was adjusted at 0.4mL/min using the binary gradient of eluent (A) which was water acidified by formic acid at (pH = 2.5), and eluent (B) which was Acetonitrile. The mobile phase consisted of 25% (B) during the initial 2min, followed by a gradient increase of the percent of solvent B up to 90% for the next 38min. Sample injection volume was 20 μL. The components were identified by comparison of their retention times and their UV absorption spectra with those of the commercial standards under the same analysis conditions.

Acetonitrile, and standards of rosmarinic acid, caffeic acid, syringic acid and carnosic acid were supplied by from Sigma-Aldrich Corporation. Standards of luteolin, luteolin-7-O-glucoside, kaempferide, apigenin, apigenin-7-O-glucoside, chlorogenic acid and kaempferol were supplied by Extrasynthese. Ferulic acid, p-coumaric acid and eriodictyol were purchased from Fluka.

2.4 Preparation of *Aspergillus flavus* for pistachio seed infection experiments

Pistachia vera seeds (cv Eginis) were primarily immersed in the plant test solutions that have been described above for 2 h and then let dry in a laminar flow cabin for 24 h. Pistachio seeds were infected by *A. flavus* by immersing them in a spore suspension (10^6 conidia/mL) for 30min. After drying seeds were transferred in sterile petri dishes and were incubated at 28°C in the dark for nine days. Furthermore, two control series were included, one (TrCt) where the plant extract was replaced by ddH_2O as the test solution, and a second control series (Control) where the pistachio seeds were not treated by any test solution and were not inoculated with *A. flavus.* Each treatment was performed in triplicate.

A. flavus strain has been isolated from naturally infected pistachios seeds and was provided by the laboratory of Phytopathology of the Agricultural University of Athens. BrK was supplied by Sigma Aldrich and ELISA test was supplied by (AgraQuant Total AF Test Kit 4-40 ppb, Romer Labs, Singapore).

2.5 ELISA test

The AFs concentration of pistachio samples was determined by the ELISA test (AgraQuant Total AF Test Kit 4-40 ppb, Romer Labs, Singapore). 10gr ground portion of each pistachio sample was mixed with 50mL 70% methanol extraction solvent for 3 minutes. Mixture was then filtered and pH was adjusted between 6-8. The filtrates were directly tested with ELISA kit according to the manufacturer's guidelines. 100μL of each extracted filtrate and the AFs standards contained in the kit were mixed with 200μL of the enzyme conjugated buffer and then were added to antibody strips and left for 15min at room temperature. Microwells were then emptied and rinsed three times thoroughly before the addition of 100μL substrated buffer and left for 5min at room temperature before adding the stop solution buffer. In contaminated samples, AFs competes for bidding sites with the enzyme conjugated buffer and in the presence of the substrate develops a blue color inversely proportional to AFs concentration. ELISA plates were then read by a dual wavelength microwell reader (Biotek 800 TS, USA).

2.6 FTIR sample preparation, measurement and analysis

A pistachio paste from treatments was prepared by grinding thoroughly the raw pistachio seeds together with KBr (Potassium bromide) in a ratio of 1:10 (W/W) until reach a homogeneous paste sample.

The IR spectra of pistachio paste samples were recorder in the Drift mode in absorbance between 4000 and 400 cm^{-1} using an FTIR spectrometer (Thermo Nicolet 6700 spectrometer which has a DTGS detector; Nichromesource; KBr beamsplitter; Thermo Electron Corporation, Madison, WI, USA). As a background was used KBr. Spectra were recorded at a 4 cm^{-1} spectral resolution and each spectrum was achieved by co-adding 100 scans. Five individual spectra were collected from each pistachio paste sample.

2.7 Statistical analysis

Data of total phenolic content of plant extracts were expressed as means ± S.E. For statistical analysis, one-way-analysis of variance (ANOVA) was applied followed by Tukey's test. Differences were considered significant at $p < 0.05$.

Data on AFs concentrations from the ELISA test were statistically compared by analysis of variance (ANOVA) and Fisher's Least Significant Difference (LSD) using the Statgraphics Plus Software.

2.8 Discriminant analysis

Pre-processed (smoothed and mean-centered) spectras of pistachio samples of different treatments, lead to production of different AFs concentration, were analyzed with discriminant analysis technique using OMNIC calibration and prediction software. Samples were grouped into two batches as contaminated and non-contaminated. Pre-processed (filtered by smoothing) spectral data of each group were analyzed with the discriminant analysis technique using the TQ Analyst software of the Turbo Quant IR calibration and prediction software package (Thermo Fisher, Madison, WI, USA). When Discriminant Analysis is applied by TQ Analyst, the software applies the spectral information in the specified regions of the unknown sample spectrum to a stored method model in order to determine which class of standards is most similar to the unknown. During calibration, the software computes a mean spectrum and then generates a distribution model by estimating the variance at each frequency in the analysis range.

When Discriminant Analysis is selected for Analysis Type, the TQ Analyst software applies the spectral information in the specified regions of an unknown sample spectrum to a stored method model in order to determine which class of standards is most similar to the unknown. A measurement of the Mahalanobis distance between the unknown sample and each reported class is also provided.

2.9 PLS-regression

PLS-regression (PLSR) is the PLS (Partial least Squares) approach in its simplest, used as (two-block predictive PLS) in chemistry and technology for relating two data matrices, X and Y, by a linear multivariate model, which goes beyond traditional regression in that it models also the structure of X and Y. PLSR has the ability to analyze data with many, collinear, noisy and even incomplete variables in both X and Y. PLSR has the desirable property that the precision of the model parameters improves with the increasing number of relevant variables and observations.

In this study, the PLSR model was developed using TQ Analyst software to quantitatively correlate spectras of contaminated pistachio samples with AFs concentration as it was estimated by ELISA. The number of factors was decided by the minimum predicted residual error sum of squares (PRESS) values. The performance of the developed PLSR model was evaluated through correlation coefficient of determination (R^2), root mean-squared error of calibration (RMSEC) and root mean-squared error of prediction (RMSEP).

3. Results and discussion

3.1 Analysis of plant extracts

Analysis of plant extracts based on their total phenolic content and qualitative analysis using HPLC coupled with photodiode array. Total Phenolic content of plant extracts was estimated and expressed in mg of caffeic acid/mL (Table 1). Estimated values of total phenolic content varies from 0.06 to 9.85mg of caffeic acid/mL. TrMf attributed to *Melissa officinalis* exhibited the higher phenolic content between the other Lamiaceae plant extracts as also reported previously in [22] and TrSf exhibited the lowest.

Table 1. Total phenolic content of plant extracts

Plant extract	mg Caffeic acid/mL
Tr Hf (*Hyssopus officinalis*)	$1.97 \pm 0.02^{d*}$
Tr Mf (*Melissa officinalis*)	9.85 ± 0.01^{f}
Tr Sv (*Salvia officinalis*)	3.31 ± 0.05^{b}
Tr Od (*Origanum dictamnus*)	3.20 ± 0.01^{c}
Tr Or (*Origanum vulgare*)	6.43 ± 0.03^{f}
Tr Sf (*Crocus sativus*)	0.06 ± 0.004^{b}

Qualitative analysis of plant extracts conducted by HPLC and corresponding chromatograms at 280nm are presented in Fig 1. The components in plant extracts were identified by comparison of related references in the literature and the retention times and the UV spectra of the corresponding analyzed standards. There compounds that could not be identified from spectra and retention times of standards analyzed were determined as hydroxycinnamic derivatives or flavonoids based on their chromatographic behavior and UV spectra their chemical class. The components that identified by comparisons to the retention times and UV spectra of standards analyzed were gallic acid in TrMf, TrHf, TrOr and TrSv as also previously reported in [23], chlorogenic acid in TrHf, TrMf, TrDt and TrSv in accordance to [23, 24], rutin in TrOr and TrDt and luteolin in TrDt in accordance to [25], p-hydrobenzoic acid in TrHf, and TrOr, luteolin-7-O-glucoside detected in TrMf, TrDt and TrSv as in [26], apigenin-7-O-glucoside in TrHf. TrMf, TrSv, TrDt, and TrOr, vanillic acid in TrHf, ferullic acid detected in in TrHf, TrMf, TrDt and TrOr as in [27-29], apigenin in TrOr as in [30] and rosmarinic acid in all extracts except TrSf [27-30].

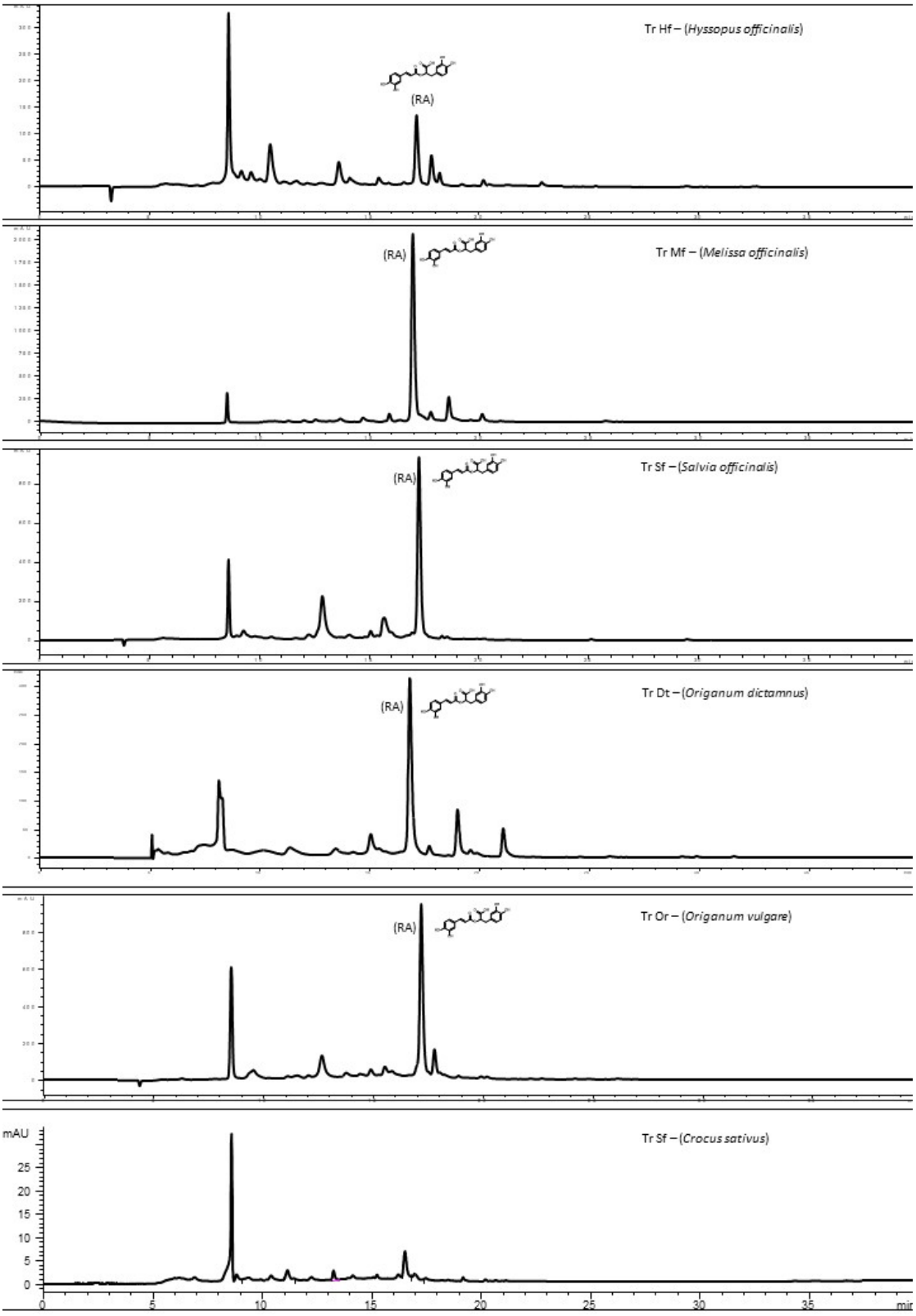

Figure 1. HPLC profiles of extracts at 280nm. Rosmarinic Acid (RA) identified in all Lamiaceae species extracts

From the compounds identified mentioned above, the major and most abundant component present in all extracts was the rosmarinic acid (RA), which reach its higher values in TrMf (*Melissa officinalis*) compared to the other extracts and also other Lamiaceae species [21]. For this reason, an additional treatment was set to the experiments as TrRs contained rosmarinic acid at a concentration corresponded to its content in the TrMf extract calculated as 8.6mg/mL according to [21]

in an attempt to attribute the biological activity identified towards this compound.

It has to be mentioned that geography, climate and modifications in extraction procedures may lead in differences of presence or absence of phenolic or flavonoid compounds in the samples tested, compared to other past compositional analysis of the same plant extracts [26, 31, 32].

3.2 Effect of different treatments to AFs production on pistachio

Treatment of pistachio seed samples with different aqueous medicinal and aromatic plants extracts resulted in variation of AFs concentration as estimated my ELISA method presented in Fig 2. As it can be seen four of the seven treatments (TrSv, TrDt, TrOr and TrRs) found to reduce AFs compared to TrCt but only the TrMf treatment was found effective to almost completely eliminate the AFs as estimated by ELISA compared to TrCt. Of the other two treatments TrSf found not to significantly influence AFs in contrast to TrHf which found to increase AFs levels compared to TrCt. The most effective extract against AFs production was TrMf, which showed the highest total phenolic content and the higher concentration in RA. But RA alone is not the reason of such effective action as TrRs treatment found not to be so effective. A synergism of other compounds in TrMf along with RA may result in elimination of AFs production by *A. flavus*. All data are the mean value of three independent experiments ± SE.

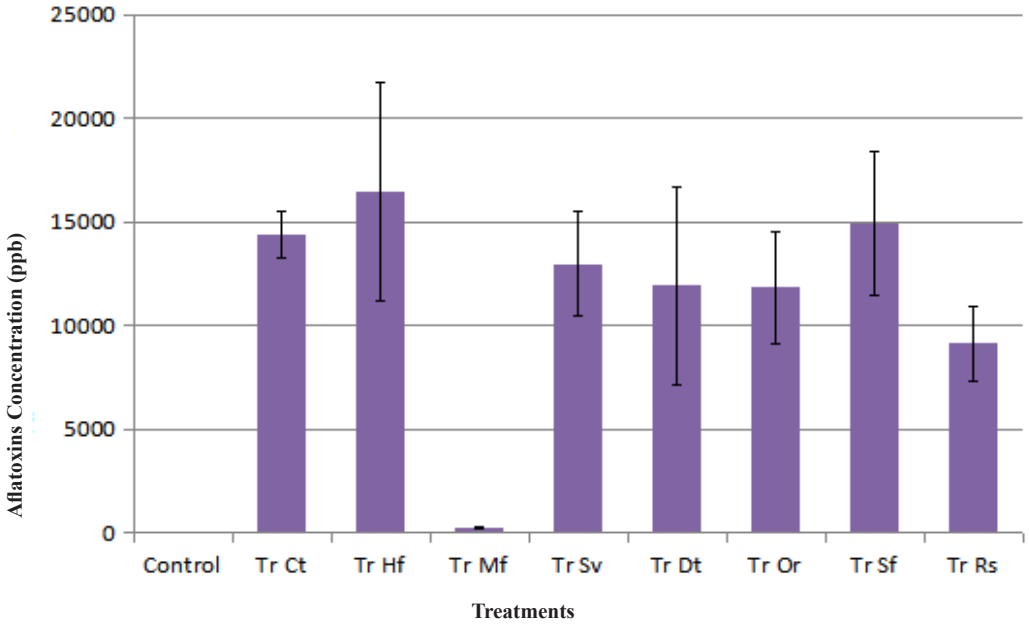

Figure 2. AFs concentration in pistachio seeds as estimated by ELISA. Standard errors displayed by vertical black lines. Values are mean of three replicates

3.3 Interpretation of FTIR spectra

In our study, the Fourier-transformed infrared (FTIR) spectroscopy was used for the detection of biochemical composition changes in pistachio samples contaminated by AFs. The results presented below show the potential of FT-IR spectroscopy for rapid, reliable and cost-effective evaluation of AFs contamination.

Despite the fact of complex structure of both *A. flavus*, plant extracts and pistachio matrices, resulted to a superposition of hundreds of infrared nodes, the FTIR spectrum of contaminated and not contaminated pistachio samples, as presented in Fig.3, was comparable to spectra derived from contaminated peanuts [33]. Strong absorptions were detected in all spectral regions that characterize the major cellular components. In detail, at region 3000-2800cm^{-1}, the peak found at 2928cm^{-1} and 2854cm^{-1} are assigned to the C-H stretching bands of the cell membrane fatty acids [20, 34-36] and identified in both contaminated and not contaminated samples. At region 1750-1500cm^{-1}, two intense peaks were found in both contaminated and non-contaminated samples identified at 1742cm^{-1}, due to enzymatic degradation of triglycerides caused a decline at fatty acid ester linkage centering at 1743cm^{-1}. The peak at 1661cm^{-1} in not contaminated samples is due to the vibrations of amide I band [26, 20], the additional strong peak at 1710cm^{-1} in contaminated samples is attributed to the increased lipid break down product of free fatty acids by the activity of lipase due to the fungal invasion progress [26, 38] where at 1553 cm^{-1} are also amide II band of proteins [39]. Peaks found at 1313 and 1255cm^{-1} attributed to amide III band components of proteins [40, 41]. In region, 1200-900cm^{-1}, absorption at 1152cm^{-1} is due to CH_3 deformations [42], at 1086

cm^{-1} due to the symmetric phosphate modes originated from the phosphodiester groups in nucleic acids [43] and absorption at 917cm^{-1} can be assigned to phosphodiesters [44]. The increased absorption peaks of proteins and decreased absorption at lipid and carbohydrate regions are due to the metabolic fungal activity [36]. Along with protein peaks, the FTIR spectra of infected and contaminated samples compared to the non-contaminated shows linear increase and/or decrease of fat associated peaks indicating lipid hydrolysis [45].

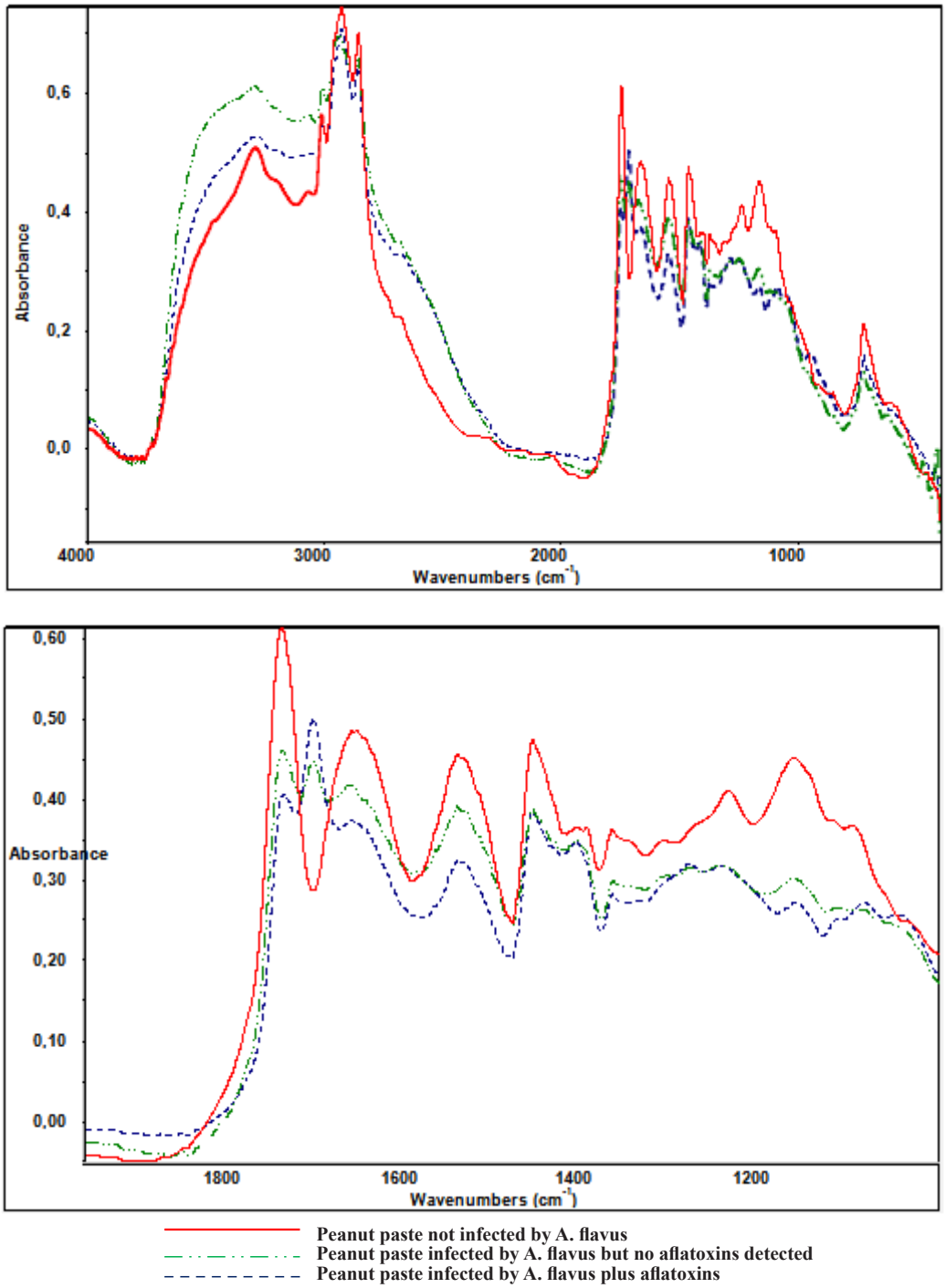

Figure 3. Drift spectra of peanut paste. Data are means of three independent experiments

3.4 Spectra region selection

While *A. flavus*, pistachio and AFs have very complex molecular structure and the spectra of their mixture are expected to contain hundreds of vibrational modes, the right choice in spectral regions is of primary importance in

order to derive the most relevant data. Under this context match values of averaged treated spectra compared to TrMf (which showed the minimum AFs values) were calculated in order to identify properly variations in global biochemical compositions from the original spectra in specific band regions and indicate the band areas where is noticed the less match compare to control. For this purpose, a library was set by spectras of not contaminated by AF pistachio samples to proceed in spectrum match with the contaminated ones. Spectra were processed using Ominc software (version 7.3, Thermo Electron Corporation, USA). The signal was smoothed and baseline corrected using the automatic functions and the algorithms built into the software mentioned above. Furthermore, the second derivative of each processed spectrum was calculated with the Savitzky-Golay derivative filter and default parameters (7-point, $4cm^{-1}$ resolution, polynomial degree 3). Spectrum match was performed using TQ Analyst software by comparing the spectral information for the specific region or regions given of an unknown sample with the spectra of a given standard, in order to determine which class of standards is most similar to the unknown. The result of this comparison is the single best match from each reported class.

The spectra regions that gave the less matching values in absolute difference for AF in pistachio paste samples were ($1705\text{-}1575cm^{-1}$) and ($1350\text{-}900cm^{-1}$) as are presented in Table 2.

Table 2. Integrated infrared Band area spectral match values in absolute difference among Pistachio treatments resulted in different AF concentration compared to TrMf treatment which inhibited the production of aflatoxins. Spectrum match was performed using TQ Analyst software

Spectral area (cm-1)	Designation	Treatments						
		TrCt	TrHf	TrSv	TrDt	TrOr	TrSf	TrRs
3000-2800	Lipids	80.89	89.13	82.20	81.91	87.12	85.90	67.55
1705-1575	Amide I	38.95[a]	40.66[a]	51.21	45.90[a]	59.72	38.65[a]	67.32
1575-1480	Amide II	87.19	84.35	73.78	77.92	73.26	84.04	83.01
1350-900	Amide III & carbohydrates	57.64	61.81	56.17	55.20	56.89	51.61	61.34
4000-400		87.51	71.67	79.65	80.27	85.00	66.61	71.30

(a) No match compared to TrMf
***Critical match value = 50.00**

3.5 Discriminant analysis

In Fig. 4 illustrated the discriminant analysis results of AFs contaminated and non-contaminated pistachio seed samples as distance values indicating that AFs in pistachio samples dominates with respect to spectral measurements and leads to segregation of samples. Discriminant analysis was conducted for full spectral range abs values as well as their transformation in 1st and 2nd derivative. Best discrimination found on full spectra range absorbance values without transformation (Fig. 4). For every treatment three different samples were prepared. For each of them 5 different spectra were collected. Pre-processed spectral data (mean centering, smoothing) were classified using discriminate analysis

In this classification method 10 principal components were used and 100% of variability described. 26 of 27 sample readings were used for calibration. In previous discriminate analysis studies, AFs amount was interpolated against *Aspergillus* sp. cell count, and then classification models on AFs contamination were based on mold concentration and the relevant spectra [21]. Besides these, discriminate analysis showed that contaminated by AFs pistachio seeds can be separated based only on AFs contamination and not fungal invasion (Fig.4).

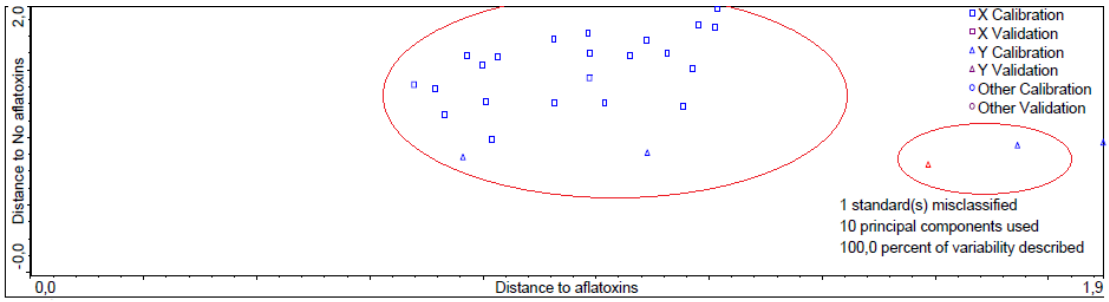

Figure 4. Discriminate analysis PCA

3.6 PLS regression

The mean centered and smoothed spectral data of 2nd derivative were loaded into a PLS tool in order to obtain a quantitative analysis method for AFs in contaminated pistachio samples. PLS regression analysis used to predict dependent variables from a set of independent variables achieved by extracting a set of variables which have the best predictive power and to obtain a relationship between predicted and actual FTIR values. The PLS model was developed simultaneously for 1350-900cm^{-1} and 1705-1575cm^{-1} band areas, as these two band areas showed the less matching score values for all treatments (Table 2). The model, as also presented in Fig. 5 fits perfectly the actual concentration values with R^2 at 99.99%. RMSEC and RMSEP values were 0.0277 and 1.55 respectively. The factor numbers were decided according to the predicted residual error sum of squares plot which decreased as the factors are added and its minimum provides the number of factors used in PLS. The low levels of errors of calibration, validation and prediction indicated the high performance of FTIR as analytical tool for estimation of AFs in pistachio samples with minimum and low-cost sample preparation.

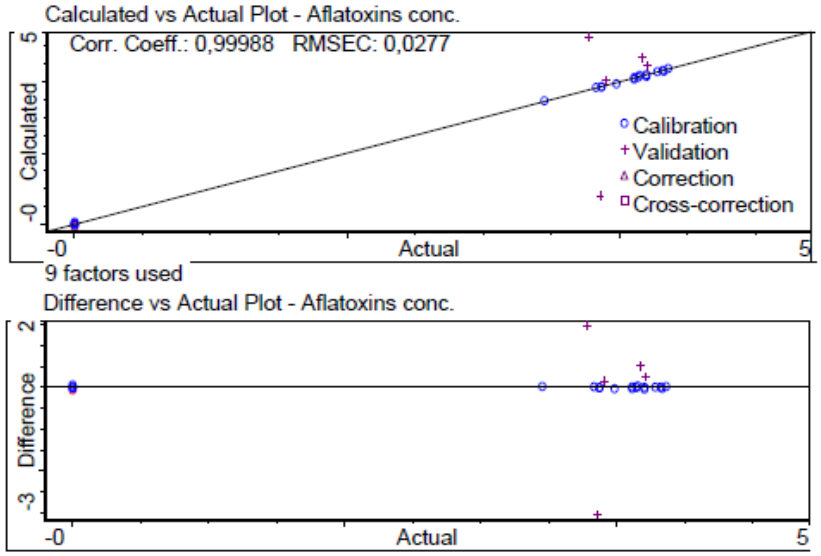

Figure 5. PLS regression statistics for 1350-900 and 1705-1575cm^{-1} band areas. Correlation Coefficient (0.99988) calculated and displayed on plot, as well as the RMSEC (0.0277)

4. Conclusions

In this study, Fourier Transform Infrared (FTIR)-drift mode has been shown to be fast, low-cost, non-destructive and highly sensitive, requiring little, easy and very quick sample preparation without use of harmful chemical agents. Pistachio paste matrix, *A. flavus* and AFs have complex structures leading to absorptions at similar frequencies which maybe masking the spectral contribution of one another. However, PLS regression pointed FTIR-drift technique as of great potential for chemometric quantitave analysis of AFs, since very high R^2 values were combined with low error percentages. Besides these, discriminate analysis indicates that contaminated by AF pistachio seeds can be separated based only on AFs contamination and not fungal invasion.

Additionally, the fact that the extract of *Melissa officinalis* was able to almost completely eliminate the AFs contamination in pistachio seeds might indicate that could act as a potential biocontrol product for AFs control. Further

in-vitro and in-situ experiments are needed in order to investigate the molecular mechanisms of *M. officinalis* extract activity on AFs production by *A. flavus* in pistachios or other crops and to also to identify the most active compounds of *M. officinalis* against AFs production.

Future additional studies should evaluate the performance of this method on different levels of AFs concentrations and different sample types, in order to validate its efficiency as a fast and precise determination tool for real-time monitoring of AFs at critical sample collection points that would ensure quality and safety of food. However, there is still a long road ahead to accomplish this full potential, with the harmonization of techniques and the selection of principal criteria for a reproducible and reliable analysis of food products, as the major aspects to be properly addressed.

Given that AFs contamination of food has become a matter of increasing concern, approaches for its analysis will continue to be developed and improved. On-site rapid quantitative determination of AFs and multi-analyte detection may become the main trends in future research.

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Conflict of interest

The authors declare no competing financial interest.

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