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Use of FT-IR Spectroscopy Combined with Discriminant Analysis for Identification of Hazelnuts Infected by *Aspergillus flavus*

Magdalena Reder ✉, Hanna Ciemniewska-Żytkiewicz, Katarzyna Sujka, Piotr Koczoń, Anna Matlingiewicz, Edyta Lipińska

Department of Chemistry, Faculty of Food Sciences, Warsaw University of Life Sciences (WULS-SGGW), Nowoursynowska St. 166, 02-787 Warsaw, Poland

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✉ *Corresponding author (Yazışmalardan Sorumlu Yazar): magdalena_reder@sggw.pl (Magdalena Reder)*
✆ +48 22 59 37 607 🖷 +48 22 59 37 635

ABSTRACT

Fourier-transform infrared (FT-IR) spectroscopy method developed for the analysis of mold presence in hazelnuts was examined in terms of controlling chemical and microbiological quality of hazelnuts. Following molds growing on hazelnuts stored for 3 months in room temperature were isolated and characterized: *Mucor, Rhizopus, Chrysosporium*, *Verticillium*, *Penicillium*, *Aspergillus* and *Alternaria*. IR spectra of hazelnuts in various times of storage were registered. Using both microbiological and spectral data, statistical model discriminating microbiologically pure hazelnuts from hazelnuts contaminated with mold *Aspergillus flavus* was calculated and validated. The developed methodology may be considered as an alternative approach for rapidly scanning hazelnuts for rapid determination of contamination of hazelnuts by *Aspergillus flavus* and further for mycotoxins determination.

Key words: FT-IR, Hazelnuts, *Aspergillus flavus*

Aspergillus flavus ile Enfekte Olmuş Fındıkların Belirlenmesi için FT-IR Spektroskopisi ve Diskriminant Analiz Kombinasyonu Kullanımı

Fındıklardaki küf varlığını analiz etmek için geliştirilen Fourier dönüşümlü kızılötesi (FT-IR) spektroskopi metodunun, fındıkların kimyasal ve mikrobiyolojik kontrollleri için kullanılıp kullanılayacağı araştırılmıştır. Oda sıcaklığında 3 ay depolanan fındıklarda küf gelişimini takiben *Mucor, Rhizopus, Chrysosporium*, *Verticillium*, *Penicillium*, *Aspergillus* ve *Alternaria* cinsleri fındıklardan izole ve karakterize edilmiştir. Farklı sürelerde depolanan fındıkların IR spektrumu kaydedilmiştir. Mikrobiyolojik ve spektral data verileri birlikte kullanılarak *Aspergillus flavus* ile kontamine ve mikrobiyolojik olarak temiz fındıkların ayrımı için istatistiksel model geliştirilmiş ve valide edilmiştir. Geliştirilen metodoloji, fındıklarda *Aspergillus flavus* ve diğer mikotoksinlerin kontaminasyonunun hızlı bir şekilde belirlenmesi ve fındıkların hızlı taranması için alternatif bir yaklaşım olarak değerlendirilebilir.

Anahtar Kelimeler: FT-IR, Fındık, *Aspergillus flavus*

INTRODUCTION

In Poland, the majority of hazelnut production comes from the Lubelskie Voivodeship, located in the eastern Poland. According to Central Statistical Office [1], the cultivation area of hazelnuts in Poland covers about 3100 ha and the average production amount assesses at 4200 tons/year, which places Poland on the 10th place in worldwide hazelnuts production ranking [2]. Most of polish plantations cultivate European hazelnut (*Corylus avellana* L.), which are in general dedicated for direct human consumption [3].

According to Codex Alimentarius, hazelnuts (tree nuts family) harvest includes trees shaking and picking up nuts from the ground. Harvest and further proceedings, such as, washing, shelling, drying, packing and storage should be carried on hygienically in order to maintain assumed parameters. One of the main parameters is moisture. According to Comission Regulation (EC) No 1284/2002 [4], hazelnuts in shell moisture content must not exceed 12% of whole hazelnut and 7% of kernel, which prevents from undesirable mold growth followed by mycotoxins production. Because of that, there are maximum levels set for mycotoxins in hazelnuts intended for direct human consumption at 10 µg/kg and for further processing at 15 µg/kg (for sum of B1, B2,G1 and G2) (Comission Regulation (EC) No 1881/2006) [5].

Mold growth, especially of *Aspergillus* species which produce mycotoxins is a serious problem for the nuts producers, distributors and dealers. In this study, an attempt was made to test FT-IR spectroscopy as a method for monitoring *Aspergillus flavus* strain growth on stored hazelnuts. The advantage of FT-IR spectroscopy is that it can be applied for food analysis without any sample pre-treatment or preparation and without external calibration. This method provides rapid, sensitive and precise results. It can be applied to detect minute amount of given chemical and therefore microbiological impact [6, 7].

The aim of this work was to isolate and identify the molds naturally growning on hazelnuts during storage and to assess the relationship of FTIR spectra with microbiological changes in hazelnuts deliberatelly infected by *Aspergillus flavus* KKP 686 strain.

MATERIALS and METHODS

Hazelnuts

Samples of hazelnuts from polish cultivations (4 different brands) were divided into two groups:

- A – Intact samples stored for 3 months, under room conditions
- B – Samples initially sterilized by UV radiation, inoculated with *Aspergillus flavus* and incubated for 6 weeks

Isolation and Identification of Microorganisms – Samples from Group A

In order to isolate microorganisms growing on hazelnuts stored for 3 months in room conditions, test samples, initial suspension and decimal dilutions for microbiological examination were prepared according to ISO 6887-1:1999 [8]. Cultures grown on Sabouraud medium were isolated with inoculation loop or areal inoculation, pure cultures were inoculated into solid broth and Czapek-Dox media. Cultivation was carried out in 28°C for 7 days.

The following microbiological media were used: Sabouraud glucose agar with chloramphenicol (acidity 5,6), PCA (plate count agar) medium (acidity 7,2), broth medium with agar (acidity 5,5) and Czapek-Dox agar (acidity 7,3). Hazelnuts were analyzed with and without shell.

Macroscopic observations were conducted for broth and Czapek-Dox media, microscopic observations were conducted for all samples with the use of OPTA-TECH light microscope.

Total Microbial Count and Total Mold and Yeast Count – Samples from Group A

Total aerobic microbial count, total mold and yeast count in samples from group "A" were determined by plate method, according to ISO 4833:2003 [9] and ISO 21527-2:2008 [10] respectively.

Sterilization, Inoculation and Incubation of Samples from Group B

Samples from group "B" were sterilized with ultraviolet (UV) light, *Aspergillus flavus* (strain KKP 686) that was grown in a Roux flask, on Wort agar with 1% of glucose. Dehulled and previously sterilized hazelnuts (B group) were placed in petri plates and sprayed with *Aspergillus flavus* spore suspension (10^5 spores/cm^3). Inoculated samples were incubated in 28°C for 6 weeks, in humidity around 90-95% and in the absence of light.

FT-IR Spectroscopy – Samples from Group B

In order to register spectra of solid state samples, thin pellets of potassium bromide (background sample) and potassium bromide mixed with hazelnut sample in the mass ratio of 10:1 were prepared.

The 2000 Perkin Elmer instrument operated by PEGRAMS software running on Win 95 platform was used to register FT-IR spectra. Transmission technique was applied to conduct 25 scans for each of studied hazelnuts, in the spectral range of 4000–370 cm^{-1}. The resolution was 4 cm^{-1} and shift velocity 2 cm/s.

FT-IR measurements were performed for of microbiologically pure hazelnuts and after each week of incubation in 10 replicates (total amount of spectra=280). Based on registered spectra, discriminant modes were calculated. Obtained models were evaluated using Performance index, which indicates how accurately a calibrated method can classify validation standards (maximum value is 100), this parameter is calculated automatically by TQ Analyst Software. The greater parameter the better model.

Spectral Analysis, Statistics and Modeling

In order to find relation between spectral data and presence of mould in hazelnuts, visual analysis focused on finding differences between spectra of sterilized hazelnuts and hazelnuts contaminated with *A. flavus* was conducted. However, more accurately, discriminant analysis in TQ Analyst software was used to calculate

statistical model enabling indirect detection of microbial contamination in studied samples. 200 spectra of hazelnut samples were used as a calibration set, whereas 80 were used as validation standards.

RESULTS and DISCUSSION

Mold Isolated from Hazelnut

After 3 months of storage total microbial as well as total yeast and mold count were determined in both kernels and shells of hazelnuts samples. The results are presented in Table 1 and Figure 1-7.

The number of yeast and mold accounted for 69% and 61% of total oxygen mezofilic microorganisms count in hazelnut shell and hazelnut kernels, respectively. Also it was noted that the total number of microorganisms in shell was greater than in kernel.

Xu et al. [11] identified 6 genera of mold growing on hazelnuts: *Penicillium*, *Alternaria*, *Paecilomyces*, *Fusarium*, *Cladosporium* and *Chrysonilia*.

Khorsavi et al. [12] reported molds form *Penicillium*, *Aspergillus*, *Mucor* and *Fusarium* genera growing in greatest amounts in hazelnuts, with percentage abundance: 36.1%; 29.7%, 14.8%; and 12.7%, respectively. Mold from genus *Paecilomyces* and yeast occurred in significantly smaller amounts (4.3% and 2.1% respectively).

Table 1. Total number of microorganisms in colony forming units per gram

Analyzed hazelnut	Total number of oxygen mezofilic microorganisms [cfu/g]	Total number of yeast and moulds [cfu/g]
Hazelnut in shell	$2.8 \cdot 10^3 \pm 0.1 \cdot 10^3$	$1.9 \cdot 10^3 \pm 0.2 \cdot 10^3$
Hazelnuts without shell	$1.3 \cdot 10^3 \pm 0.1 \cdot 10^3$	$0.8 \cdot 10^3 \pm 0.1 \cdot 10^3$

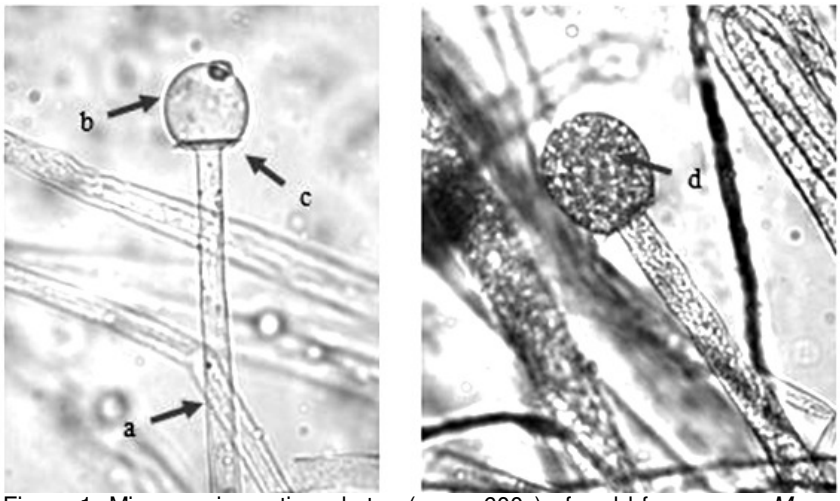

Figure 1. Microscopic section photos (zoom 600x) of mold from genus *Mucor* cultivated on broth medium (a - sporangiophore, b – columella, c – collarette, d –spores)

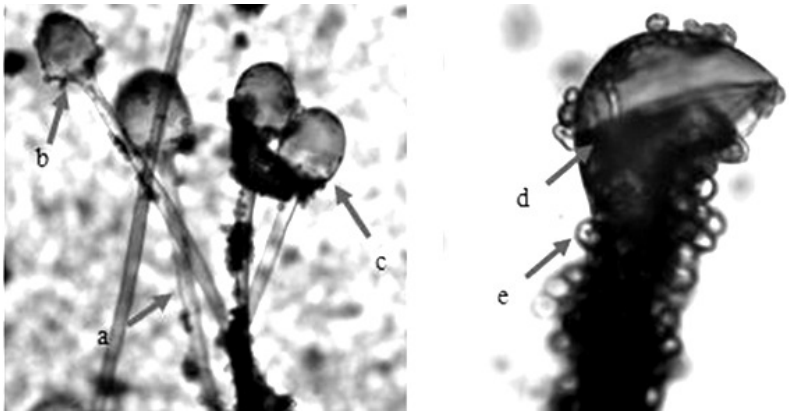

Figure 2. Microscopic section photos (left: zoom 400x, right: zoom 600x) of mold from genus *Rhizopus* cultivated on broth medium (a – sporangiophore, b – apophysis, c – columella, d – sporangium, e – spores)

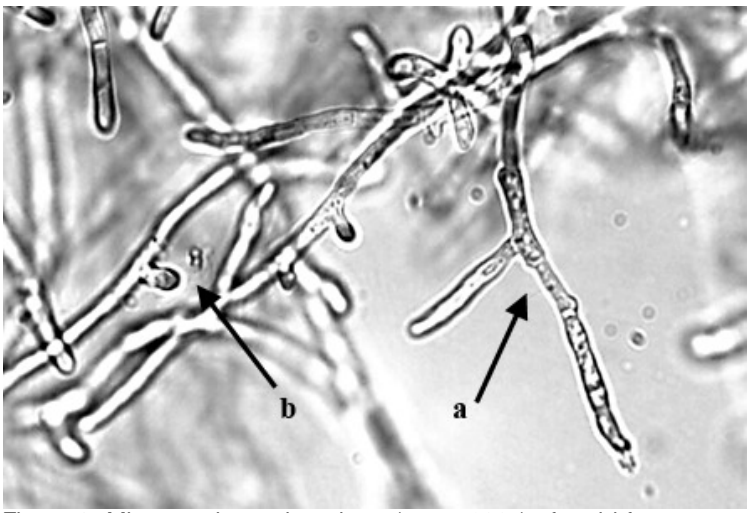

Figure 3. Microscopic section photo (zoom 600x) of mold from genus *Chrysosporium* cultivated on broth medium (a - conidiophore, b – aleurioconidia).

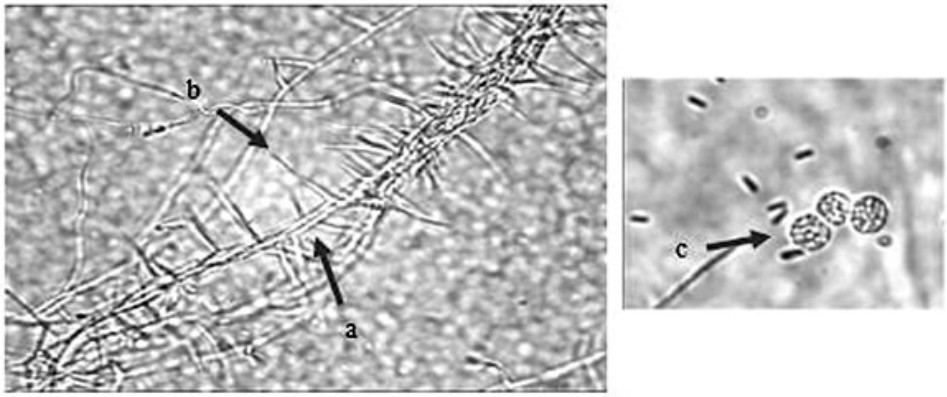

Figure 4. Microscopic section photo (left: zoom 400x, right: zoom 600x) of mold from genus *Verticillium* cultivated on broth medium (a - conidiophore, b – phialide, c - conidia).

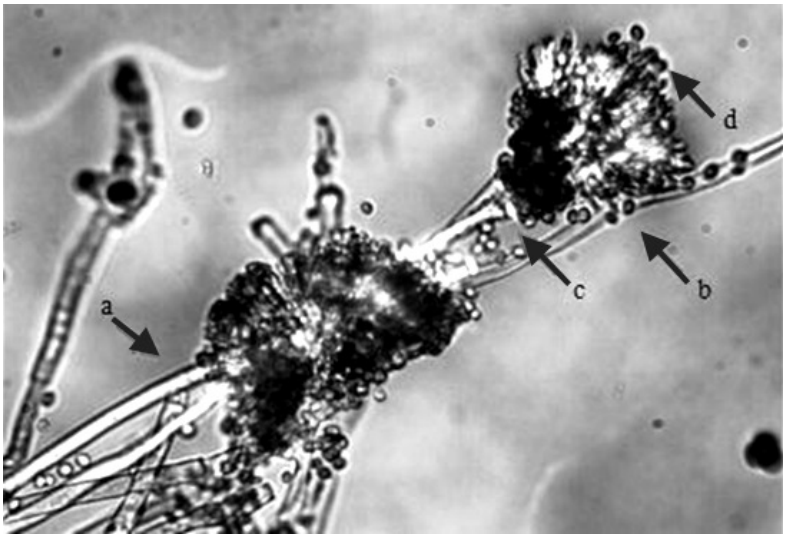

Figure 5. Microscopic section photo (zoom 600x) of mold from genus *Penicillium* cultivated on broth medium (a - conidiophore, b – penicilli, c – conidiophore ending branching into penicilli, d - conidia).

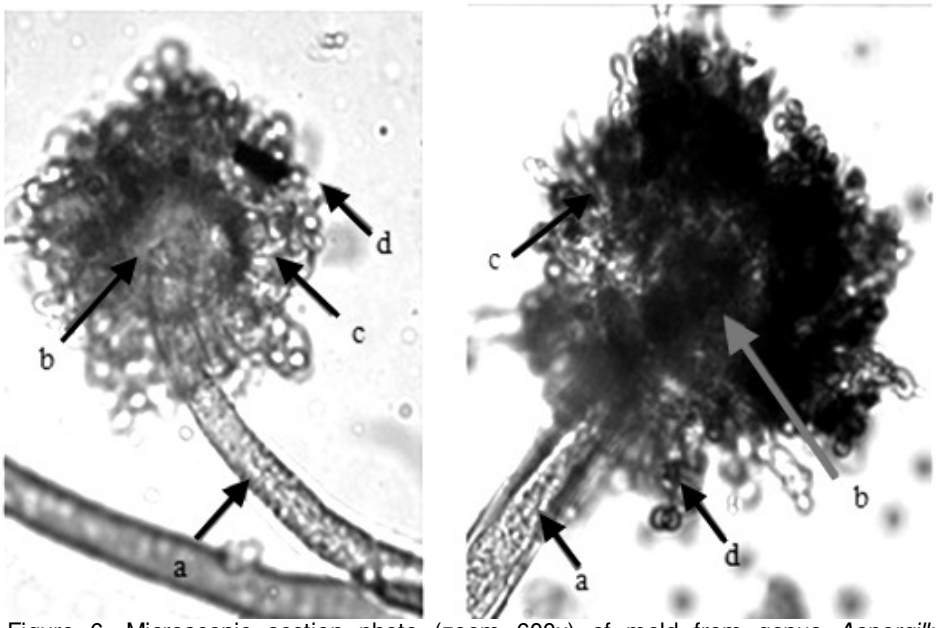

Figure 6. Microscopic section photo (zoom 600x) of mold from genus *Aspergillus* cultivated on broth medium (a – conidiophore, b – conidial head, c – metulas and phialides, d - conidia).

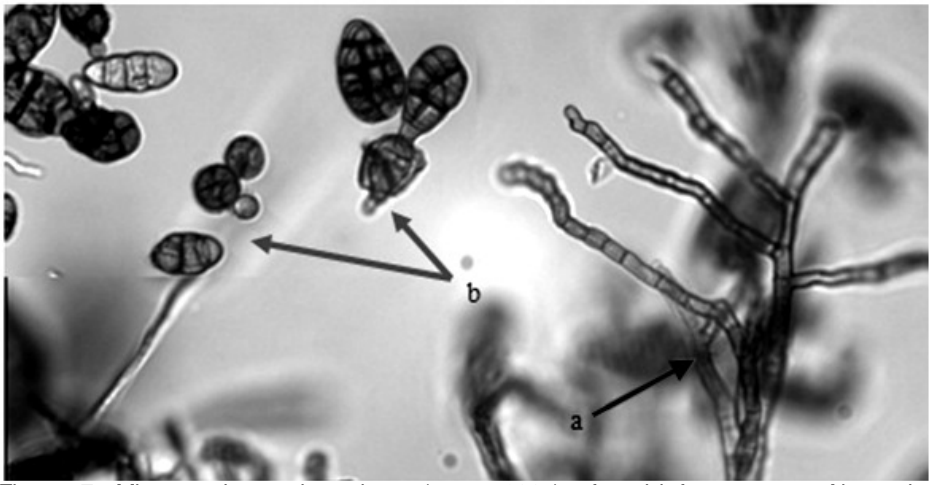

Figure 7. Microscopic section photo (zoom 600x) of mold from genus *Alternaria* cultivated on broth medium (a – conidiophore, b - conidia).

Spectral Analysis

The following spectral regions were found to be the most relevant in differentiation between pure and contaminated hazelnuts:

- 1770–1440 cm^{-1} (Figures 8 and 9): in contaminated hazelnuts the appearance of two bands was observed, the first one at 1711 cm^{-1} and the second one less intense at 1648 cm^{-1} (Fig. 8). Additionally, after five and six weeks of incubation, disappearance of band at 1535 cm^{-1} was noted (Fig. 8). One of the most important differences between spectra of pure and contaminated hazelnuts was the change in intensity and shape of band at 1466 cm^{-1} (Fig. 9). Furthermore, within this spectral region, small additional bands appeared during 6 week incubation which evidences the change in structure, formation or degradation of chemical compounds, most probably metabolites of *A. flavus*.

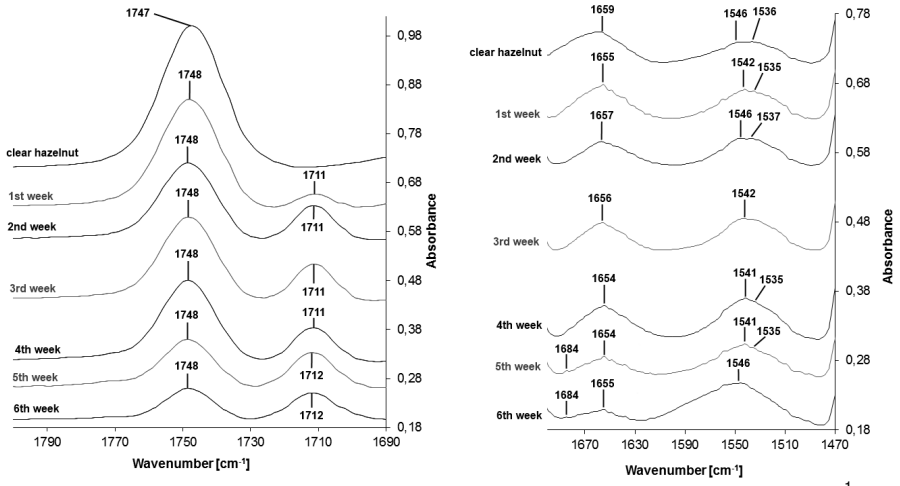

Figure 8. Infrared spectra of studied hazelnuts in the region 1800 – 1470 cm^{-1}

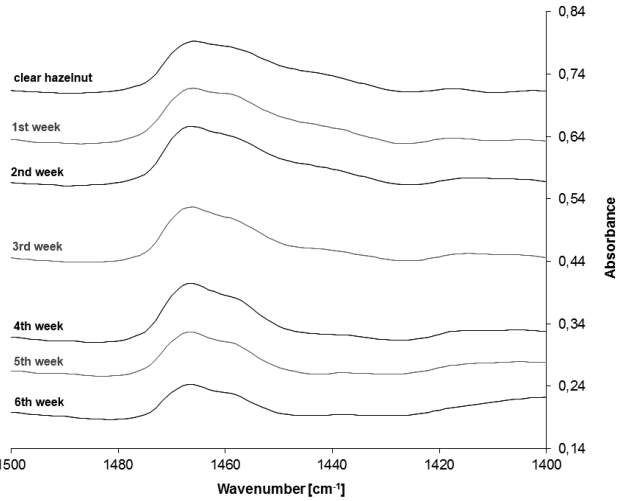

Figure 9. Infrared spectra of studied hazelnuts in the region 1500 – 1400 cm^{-1}.

- 1333–880 cm^{-1} (Fig. 10.): after six weeks of incubation intensity of bands at 1243 cm^{-1} and 1164 cm^{-1} notably decreased significantly. Additionally, disappearance of weak but characteristic spectral bands occurring in the region 1072 – 967 cm^{-1} was observed throughout the incubation time.

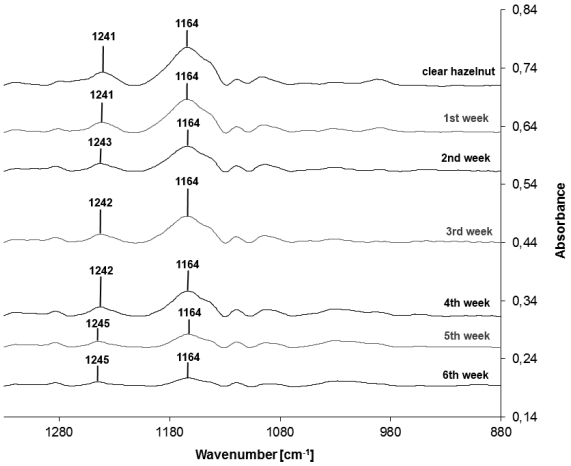

Figure 10. Infrared spectra of studied hazelnuts in the region 1330 – 880 cm^{-1}

Basaran and Demirbas [13] analyzed spectra of hazelnuts contaminated with *Aspergillus parasiticus.* During 20-day incubation time some changes were observed at 1163 cm^{-1}, 1118 cm^{-1}, 850 cm^{-1} and 587 cm^{-1} however, differences between the spectra of pure and contaminated hazelnuts were dissimilar to our results. Furthermore authors concluded that FT-IR method was not sufficient for proper characterization of chemical changes caused by microorganisms.

Discriminant Analysis

The attempt was made to distinguish contaminated from pure hazelnuts based on spectral data exclusively. Moreover, we tried to use spectral data to differentiate hazelnuts due to time of microbial growing. Data from spectral regions discussed in the previous section were used for creating discriminant models. Calibration based on unprocessed spectral data resulted in relatively high value of performance index (88.3), the interpretation of pairwise distance plot (Fig. 11.) suggested however, that the model did not sufficiently distinguished each class. When the data of the 1st derivative spectra were used for constructing statistical model, greater value of performance index (90.2) and better discrimination between pure and contaminated hazelnuts was noted (Fig. 12.). Microbiologically-free hazelnuts were perfectly differentiated from those infected, however calibrated models did not allow to distinguish hazelnuts due to the time of storage.

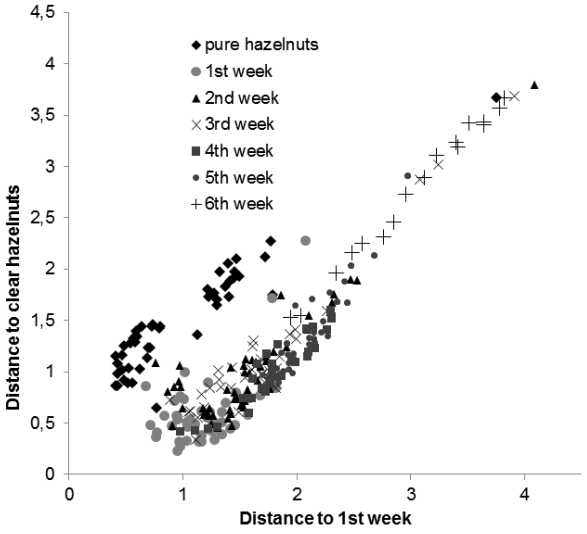

Figure 11. Pairwise distance plot for model based on unprocessed spectra. The plot shows graphically the Mahalanobis distance between each standard and the two classes selected for the X- and Y-axis

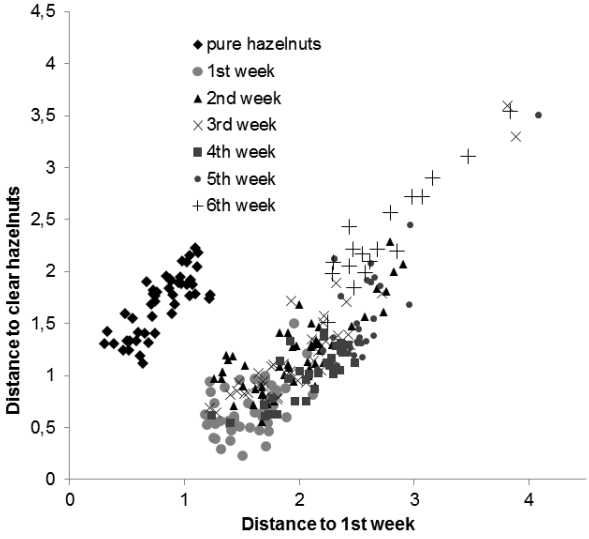

Figure 12. Pairwise distance plot for model based on first derivative spectra. The plot shows graphically the Mahalanobis distance between each standard and the two classes selected for the X- and Y-axis.

CONCLUSION

Macroscopic and microscopic observations showed that hazelnuts stored for 3 months in ordinary, room conditions can be infected with 7 different genera of mold: *Mucor*, *Rhizopus*, *Chrysosporium*, *Verticillium*, *Penicillium*, *Aspergillus* and *Alternaria* that were identified within this study.

Statistical parameters calculated in discriminant analysis evidenced high relationship between spectral data and presence of *Aspergillus flavus* in hazelnuts. First derivative spectra were found to be statistically more significant than unprocessed spectra to discriminate contaminated samples from pure ones. Results clearly showed that in the near future FT-IR spectroscopy could become a useful tool for controlling microbiological quality of hazelnuts.

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