



Comparison of fluorescence spectroscopy and FTIR in differentiation of plant pollens

Monika Mularczyk-Oliwa^a, Aneta Bombalska^{a,*}, Miron Kaliszewski^a, Maksymilian Włodarski^a, Krzysztof Kopczyński^a, Mirosław Kwaśny^a, Małgorzata Szpakowska^b, Elżbieta A. Trafny^b

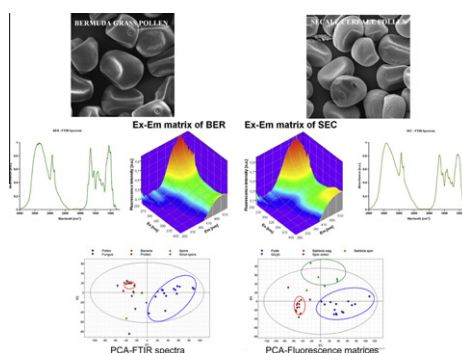
^a Institute of Optoelectronics, Military University of Technology, Gen. S. Kaliskiego 2, 00-908 Warsaw, Poland

^b Department of Microbiology, Military Institute of Hygiene and Epidemiology, Kozielska 4, 01-163 Warsaw, Poland

HIGHLIGHTS

- Fluorescence and FTIR methods are suitable to classify the biological material.
- Both techniques allow discrimination of pollens from other samples.
- Combined FTIR and fluorescence data did not improve bio-particles classification.
- Sensitivity of fluorescence techniques allow signal collection from single particle.
- Fluorescence technique might be used for early detection systems.

GRAPHICAL ABSTRACT



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ABSTRACT

Spectroscopic techniques are under investigation on possibility of differentiation of airborne particles. This paper describes pollen discrimination among other bio-particles in laboratory conditions. Pollen samples were characterized with UV–Vis fluorescence, drift and KBr pellet techniques of infrared spectroscopy. Principal Component Analysis of UV–Vis fluorescence and FTIR spectra revealed that pollens can be distinguished from other bio-materials with use of these methods. Both methods resulted in similar classification capability. Combined FTIR and fluorescence data analysis did not improve the discrimination between pollen allergens and other airborne biological materials.

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Introduction

Environmental monitoring recently attracts worldwide interest. A number of biological particles of different origin are constantly present in the environment and they may eventually affect human and animal health. Immune system discriminates between harmless substances and hazardous ones and provides an adequate response.

Plants produce enormous number of pollen grains during pollination season. Some people can suffer strong allergic response that is induced by specific peptide antigens present on the surface of

pollen. Some people suffer from skin and mucosa itching, while others have strong respiratory dysfunction related to breathlessness [1,2].

Current methods of pollen identification rely on their morphology inspection under light microscope. However, it does not provide any rapid warning systems for allergist [3,4]. Spectral methods such as fluorescence spectroscopy [5,6], Raman spectroscopy [7,8], infrared spectroscopy [9–12] and laser-induced breakdown spectroscopy (LIBS) [13] are modern approaches to discrimination and classification of pollens and other bio-materials, but were not yet applied in aerobiological monitoring stations. Until recently these methods were used only in laboratory conditions for characterization and distinguishing pollen grains from other airborne particles. Latest reports showed that advances in discrimination were

* Corresponding author. Tel.: +48 22 683 75 14; fax: +48 22 666 89 50.

E-mail address: abombalska@wat.edu.pl (A. Bombalska).

expanded to pollen belonging to the same genus. Some papers that have been published, showed usefulness of the Fourier transform infrared spectroscopy in pollen studies. Pappas et al. [14] showed that IR method was able to discriminate pollen species belonging to the same genus, such discrimination cannot be done by microscopic studies. Gottardini et al. [9] confirmed results of [14] and also demonstrated the discrimination potential of FTIR by examine the mixture of two different pollen taxa. Dell'Anna et al. [11] showed the application of FTIR together with statistical analysis (HCA, K-NN) in the process of pollen discrimination. Fluorimetric methods are common in bio-analyses and found application in distinguishing between biological and non-biological particles. Recently fluorescence microscopy was demonstrated as a tool in pollen analysis [16].

The present work shows the application of spectroscopic methods for detection and discrimination of pollens from the other biological substances contained in the sample. Our spectroscopic work was a part of larger project aiming detection and identification of biological particles with use of advanced optoelectronic methods. Different analytical methods were used to characterize biological material as absorption spectroscopy (UV–Vis, IR), fluorescence spectroscopy including LIF (Laser Induced Fluorescence). In presented study fluorescence and FTIR techniques along with PCA analysis were used for pollen characterization and discrimination. The aim of this experiments was the comparative analysis of the differentiation potential of FTIR and fluorescence techniques.

Materials and methods

Pollens, proteins, standards

All materials used are listed in Table 1).

Bacteria, yeast and filamentous fungi

The following bacterial strains were used throughout the study: *Bacillus atrophaeus* var. *globigii* ATCC 9372 (BG), *Bacillus thuringiensis* ATCC 10792, *Bacillus cereus* ATCC 14579, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Micrococcus luteus* ATCC 4698, *Bacillus megaterium* ATCC 14581 and *Pantoea agglomerans* ATCC

27155. The working cultures of each strain were prepared from the reference stock and the bacteria were grown in the appropriate liquid media for 18 h at 37 °C [17]. Endospores from *Bacillus* species were purified according to Caipo et al. 2002 [18]. *Candida albicans* ATCC 18804 and four strains of filamentous fungi: *Penicillium brevicompactum* ATCC 9056, *Penicillium chrysogenum* ATCC 9179, *Alternaria alternata* ATCC 6663 and *Cladosporium herbarum* ATCC 289877 were also used. The *C. albicans* strain was grown in liquid Yeast Extract–Peptone–Dextrose Medium (YPD medium), and filamentous fungi were cultured in MEA medium, respectively.

Methods

Fluorescence measurements

Spectra were recorded using fluorimeter FS 900 (Edinburgh Instruments) provided with front surface holder. Such experimental setup allows measurement of powders. Pollen powders were pressed to the cuvette surface and protected from sliding with an additional glass holder. An empty cuvette was used as a blank. The slits of both monochromators were set to 2 nm of spectral resolution. Excitation was measured from 250 to 400 nm in 5 nm steps, and emission from 260 to 550 nm in 2 nm steps. Raman signal was subtracted from the sample signal in post processing. Fluorescence matrices were normalized to a maximum value. Excitation–Emission (EX-EM) matrices were plotted with KyPlot, Kyenslab Inc., Japan.

FTIR spectra

FTIR spectra were obtained using Perkin-Elmer Spectrum GX Optica FTIR spectrometer. Measurement range was 4000–650 cm^{-1} (2.5–12.5 μm) with 4 cm^{-1} resolution. Three methods of measurement were applied:

- Conventional Transmission Mode using KBr pellet,
- Diffuse Reflectance Infrared Fourier Transform – DRIFT,
- Horizontal Attenuated Total Reflectance – HATR.

Conventional KBr pellet spectra were recorded from the sample (about 2 mg), which was grinded and mixed with dry KBr powder (about 200 mg), and then compressed with an Evacuable Pellet Die to produce a 13 mm diameter pellet. The IR light passes the pellet before it reaches the detector.

DRIFT method provides a convenient and rapid method of the sample preparation. It is not necessary to prepare the sample with KBr in the form of pellet. If the sample is not compressed, no polymorphic changes in crystalline structure occur. The principle of this method is the measurement of spectrum of radiation scattered from the sample, minimizing influence of specular reflectance. The collection angle of the diffuse reflectance accessory is full π steradians. The angle of incidence is 38°, which reduces the specular reflectance component from KBr to no more than 5%.

HATR spectra were recorded from the film of the sample prepared on the ZnSe crystal surface. Pollens were suspended in water, stirred and placed on ZnSe crystal. When suspension was desiccated the spectrum was recorded. Twenty scans were recorded for each sample. Infrared beam enters a crystal made from material with high refractive index. ZnSe crystal supplied with the HATR by Perkin-Elmer has 12 reflections at the crystal angle of 45°. The sample was held in close contact with the crystal. At each internal reflection the beam penetrates the sample. The penetration depth is typically of the order of a few microns, allowing measurement of thin samples. The spectra were recorded with use of mid infrared FR-DTGS detector with KBr window.

Pollens with use of FTIR were examined with pellet and DRIFT techniques. HATR was not applied due to the insolubility of pollens and potential influence of water on their chemical stability. Also

Table 1
List of biological samples.

Material	Abbrev.	Source
AC Fine Test Dust standard	DUST	Duke ^a
Artemisia absinthium,	ARTA	Sigma–Aldrich ^b
Artemisia tridentata	ARTT	Sigma–Aldrich
Bermuda grass	BER	Duke
Bermuda grass smut spores	BERs	Duke
Black walnut	WAL	Duke
Corn	COR	Duke
Iva xantifolia	IVA	Duke
Johnsons grass smut spores	JONs	Duke
Kochia scoparia	KOS	Sigma–Aldrich
Lycopodium	LYC	Sigma–Aldrich
Paper mulberry	PAP	Duke
Pecan	PEC	Duke
Poa pratensis	POA	Sigma–Aldrich
Populus nigra italica	NIG	Sigma–Aldrich
Populus tremuloides	ASP	Sigma–Aldrich
Ragweed	RAG	Duke
Secale cereale	SEC	Sigma–Aldrich
Ovalbumin	OVA	POCH ^c
Bovine Serum Albumin	BSA	Sigma–Aldrich
Syloid 244 FP	SYL	Grace ^d

^a Duke – Duke Sci. Corp, Faber Place, Palo Alto, CA.

^b Sigma–Aldrich – Sigma–Aldrich, Poland.

^c POCH – Joint-Stock Company, Poland.

^d Grace – Grace GmbH&Co KG.

adhesion of pollen grains might be insufficient for that kind of analysis. Bacteria and fungi were examined with HATR due to fragile character of the bio-material. Other techniques would destroy the cells.

Analysis

The FTIR data were pre-processed with algorithm of baseline correction. Background was recorded each time before the measurement, and subtracted automatically by the software. Also all numerical data were normalized to the value of one. HATR data were processed by a function of ATR correction. ATR Correction is used to make spectra collected with an Attenuated Total Reflection accessory similar to transmittance spectra. This procedure allows to compare spectra of the two types. The band intensities in internal reflectance spectra differ from those in transmittance spectra because the depth of the penetration of the radiation into the sample varies with the wavelength. If there is perfect contact between the sample and ATR crystal, the penetration depth is proportional to the wavelength. Poor contact with the crystal results in fractional penetration depth of the wavelength. ATR correction compensates this effect and obtained spectra has closest possible match with the intensities in a reference transmittance spectrum. Even after correction, the band in internal reflectance spectra will be slightly different from those obtained by transmittance measurements, but not by enough to affect qualitative interpretation [19].

Principal Component Analysis (PCA) was performed with SIMCA-P program, from Umetrics AB, Umeå, Sweden. Analyzed data was mean-centered and scaled to unit variance. Percentage of variance modeled by Principal Components (PC) is listed in axis titles of each score plot.

Results and discussion

Three dimensional EX-EM plots provide very useful tool for visual representation of material fluorescence properties. Sample

EX-EM matrices of three biological materials are shown in Fig. 1. The common feature of biological samples is fluorescence in the region characteristic for tryptophan – an amino acid present in proteins. The emission maximum for ovalbumin and *E. coli* ranges from about 310–360 nm. Similarly, *P. brevicompactum* fluoresces at this region but main peak is placed at 360–500 nm. Presented samples show the highest fluorescence signals at excitations ranging from 250 to 280 nm.

Analysis of 15 different pollens and two smut spores revealed that dry pollens are characterized with emission peaks at longer wavelengths compared to the biological material of other origin. This gives possibility of discrimination of pollens from other bio-particles. EX-EM matrices of three pollens and one smut spore reveal that dry pollens fluoresces at wavelengths over 380–400 nm, and wide excitation region spreads over the whole measurement range (Fig. 2). No fluorescence was observed in Bermuda grass smut spores (BERs) thus main graph area remains nearly flat. Sharp peaks appearing on BERs graph are characteristic artifacts of fluorimeter.

The following regions of matrices were selected for PCA: excitation 260–400 nm and emission 300–500 nm. It allowed to exclude artifacts mentioned above that could influence the results of statistical analysis. Two-dimensional PCA plot is presented in Fig. 3. Pollens, although spread over half of the graph area, can easily be distinguished from other bio-materials. Points representing fungi and bacterial spores are scattered widely across the plot area thus they are difficult to separate. Vegetative bacteria form relatively narrow group that is interlaced by points corresponding to some fungi, proteins and bacterial spores.

Bacterial spores were grouped out of confidence ellipse for vegetative bacteria, the reason of such classification is presence of DPA (dipicolinic acid) in their structure and the fact that some of spores were technical ones, which contained different kinds of admixtures. That features influence fluorescence characteristics.

Surprisingly, single emission spectra provide qualitatively similar information for discrimination process. Excitation wavelengths 280, 340 and 355 nm were selected to examine possibility of use

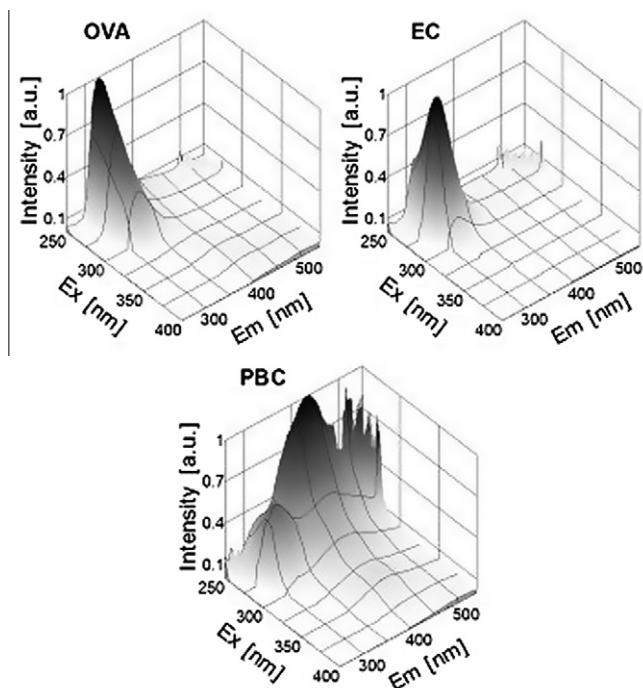


Fig. 1. Sample EX-EM matrices of dry Ovalbumin (OVA), *E. coli* (EC) and *P. brevicompactum* (PBC).

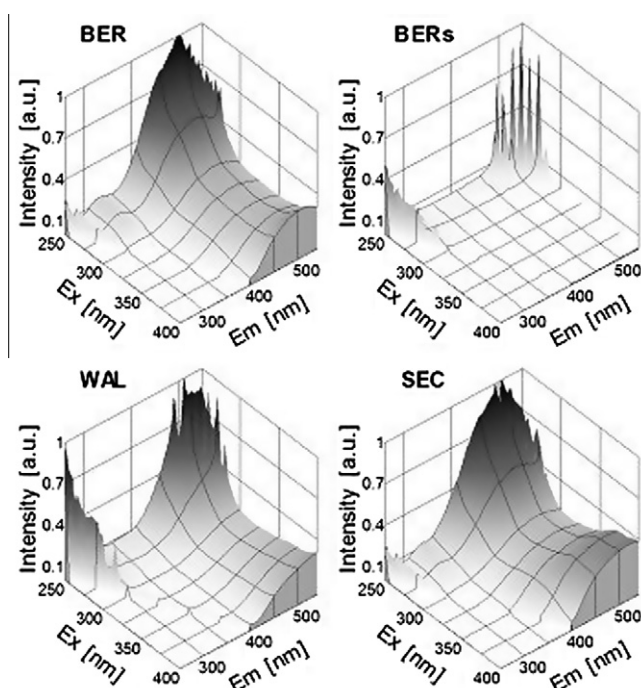


Fig. 2. EX-EM matrices of dry pollens: Bermuda grass pollen (BER), Bermuda grass smut spores (BERs), Black walnut pollen (WAL), *Secale cereale* pollen (SEC).

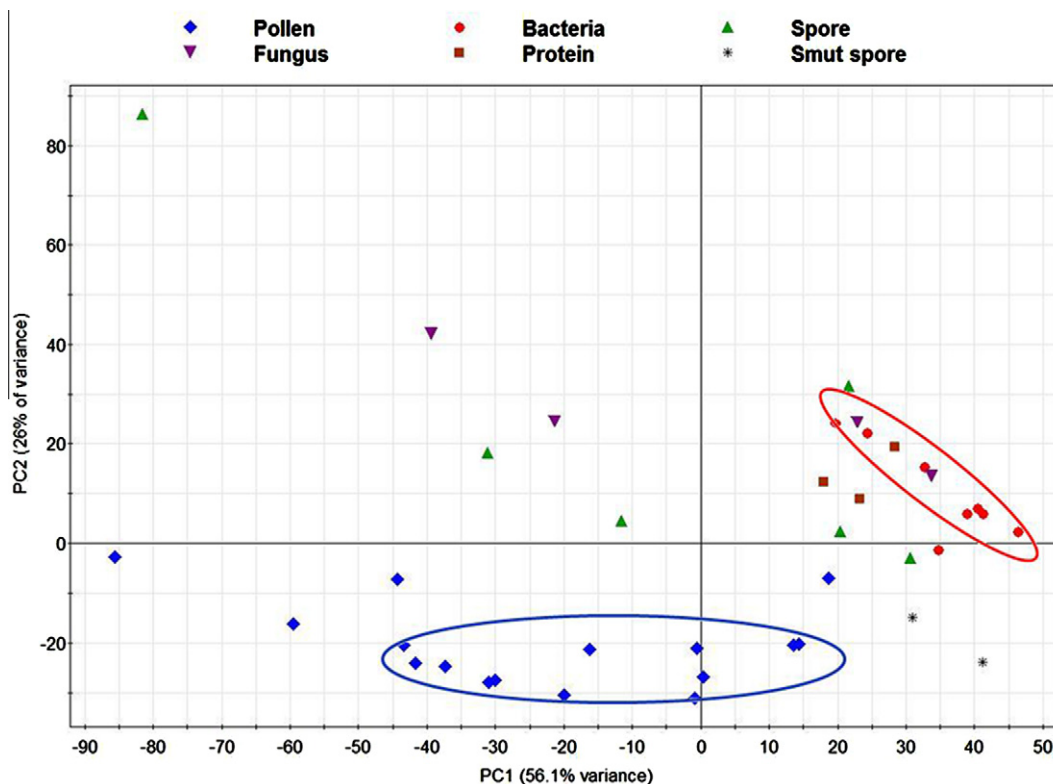


Fig. 3. PCA on whole EX-EM matrices. The fluorescence spectra were analyzed in a range of 250–400 nm for excitation and 260–550 nm for emission. The purpose of this analysis was to distinguish pollens from the other bio-particle background. The emission characteristics differs among classes of compounds allowing their general classification on PCA graph.

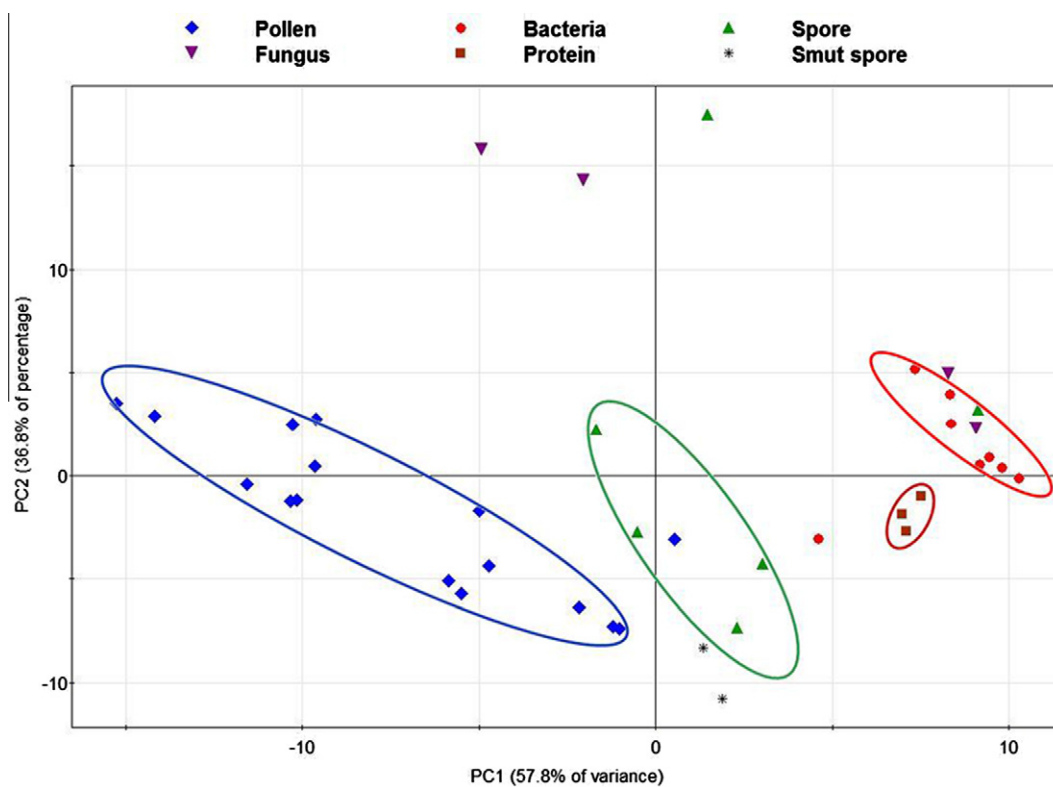


Fig. 4. PCA on spectra from 280 nm excitation. Excitation at 280 nm is characteristic for protein content in the sample. Since all examined particles were organic ones, the discrimination at this wavelength surprisingly was characteristic one.

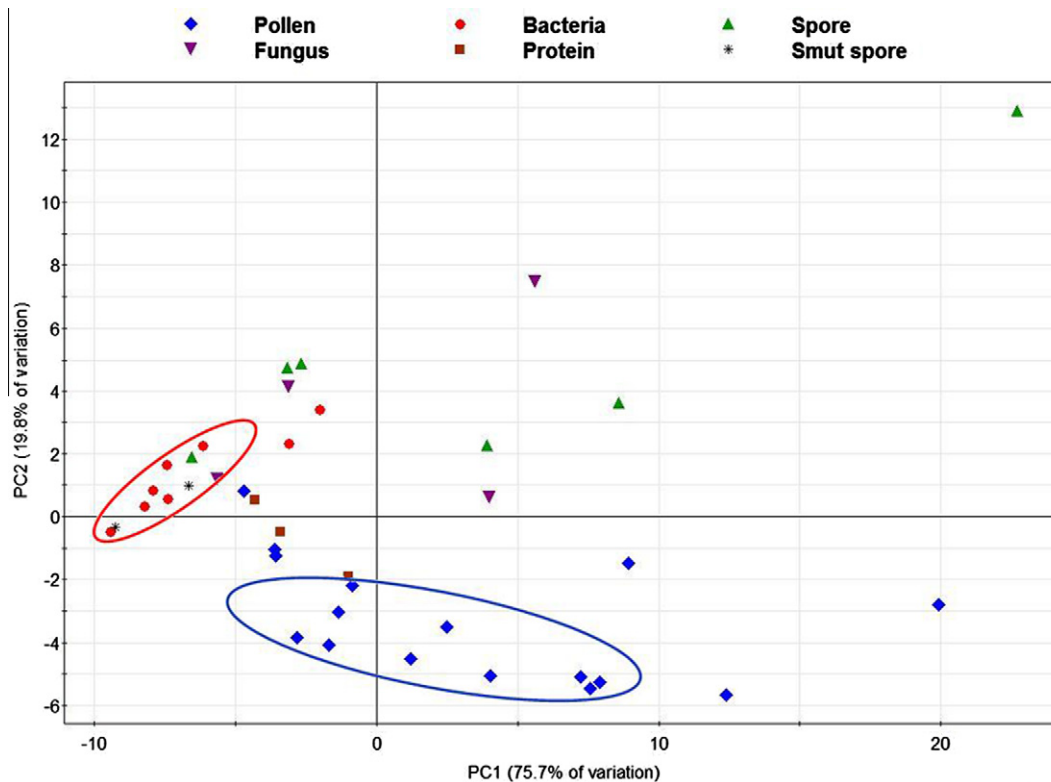


Fig. 5. PCA on spectra from 340 nm excitation.

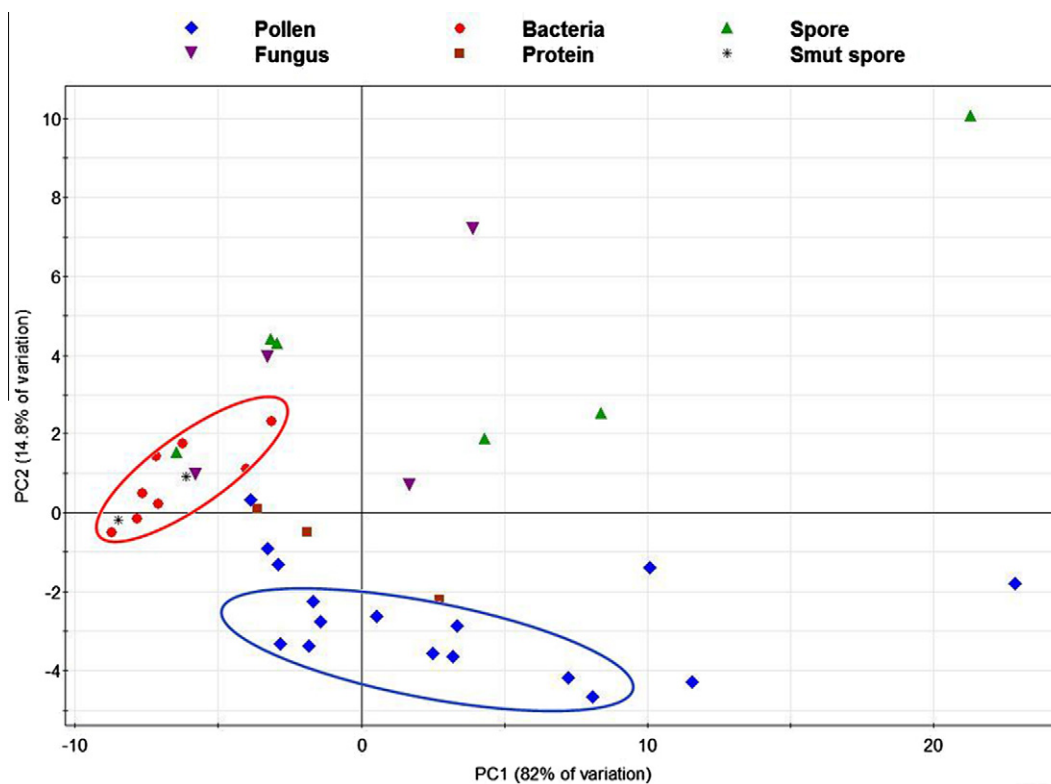


Fig. 6. PCA on spectra from 355 nm excitation.

typical laser sources for discrimination of various bio-materials. Laser excitation enables analysis on the level of single particle.

Fig. 4 shows PCA results of biological material excited with 280 nm. Pollens are well separated from other groups, but slight

overlapping occurs with bacterial spores. Among vegetative particles two fungi (CLH, PBC) showed wide emission characteristic for 250 nm excitation. Such result was not observed for any other substances. That is probable reason why CLH and PBC were classified

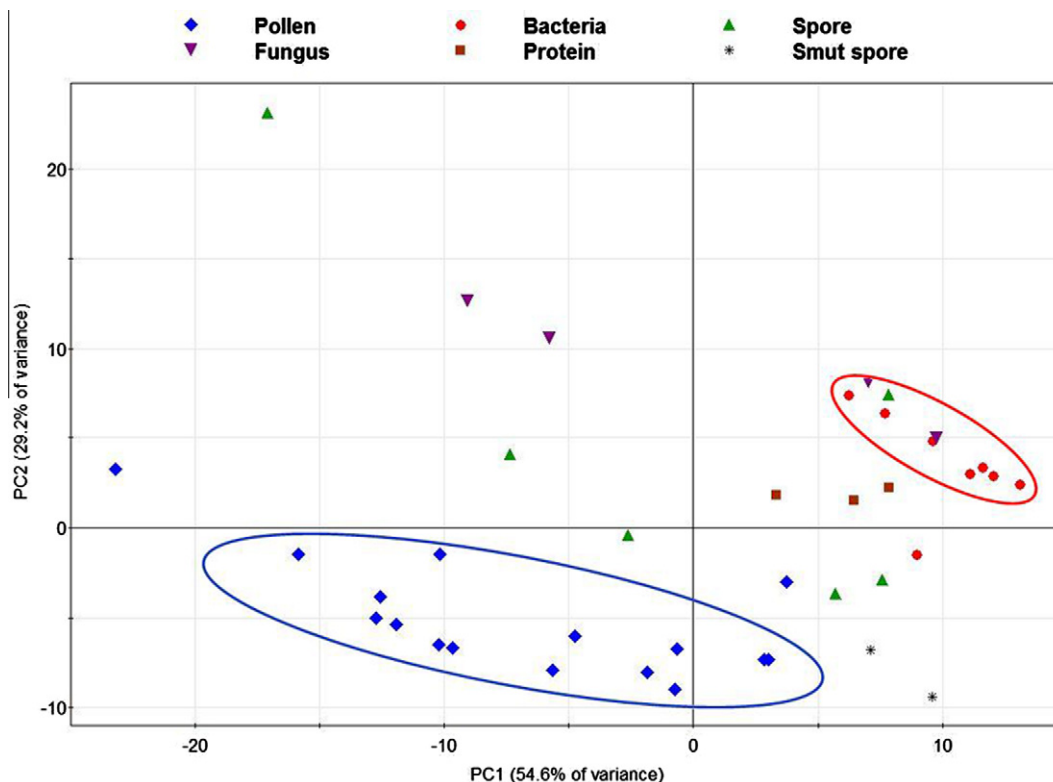


Fig. 7. PCA on spectra from 280 and 355 nm excitation.

away from other fungi. Also BT spore differs dramatically from other spores when examined with all excitation lines.

Excitation at 340 and 355 nm (Figs. 5 and 6, respectively) reveals that points representing pollens overlap partly those of proteins.

Fig. 7 presents analysis of data including excitation at 280 and 355 nm. The results are very similar to that from analysis of whole set of data (Fig. 3). It shows that two wavelengths excitation is sufficient for discrimination of pollens from other bio-particles. This is beneficial due to simplification of discrimination techniques.

The same samples of biological material were also examined with FTIR technique. The method allows fast and reliable sample analysis. Absorption spectrum of pollens is represented by peaks that are characteristic for the bonds occurring in carbohydrates (sugar and cell wall material), lipids and proteins [20]. Infrared spectra of 15 different pollens and two smut spores were obtained using both pellet technique and diffuse reflectance mode. (see Table 1).

Spectra of pollens and smut spores obtained with DRIFT and pellet techniques are compared in Fig. 8.

Four regions are revealed in the FTIR spectra. The first one between 4000 and 3000 cm^{-1} presents a broad band centered at about 3415 cm^{-1} corresponding to the absorption due to stretching of $-\text{OH}$ bands. The second region including two sharper peaks at about 2925 and 2855 cm^{-1} are primarily out-of-phase CH_2 stretching and in-phase CH_3 stretching respectively [21,22]. The third region between 1800 and 1500 cm^{-1} discloses $\text{C}=\text{O}$ and the $\text{C}=\text{C}$ double bonds. The peak at about 1738 cm^{-1} corresponds to the $\text{C}=\text{O}$ stretching vibration of alkyl-esters [23,14]. The one shown at about 1675 cm^{-1} arises from the amide I band of proteins [23,24]. The peak between 1600 and 1640 cm^{-1} is a complex band due to the absorption of water, $\text{C}=\text{C}$ double bonds and the bending COO^- group [22,25,26]. The peak that is shown at 1520–1508 cm^{-1} is assigned to lignin [14]. This is also with agreement of our own studies, when compared the spectra of lignin with aminoacids with

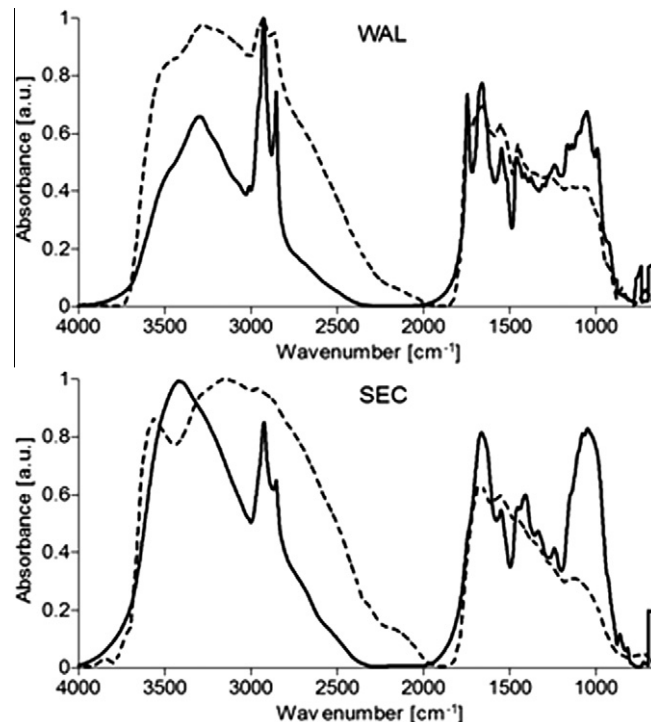


Fig. 8. FTIR spectra of pollen samples: Black walnut (WAL) and *S. cereale* (SEC), recorded using the KBr pellets (solid line), and DRIFT technique (dotted line).

pollens. The fourth region between 1500–700 cm^{-1} is associated with the skeletal vibrations of the components and it is characterized as the fingerprint region. This region play significant role in differentiation of the peaks signals and therefore in the identifica-

Table 2

The most important chemical bonds observed using FT-IR.

Chemical bond	Peak frequency (cm ⁻¹)
COO ⁻ stretching; CH ₂ , CH ₃ deformation	1445–1420
OH, COH, COO ⁻ , CH ₃ bending; CH ₂ wagging and twisting	1383–1357
C–C; C–O skeletal	1337–1308
C–OH skeletal; C–O–C of sugar ring	1107–1036
β-Glycosidic	920–883
α-Glycosidic	838–810

tion process, however is difficult to interpretation. The sugars of natural products absorb in this region. The most important chemical bonds and the typical frequencies observed in this region are listed in Table 2.

As shown in Fig. 8 the FTIR spectra recorded using the pellet technique present higher spectral resolution than those of DRIFT technique. In pellet technique IR radiation penetrates the samples with little or no diffusion, while in DRIFT technique only reflected part of the IR radiation provides information. An advantage of DRIFT technique is rapidity and elimination of the sample pretreatment. However, the KBr pellet technique results in spectra with a finer profile especially in the fingerprint region. For the comparative analysis spectra recorded in pellet techniques were used. Authors compared all three techniques based on aminoacids and proteins. As a result KBr and HATR turned to be superior to DRIFT due to peak intensities and lack of their deformation. Some differences were also observed between pellet and HATR technique. The changes have their source in solubility of materials. Non soluble proteins, as collagen and fibrinogen, did not form the thin film on ZnSe surface according to method requirements. In order to eliminate the water influence on pollen structure and composition

(germination) the pellet technique was chosen for further analysis. For other biological materials used in this study the HATR techniques was used due to the cells fragility of fungi and bacteria.

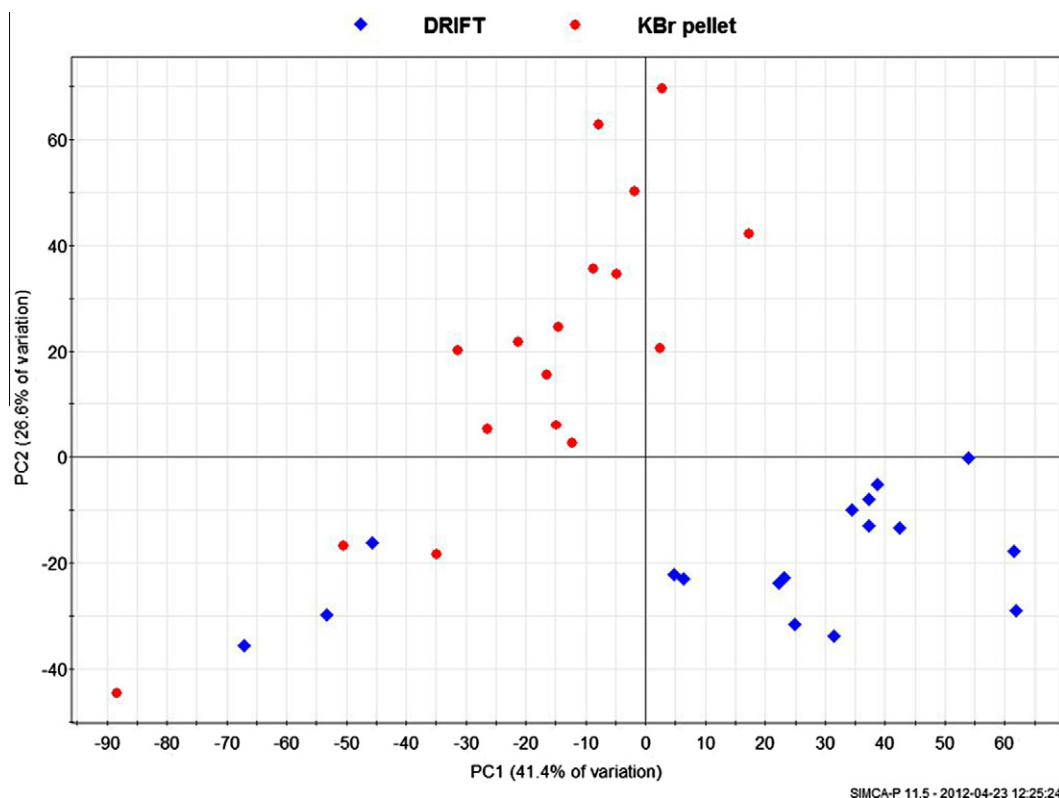
Comparison of results of pollen analysis with two different techniques is presented in PCA plot (Fig. 9). It acknowledges that points representing pollens acquired with both techniques form two separate groups. This proves that those two techniques are not interchangeable, and all measurements should be made with the method providing better results.

FTIR spectroscopy combined with PCA method appears to be a valuable method for classification pollens from other biological samples. Fig. 10 shows PCA results of pollens and different kinds of biological material. Proteins, vegetative bacteria, spores and fungi were measured using HATR technique and their spectra were corrected using a function of ATR correction. This function makes a spectrum collected with an HATR accessory similar to a transmittance spectrum so one can compare these two types of spectrum. Pollens, although spread over half graph area, can be easily distinguished among other bio-materials.

The results presented here lead to conclusion that FTIR technique allows rapid distinguishing of pollens from other biological samples. However, it seems to be very difficult to distinguish between pollens of different species and also between pollens and smut spores. Spectra of various species of pollens and smut spores reveal general similarity.

Due to more information carried by numerous peaks in infrared spectra, FTIR technique, when compared to fluorescence at excitations 280 and 355 nm, results in slightly better discrimination. However, no vital difference can be depicted (Figs. 7 and 10).

PCA was also performed on combined fluorescence (excitation at 280 and 355 nm) and FTIR data. Nevertheless, the pollens still formed separate, but widely spread group and we did not noticed distinct classification improvement (data not shown).

**Fig. 9.** PCA on spectra from pollens recorded using KBr pellet and DRIFT technique.

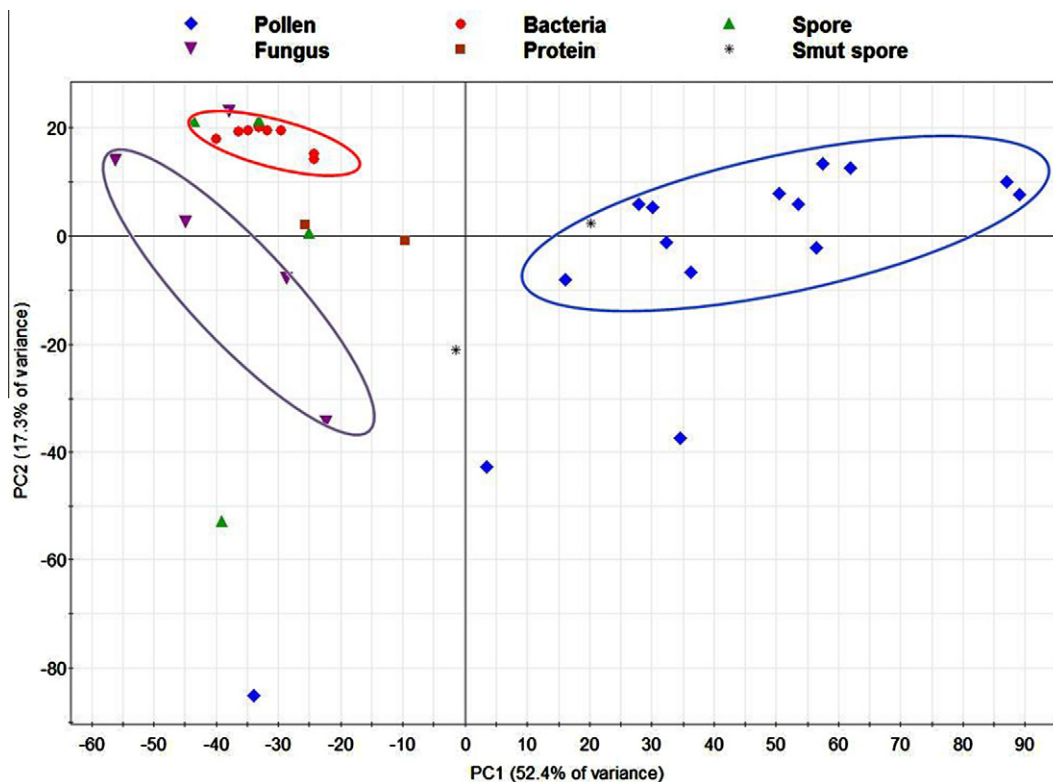


Fig. 10. PCA on FTIR spectra collected using HATR and KBr pellet technique in a range 4000–400 cm^{-1} .

Conclusion

Differentiation of the organic matter, with use of optical techniques, has a wide implication in medicine, food safety or air pollution. Application of the IR spectroscopy allows characterize pollens as chemical individuals, with high accuracy and precision. Depending on a measurement technique (HATR, KBr, DRIFT), preparation of the sample for measurement differs in difficulty and time of the preparation process. The data collection however is fast for each technique. Fluorimetric techniques found their application in photodynamic diagnosis, therapy (PDT, PDD) and caries diagnosis. Recent research are pointed towards analysis of biological specimens as bacteria, fungi, viruses and other biological interferents present in ambient air or water. The sample pre-treatment is not necessary, requires only dilution in a solvent, preferably in non-fluorescent one, in other case subtraction of the background is needed. The time of the measurement is longer than for FTIR, and come to 20 min for full EX-EM map. Time of measurement with single excitation line is comparable to FTIR.

The results in presented study show the possibility of application optical methods, as FTIR and fluorimetry, in differentiation of the plant pollens. Based on preformed experiments authors concluded that fluorescence and FTIR techniques are suitable for classification of biological material. Surprisingly, both techniques allow discrimination of pollens from other samples. For that reason, spectroscopic techniques are promising on the way to classify biological materials in allergen warning system. Such experiments are important due to creation of fluorescence data base of biological particles. Such characteristics will be used for on line systems as library of such apparatus. The more spectra are collected, if not discriminative potential, the better ratio false/positive can be obtained. So far on-line air monitoring, based on fluorimetric measurements with excitation lines at 266 and 355 nm, was performed in laboratory conditions, but the results are promising and indicate

the possibility of discrimination [27,28]. Our early studies concerning real-time measurements showed the possibility of biological particle detection with 355 nm excitation line but so far without identification. Increasing number of fluorescent signatures of interferants improve performance of such experiments.

PCA analysis was applied as a supporting method in differentiation of biological specimens with use of FTIR and fluorimetry. PCA analysis revealed that FTIR technique results in slightly better discrimination of pollens than fluorescence technique. However, combination of FTIR and fluorescence data did not improve classification of bio-particles. Out of applied excitation lines the most promising was 280 nm, which allowed to separate pollens from bacteria and fungi. Further analysis is carried out on combining more than one excitation line for improvement of the discrimination results. Further investigation need a database covering wider group of bio-particles and comparison with presented results.

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References

- [1] H. Behrendt, Wolf-Meinhard Becker, Localization, release and bioavailability of pollen allergens: the influence of environmental factors, *Curr. Opin. Immunol.* 13 (2001) 709–715.
- [2] W.M. Becker, Characterization of allergens from plants, in: G. Eisenbrand et al. (Eds.), *Food Allergy and Intolerances*, VCH, Weinheim, 1996, pp. 119–129.
- [3] M. Laaidi, *Int. J. Biometeorol.* 45 (2001) 1–7.
- [4] J. Emberlin, *Post. Derm. Alerg.* XX; 4 (2003) 196–199.
- [5] J.R. Lakowicz, *Principles of Fluorescence Spectroscopy*, 3rd ed., Springer, New York, 2006.
- [6] M. Włodarski, M. Kaliszewski, M. Kwaśny, K. Kopczyński, Z. Zawadzki, Z. Mierczyk, J. Młyńczak, E. Trafny, M. Szpakowska, *Proc. of SPIE*, Vol. 6398, 2006.

- [7] P. Rösch, M. Harz, K.D. Peschke, O. Ronneberger, H. Burkhardt, A. Schüle, G. Schmauz, M. Lankers, S. Hofer, H. Thiele, H.W. Motzkus, J. Popp, *Anal. Chem.* 78 (2006) 2163–2170.
- [8] F. Schulte, J. Lingott, U. Panne, J. Kneipp, *Anal. Chem.* 80 (2008) 9551–9556.
- [9] E. Gottardini, S. Rossi, F. Cristofolini, *Aerobiologia* 23 (2007) 211–219.
- [10] C.S. Pappas, P. Rodis, P.A. Tarantilis, M. Polissiou, *Appl. Spectrosc.* 53 (1999) 805–809.
- [11] R. Dell'Anna, P. Lazzeri, M. Frisanco, F. Monti, F. Malvezzi Campeggi, E. Gottardini, M. Bersani, *Anal. Bioanal. Chem.* 394 (2009) 1443–1452.
- [12] A. Bombalska, M. Mularczyk-Oliwa, M. Kwaśny, M. Włodarski, M. Kaliszewski, K. Kopczyński, M. Szpakowska, E.A. Trafny, *Spectrochim. Acta A* 78 (2011) 1221–1226.
- [13] A.R. Boyain-Goitia, D.C.S. Beddows, B.C. Griffiths, H.H. Telle, *Appl. Opt.* 42 (2003) 6119–6132.
- [14] C.S. Pappas, P.A. Tarantilis, P.C. Harizanis, *Appl. Spectrosc.* 57 (2003) 23–27.
- [16] K. Mitumoto, K. Yabusaki, H. Aoyagi, J. Biosci. Bioeng. 107 (2009) 90–94.
- [17] H.D. Isenberg, *Clinical Microbiology Procedures Handbook*, ASM Press, 2004.
- [18] M.L. Caipo, S. Duffy, L. Zhao, D.W. Schaffner, *J. Appl. Microbiol.* 92 (2002) 879–884.
- [19] Perkin Elmer User's Guide.
- [20] W.F. Wolters, F.A. Hoekstra, *Plant Physiol.* 109 (1995) 907–915.
- [21] K.V. Sarkanen, H.-M. Chang, G.G. Allan, *Tappi* 50 (1967) 583–587.
- [22] A.K. Chatjigakis, C. Pappas, N. Proxenia, O. Kalantzi, P. Rodis, M. Polissiou, *Carbohydr. Polym.* 37 (1998) 395–408.
- [23] C.F.B. Sene, M.C. McCann, R.H. Wilson, *Plant Physiol.* 106 (1994) 1623–1631.
- [24] M. Kacurakova, N. Wellner, A. Ebringerowa, *Food Hydrocolloids* 13 (1999) 35–41.
- [25] T.P. Abbot, I.W. Tjarks, M.O. Bagby, *Proc. Tappi Pulping Conf. Book 1* (1987) 177–183.
- [26] H.R. Constantino, K. Griebenow, R. Langer, A.M. Klibanov, *Biotechnol. Bioeng.* 53 (1997) 345–348.
- [27] V. Sivaprakam, A.L. Houston, C. Sotto, *Opt. Express* 12 (2004) 4457–4466.
- [28] V. Arganowski, Z. Ristovski, M. Hargreaves, *Aerosol Sci.* 34 (2003) 301–317.