ANALYSIS OF EUROPEAN HONEYBEE (*APIS MELLIFERA*) WINGS USING ATR-FTIR AND RAMAN SPECTROSCOPY: A PILOT STUDY

V. Machovič[1,2], L. Lapčák[2], M. Havelcová[2], L. Borecká[2], Mi. Novotná[1], Ma. Novotná[1], I. Javůrková[1], I. Langrová[4], Š. Hájková[4], A. Brožová[4], D. Titěra[3,4]

[1]University of Chemistry and Technology Prague, Prague, Czech Republic
[2]Institute of Rock Structure and Mechanics of the Czech Academy of Sciences, Prague, Czech Republic
[3]Bee Research Institute, Dol, Czech Republic
[4]Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resources, Prague, Czech Republic

The infrared (ATR-FTIR) and Raman spectroscopy was used for the structural characterization of honeybee wings. Protein, chitin, and lipid characteristic spectral features were detected using both methods. The protein secondary structure was predominantly composed of the β-sheet molecular conformation with β-turns or coil contributions. The vibration modes of the side-chain aromatic amino acid residues (tyrosine, phenylalanine, tryptophan) occurred in the wing spectra. The results of discriminant analysis showed that the infrared spectroscopy of the wing in combination with a multivariate analysis seemed promising for a resolution of the chemical structure of the wings based on lipid, proteins, and chitin content.

vibrational spectroscopy, protein, lipid, chitin

DE GRUYTER OPEN

doi: 10.1515/sab-2017-0004
Received for publication on January 7, 2016
Accepted for publication on October 6, 2016

INTRODUCTION

The wings of insects such as the cicada, butterfly, and dragonfly have been intensively studied at the micro- and nano-scale in relation to their superhydrophobic behaviour, self-cleaning wettability, stiffness, flexibility, and lightness properties (Tobin et al., 2013).

The insect wing consists of a three-dimensional skeletal network of relatively stiff veins, which are interconnected through thin membranous areas called cells. The wings are composed of cuticle, a composite material consisting of arrangements of highly crystalline chitin nanofibres embedded in a matrix of protein, polyphenols, and water, with small amounts of lipid. The crystalline structure of α-chitin is important for the interaction of chitin with the protein matrix (Neville, 1975; Vincent, Wegst, 2004).

The interaction of cuticular proteins with chitin fibres and detailed structure of insect cuticle have not yet been resolved. Recently, β-sheet–chitin chain interactions of the cuticular proteins with the chitin filaments have been proposed. The structure contains aromatic residues, namely tyrosines (Tyr) and

* Supported by the Technology Agency of the Czech Republic, Project No. TA04020317.

phenylalanines (Phe) (Iconomidou et al., 2001; Hamodrakas et al., 2002).

Infrared spectroscopy is a very fast analytical method for determining the chemical structure of insect wings (Barker, 1970; Yoshihara et al., 2012; Tobin et al., 2013). Unfortunately, only little information about the chemistry of honey bee wings can be found. Recently, a chitin content of five different body parts of a honeybee (*A. mellifera*) was determined by Kaya et al. (2015). The highest chitin content was observed for the leg (13.25%), while the lowest chitin content was recorded from the thorax (6.79%). Chitin contents of the other parts were stated as 8.9% for head, 8.61% for abdomen, and 7.64% for wings. Infrared spectra of chitins from different parts were very similar to each other. The results indicated that all the chitins extracted from different parts of honeybee were of alpha-form. Recently, Japan authors (Yoshihara et al., 2012) showed that small dragonflies species could be distinguished from medium and large dragonflies using a Principal component analysis (PCA) of infrared spectra of the dragonfly wings. Near-infrared spectroscopy has been used for the identification of stored-grain insects (Dowell et al., 1999).

The present work was aimed at providing the structural spectral analysis of honeybee wings using infrared and Raman spectroscopy. The ultimate goal of this preliminary study was the possible discrimination of honeybee subspecies using infrared spectroscopy of the honeybee wing in combination with a multivariate analysis. The developed spectroscopic method could complement the existing methods of classification of bee subspecies (e.g. geometric morphometrics of forewing venation) that are important for their protection and preservation of some breeding lines. This pilot study was performed on a small sample collection. To our knowledge, no vibrational spectroscopic studies on the honeybee wings have been reported in literature so far.

MATERIAL AND METHODS

The honeybee wing samples (w1–w7) were obtained from individuals catched on plants in the area with a highly morphologically variable bee population.

Fourier transform infrared (FTIR) spectra of the bulk samples were collected on a Nicolet 6700 FTIR spectrometer (Thermo Nicolet Instruments Co., Waltham, USA) with a N_2 purging system. Spectra were acquired using a single reflection ATR (Attenuated Total Reflection) GladiATR accessory (PIKE Technologies, Madison, USA) equipped with a single bounce diamond crystal (angle of incidence 45°). Each sample spectrum averaged 64 scans and the resolution was 2 cm^{-1}. The spectra were rationed against a single-beam spectrum of the clean ATR crystal and converted into the absorbance units by ATR correction. Data were collected over a wavenumber range of 4000–400 cm^{-1}.

The Raman spectrum of the sample was collected using a DXR dispersive Raman spectrometer (Thermo Fisher Scientific, Waltham, USA) equipped with a confocal Olympus microscope (Olympus, Tokyo, Japan). The spectrum was measured in the range 50–3500 cm^{-1} with a spectral resolution of 2 cm^{-1}. The Raman signal was excited by a 532 nm laser and detected by a cryogenically-cooled charge-coupled device (CCD) detector. Spectral parameters: bleaching time: 2 h, exposure time: 30 s., number of exposures: 100, grating: 900 lines per mm, spectrograph aperture: 25 µm pinhole, camera temperature: –50°C, laser power level: 8.0 mW, 50× objective.

The discriminant analysis was carried out using TQ Analyst software (Version 9.3.107; Thermo Fisher Scientific). The infrared spectroscopic data for discriminant analysis were fitted by mean-centering. One-point baseline was used for the wavenumber region of 3135–885 cm^{-1}. Constant pathlength was used for calculations. The Mahalanobis distance was used to formulate the distance between clusters. The number of principal components (PC) used for calculation was 10. The elemental organic composition was determined using a Thermo Finnigan FLASH EA 1112 Series CHNS/O microanalyzer (Thermo Fisher Scientific, Waltham, USA). A scanning electron microscope Quanta 450 (Thermo Fisher Scientific, Waltham, USA) was used for detailed studies and documentation.

RESULTS

The elemental composition of bee wings (sample w1) was as follows: C 57.62%, H 8.32%, and N 15.34%. Generally, the nitrogen content was mainly distributed in protein and chitin. In Fig. 1, scanning electron micrographs of the whole wing and cross-section of the wing are shown. From the cross-section micrograph of the wing its fibrous structure can nicely be seen.

ATR-FTIR

The infrared spectra of wing samples were measured by ATR technique. The wing samples were pressed on a diamond crystal by a stainless steel anvil without any pretreatment. The spectra were measured on the upper side of the wing on three different locations (spot size 2.2 × 3.0 mm). ATR-FTIR is a surface specific method and the depth of the analysis is known to be less than 1.5 µm at 1600 cm^{-1} for keratinized fibres. A representative spectrum of the bee wing is shown in Fig. 2. The band maxima of the studied bee wings spectra and the bee wing chitin spectra from literature (Kaya et al., 2015) are compiled in Table 1, together with tentative assignments of the main spectral features. The band maxima of Amide I and Amide II

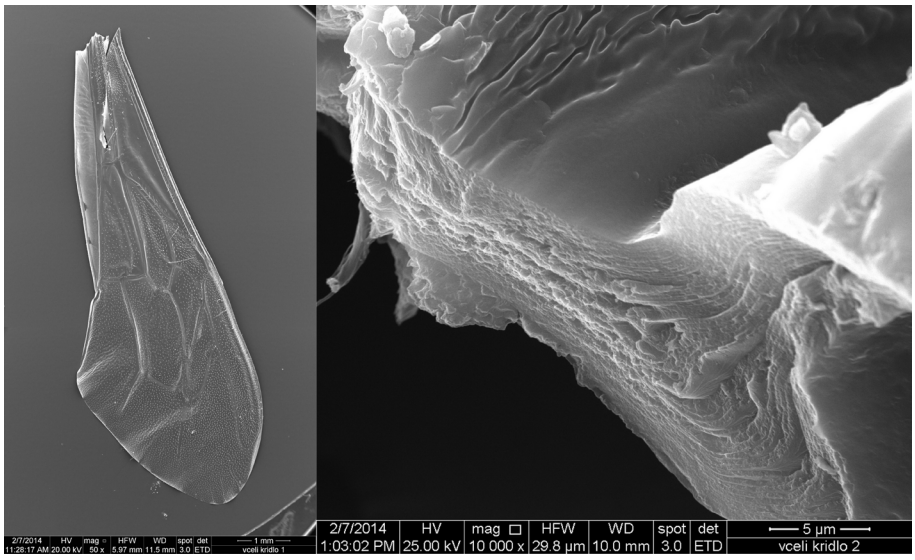

Fig. 1. Scanning electron micrographs of a whole honeybee wing (left) and cross-section of the wing at its widest part near the body (right)

were determined on the basis of second derivatives of the spectra.

Raman spectroscopy

Raman spectrum of the virgin wings could not be measured due to the strong fluorescence background but after the chloroform extraction of surface lipids, good quality Raman spectrum could be recorded. The Raman spectrum of the wing is shown in Fig. 3 and the assignment of the Raman band maxima is listed in Table 2.

DISCUSSION

ATR-FTIR

The prominent band at 3271 cm^{-1} can be assigned to the asymmetric stretching of N–H bond vibrations (see Fig. 2 and Table 1). The shoulders at 3420 cm^{-1} and at 3210 cm^{-1} can be ascribed to the O–H stretching and to the N–H symmetric stretching, respectively. FT-IR spectra of dragonflies exhibited two badly resolved bands at 3414 and 3290 cm^{-1} (Yoshihara, 2012). The band at 3087 cm^{-1} may be assigned to NH stretching modes with some contributions of the aromatic CH stretching vibrations. The bands belonging to the antisymmetric and symmetric stretching vibrations of the aliphatic C–H bonds of methyl, methylene, and methine groups can be found in the wavenumber region between 3000–2700 cm^{-1}. The bands of bending vibrations of CH_2 and CH_3 groups can be found at about ~1450 and ~1370 cm^{-1}.

Infrared spectroscopy is one of the oldest and well established experimental methods for the analysis of secondary structure of proteins. The protein repeated units give rise to nine characteristic infrared absorption bands, namely Amide A, B, and I–VII. The most sensitive spectral region of the protein

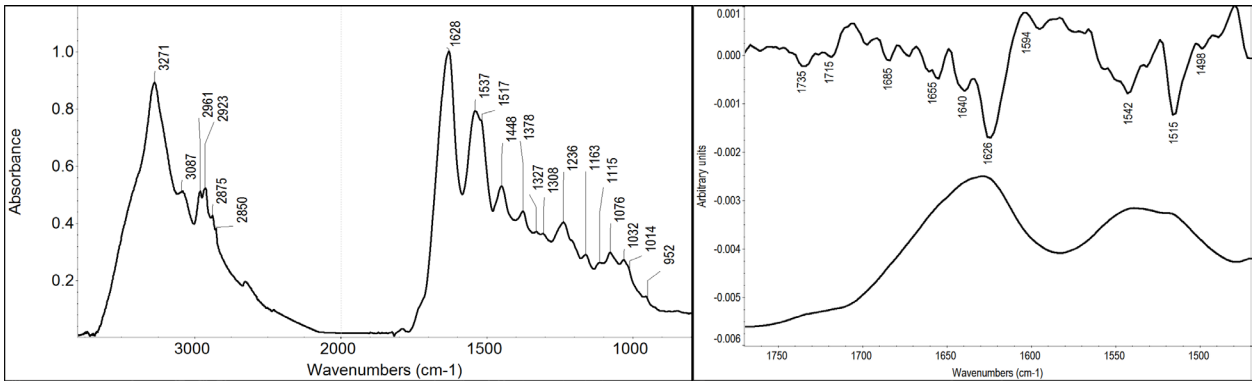

Fig. 2. Representative ATR-FTIR spectrum of the honeybee wing sample w1 (left) and the same spectrum in the wavenumber region of 1770–1470 cm^{-1} with its second derivative spectrum (right)

Table 1. Main ATR-FTIR band maxima of the studied bee wing samples and the honeybee wing α-chitin (Kaya et al., 2015); tentative assignments are included

Bee-wing (cm^{-1})	Assignment	α-Chitin (cm^{-1})	Assignment
3420sh	ν(OH)	3441	O–H stretching
3271	N–H stretching	3262–3098	N–H stretching
3190sh	ν(NH)		
3087	ν(NH), $\nu(CH)_{ar}$		
2961	$\nu^{as}(CH_3)$, lipids (2958)		
2932	$\nu^{as}(CH_2)$, lipids (2925)		
2918sh	$\nu^{as}(CH_2)$	2920	$\nu_{as}(CH_3, CH_2)$
2895sh	ν(CH)		
2875	$\nu^{s}(CH_3)$		
2850	$\nu^{s}(CH_2)$, lipids (2853)	2851	$\nu_{s}(CH_3)$
2820sh	ν(CH)		
1735, 1715	ν(C=O), lipids		
1685	Amide I, ν(C=O), antiparallel β-sheet		
1655	Amide I, ν(C=O), random coil	1658	ν(C=O)
1640	Amide I, ν(C=O), β-sheet		
1626	Amide I, ν(C=O), β-sheet/chitin	1621	ν(C=O)
1594	ν(C=C), Tyr side chains		
1542	Amide II, ν(C–N), δ(N–H), random coil/chitin	1553	N–H bend, C–N stretch
1515	ν(C=C), Tyr rings in side chains		
1498	ν(C=C), Tyr side chains		
1448	CH_2 scissoring		
		1431	CH_3, CH_2 bend
1378	CH wag/chitin	1376	CH_3, CH_2 bend
1327			
1308	Amide III, ν(C–N), δ(N–H)/chitin		CH_2 wagging
1285	Amide III, ν(C–N), δ(N–H)	1309	
1263sh	Amide III, ν(C–N), δ(N–H), random coil		
1236	Amide III, ν(C–N), δ(N–H), β-sheet		
			antisymmetric C–O–C stretching
1163	chitin	1154	in glycosidic bonds
1115	chitin	1113	antisymmetric in-phase ring stretching mode
1076	chitin	1060	C–O–C antisymmetric stretch in phase ring
1032	lipids?		
1014sh	chitin	1010	C–O antisymmetric stretch in phase ring
952	chitin	952	CH_3 wagging
893	chitin	896	CH ring stretching

ν = stretching vibration, δ = bending vibration

secondary structural components is the Amide I band (1700–1600 cm^{-1}), which belongs almost entirely to the C=O stretch vibrations (Kong, Yu, 2007, and references cited therein). The strong Amide I band at 1626 cm^{-1} and the strong Amide III band at 1236 cm^{-1} in the spectrum of studied wings are due to a β-sheet structure (Iconomidou et al., 2001), although some contributions of the α-chitin may be present in this frequency region. Bands at 1655 cm^{-1} in the Amide I, 1542 cm^{-1} in the Amide II, and 1263 cm^{-1} in the Amide III are probably due to a random coil structure (Iconomidou et al., 2001). The high-frequency band in the 1670–1695 cm^{-1} region can be assigned to an antiparallel β-sheet

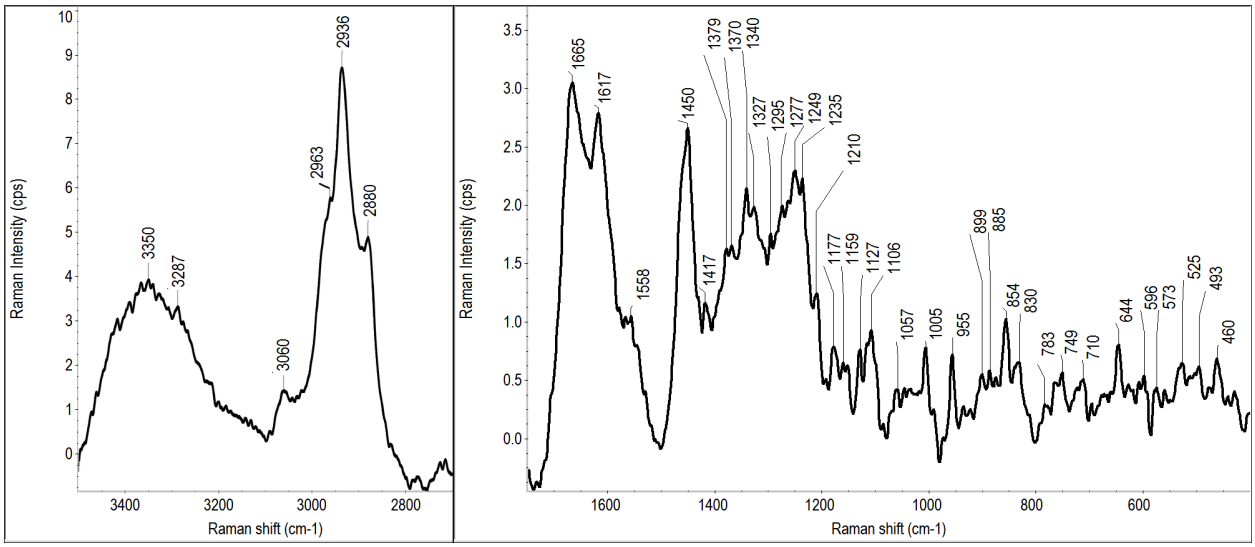

Fig. 3. Representative Raman spectrum of the honeybee wing sample w1

component (I c o n o m i d o u et al., 2001). The bands at 1515 and 1498 cm^{-1} can be ascribed to Tyr ring vibrations and the bands at 1163, 1115, 1076, 1032, 952, and 848 cm^{-1} can be assigned to the α-chitin (K a y a et al., 2015).

Raman spectroscopy

The major features of α-chitin can be found at 454, 504, 566, 647, 709, 898, 952, 1060, 1110, 1148, 1206, 1264, 1326, 1377, 1414, 1449, 1626, and 1657 cm^{-1} (I c o n o m i d o u et al., 2001).

The broad band at 3350 cm^{-1} can be assigned to NH and OH stretching modes, whereas band at 3050 cm^{-1} can be ascribed to NH and/or aromatic CH stretching modes (see Fig. 3 and Table 2). Aliphatic stretching features can be found at 2963 and 2936 cm^{-1}, corresponding bending modes at about 1450 and 1380 cm^{-1}.

Several narrow bands of the side-chain vibrations of aromatic amino acid residues occur in the spectrum. Typical bands of the Tyr can be found at 644, 830, 854, 1177, 1210, and 1611 cm^{-1}. The bands corresponding to the Phe are at 596, 620, 644, 1005, 1033, 1210, and 1611 cm^{-1} and the two

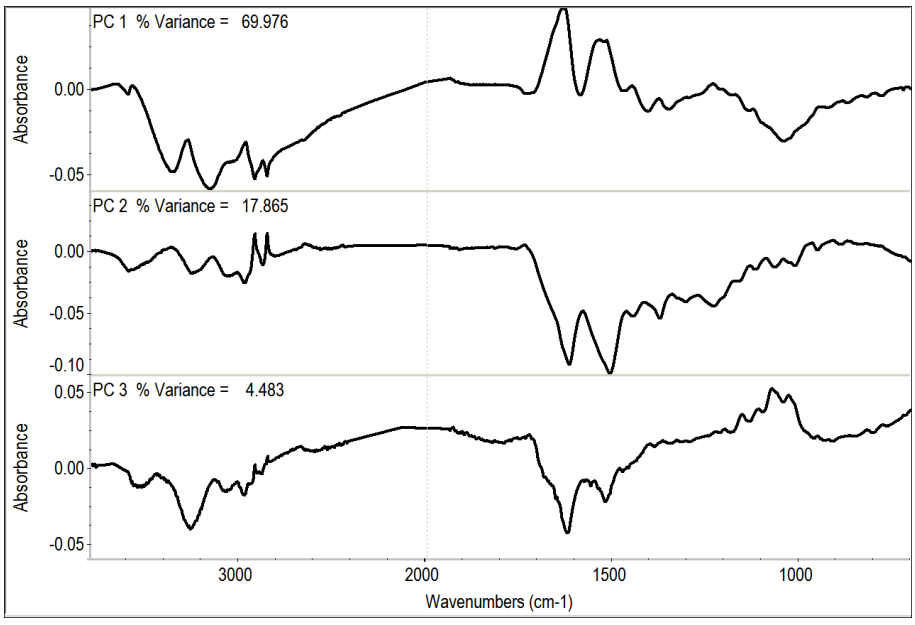

Fig. 4. Infrared spectra of the first three principal components

bands at 749 and 885 cm^{-1} can be assigned to the tryptophan (Trp). The bands in the 500–550 cm^{-1} region are typically associated with the S–S stretching mode of the C–C–S–S–C–C structural unit of disulfide bonds. The bands in the amide regions at 1665, 1558, 1249, and 1235 cm^{-1} indicate that dominant cuticular protein molecular conformation is the β-sheet. The band 1277 cm^{-1} is assigned to β-turns or coil (Iconomidou et al., 2001).

Discriminant analysis

The discriminant analysis was chosen as a tool for condensation of the spectral information into relatively small numbers of variables. PCA is one of the most common multivariate methods widespread also in infrared spectroscopy. PCA reduces multivariate data by transformation into orthogonal components, which are linear combinations of the origin variables. Produced synthetic variables, designated as principal components (PC), are set so as to describe the maximum variation in the data set. The first principal component PC1 contains the most important information about the samples. The remainder of the PCs include more specific information about analyzed samples.

The ATR-FTIR spectra of honeybee wings were measured on the upper side of the forewings at three different locations (spot size 2.2 × 3.0 mm) in the wavenumber range of 3135–885 cm^{-1}. All seven wing samples w1 to w7 were used for discriminant analysis. The infrared spectra of the first three principal components (see Fig. 4, PC1, PC2, PC3) described the most important structural information. The first principal component described 69.98% of the variance, the second 17.87%, and the third 4.48% of spectral variance. The prominent positive bands in the spectrum of PC1 (due to Amide I and Amide II) indicated changes in the protein content and negative bands in the region between 3000–2800 cm^{-1} were a consequence of varying concentrations of the lipid portion. The PC2 spectrum was very similar to the PC1 spectrum but both protein and lipid bands had an opposite trend. In the spectrum of PC3 also the chitin spectral features were present in the range of 1200–1000 cm^{-1}.

The Mahalanobis distance is a very useful way of determining the similarity or dissimilarity of a set of samples and an unknown sample. The closer each Mahalanobis distance value is to zero, the better the match (Mark, Tunnel, 1985). Results of the honeybee wing analysis were presented as distance plots in Mahalanobis distance units in Fig. 5. The *x*-axis shows the Mahalanobis distance to the w1 class while the *y*-axis shows the distance to the w2 class. The results showed that the analyzed samples could be approximately divided into two clusters. The first was represented by the samples w1, w5 and w7 and the second by the samples w2, w3, w4, and w6. Classes w1 and w2 exhibited the largest value of the Mahalanobis distance and hence the maximal structural variations. The infrared spectrum of the

Table 2 Main Raman band maxima of studied honeybee wing sample w1 and the 'soft' cuticle α-chitin (Iconomidou et al., 2001). Tentative assignments are included

Bee wing	α-chitin	Assignment
3350	3440	ν(OH/NH)
3060		$\nu(NH), \nu(CH)_{ar}$
2963	2963	$\nu^{as}(CH_3)$
2936	2935	$\nu^{as}(CH_2)$
2880	2881	$\nu^{s}(CH_3)$
1665	1657	beta-sheet
1617	1626	Amide III
1611sh		Tyr, Phe
1558		beta-sheet
1450	1449	CH_2 def
1417	1414	chitin
1379		
1370	1372	
1340		Amide III
1327	1326	
1295		
1277	1264	beta-turns/random coil
1249		beta-sheet
1235		beta-sheet
1210	1206	Tyr, Phe
1177		Tyr
1159	1148	C-O stretching
1127		C-O stretching
1106	1110	C-O stretching
1057	1060	C-O stretching
1005	1006	Phe
955	952	
899	898	C1H bending of beta-anomeric form of GlcNAc in chitin
885		Trp
854		Tyr
830		Tyr
783		
749		Trp
710	709	
644	647	Tyr, Phe
596		Phe
573	566	
525	504	S-S
493		
460	454	

sample w2 showed more intense lipid bands at 2918 and 2850 cm^{-1}, while the spectrum of the sample w1 had prominent Amide I and Amide II bands. The results of the discriminant analysis showed that the method seems to be promising for a resolution of the chemical structure of the wings.

CONCLUSION

The study was aimed at providing the structural spectral analysis of honeybee wings using infrared and Raman spectroscopy. The goal of this preliminary work was the possible discrimination of honeybee subspecies using infrared spectroscopy of the honeybee wing in combination with a multivariate analysis.

The following conclusions were possible to draw from the results. Protein, chitin, and lipid characteristic spectral features were detected using both methods. The protein secondary structure was predominantly composed of the β-sheet molecular conformation with β-turns or coil contributions. The vibration modes of the side-chain aromatic amino acid residues (Tyr, Phe, Trp) occurred in the wing spectra.

The results of discriminant analysis showed that the infrared spectroscopy of the wing in combination with a multivariate analysis seemed promising for a resolution of the chemical structure of the wings based on lipid proteins and chitin content. Further work will focus on larger, well-defined collection of honeybee wing samples.

REFERENCES

Barker RJ (1970): Infra-red absorption spectrum of the cuticular surface of drone honey bee wings as revealed by attenuated total reflectance. Journal of Insect Physiology, 16, 1921–1926.

Dowell FE, Throne JE, Wang D, Baker JE (1999). Identifying stored-grain insects using near-infrared spectroscopy. Journal of Economic Entomology, 92, 165–169.

Hamodrakas SJ, Willis JH, Iconomidou VA (2002): A structural model of the chitin-binding domain of cuticle proteins. Insect Biochemistry and Molecular Biology, 32, 1577–1583.

Iconomidou JA, Chryssikos GD, Gionis V, Willis JH, Hamodrakas SJ (2001): 'Soft'-cuticle protein secondary structure as revealed by FT-Raman, ATR FT-IR and CD spectroscopy. Insect Biochemistry and Molecular Biology, 31, 877–885.

Kaya M, Mujtaba M, Bulut E, Akyuz B, Zelencova L, Sofi K (2015): Fluctuation in physicochemical properties of chitins extracted from different body parts of honeybee. Carbohydrate Polymers, 132, 9–16.

Kong J, Yu S (2007): Fourier transform infrared spectroscopic analysis of protein secondary structures. Acta Biochimica et Biophysica Sinica, 39, 549–559.

Mark HH, Tunnel D (1985): Qualitative near-infrared reflectance analysis using Mahalanobis distances. Analytical Chemistry, 57, 1449–1456.

Neville AC (1975): Biology of the arthropod cuticle. Springer-Verlag, New York.

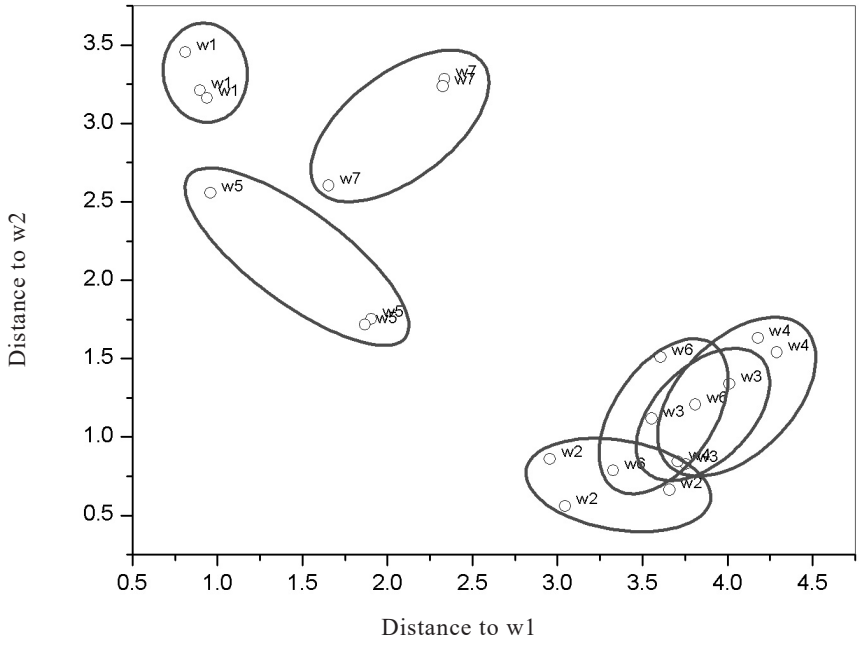

Fig. 5. Mahalanobis distance plot for seven classes of honeybee wings

Tobin MJ, Puskar L, Hasan I, Webb HK, Hirschmugl CJ, Nasse MJ, Gervinskas G, Juodkazis S, Watson GS, Watson JA, Crawford RJ, Ivanova EP (2013): High-spatial-resolution mapping of superhydrophobic cicada wing surface chemistry using infrared microspectroscopy and infrared imaging at two synchrotron beamlines. Journal of Synchrotron Radiation, 20, 482–489.

Vincent JFV, Wegst UGK (2004): Design and mechanical properties of insect cuticle. Arthropod Structure and Development, 33, 187–199.

Yoshihara A, Miyazaki A, Maeda T, Imai Y, Itoh T (2012): Spectroscopic characterization of dragonfly wings common in Japan. Vibrational Spectroscopy, 61, 85–93.

Corresponding Author:

Ing. Dalibor T i t ě r a , CSc., Czech University of Life Sciences Prague, Faculty of Agrobiology, Food and Natural Resources, Department of Zoology and Fisheries, Kamýcká 129, 165 00 Prague-Suchdol, Czech Republic, phone: +420 607 985 393, e-mail: titera@beedol.cz