

Infrared (FTIR) spectral features of honey bee (*Apis mellifera* L.) hemolymph

Infracrvene (FTIR) spektralne značajke hemolimfe medonosne pčele (*Apis mellifera* L.)

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Received: January 13, 2020; accepted: February 26, 2020

ABSTRACT

Hemolymph is a liquid of the open circulatory system of the honey bee (*Apis mellifera* L.) which represents a functional equivalent of vertebrate blood and lymph. The aim of this study was to determine the overall biochemical composition of honey bee hemolymph based on chemical characterization using Fourier transform infrared (FTIR) spectroscopy. Individual hemolymph samples of two age groups of workers (newly emerged bees-1 day old; foragers-42 days old) were collected by extraction from the thorax, and analysed by FTIR-ATR (Attenuated Total Reflectance) recording technique. The results have revealed a unique IR chemical fingerprint of honey bee hemolymph. Characteristic age-related spectral features indicating compositional variations between newly emerged bees and foragers were observed.

Keywords: chemical characterization, FTIR-ATR spectroscopy, hemolymph, honey bee

SAŽETAK

Hemolimfa je tekućina otvorenog sustava cirkulacije medonosne pčele (*Apis mellifera* L.) koja predstavlja funkcionalni ekvivalent krvi i limfe kralježnjaka. Cilj je ovog rada bio utvrditi ukupni biokemijski sastav hemolimfe pčela temeljem kemijske karakterizacije primjenom infracrvene spektroskopije s Fourierovom transformacijom (FTIR). Pojedinačni uzorci hemolimfe dviju dobnih skupina pčela radilica (mlade pčele u dobi od jednog dana; skupljačice u dobi od 42 dana) prikupljeni su ekstrakcijom iz prsišta i analizirani FTIR-ATR (prigušena totalna refleksija) tehnikom snimanja spektara. Rezultati su pokazali jedinstveni IR kemijski „otisak prsta“ hemolimfe pčela. Uočene su karakteristične spektralne značajke analiziranih dobnih skupina pčela koje ukazuju na razlike u sastavu hemolimfe između mladih radilica i skupljačica.

Ključne riječi: FTIR-ATR spektroskopija, hemolimfa, medonosna pčela, kemijska karakterizacija

INTRODUCTION

Honey bees play an important role in the ecosystem, and the increasing losses of honey bee colonies in the last decade have induced numerous discussions about their ecological and economic importance. Hemolymph represents a functional equivalent of vertebrate blood and lymph (Svečnjak et al., 2012). Understanding the biochemical features of the honey bee hemolymph (*Apis mellifera* L.) is of great importance as it is closely related

to the immune response of honey bees on various negative factors, thus reflecting the physiological state of honey bee organism. Hemolymph is a colorless or slightly yellowish liquid of open circulatory system of honey bees consisted of water plasma and free-floating cells - hemocytes (Wyatt, 1961) which are important for immune response (El Mohandes et al., 2010) along with antimicrobial peptides (AMPs) (Daníhlík et al., 2015). It has

a complex chemical composition and contains numerous proteins (Chan et al., 2006), sugars (Blatt and Roces, 2001) and lipid-based components (Bounias, 1986). Most of the studies on honey bee hemolymph composition were focused on the analysis of selected individual biochemical compounds (carbohydrates, proteins, AMPs, aminoacids and lipids), but it has not yet been explored on comprehensive molecular level (Svečnjak et al., 2012). Previous studies revealed certain differences in the hemolymph composition (mainly protein-based) between different honey bees castes (queen, workers, drones) and different development stages (larvae, pupae, adult bees) (Chan et al., 2006; Cremonese et al. 1998). However, a detailed chemical characterization of overall hemolymph composition of individual honey bees has not yet been investigated sufficiently. As opposed to classical chemical and molecular analytical methods, analysis of compounds by infrared (IR) spectroscopy provides a unique fingerprint of a sample based on overall biochemical composition thus enabling low-cost and multicomponent measurement. Therefore, the aim of this study was to determine the overall biochemical composition of the honey bee hemolymph based on the hemolymph chemical fingerprinting using FTIR-ATR spectroscopy.

MATERIALS AND METHODS

Newly emerged honey bees collected from experimental capped brood comb incubated overnight at 34.5 °C were marked on the thorax with honey bee marking pen and placed back in the honey bee colony to obtain foragers of defined age (42 days old). Marking was performed during the production season (June). Individual hemolymph samples were extracted from newly emerged workers (n=31) immediately after 24h incubation (1 day old workers), while the hemolymph of foragers (n=18) was obtained by extraction 42 days after marking the newly emerged bees (42 days old foragers). Hemolymph samples (~4-5 µL/bee) were collected by extraction from the thorax (after removing the legs - to avoid contamination with nectar or gut content which often occurs when other extraction methods are applied) using 10 µL glass microcapillary tubes. The

infrared spectra of individual hemolymph samples were recorded using Cary 660 Fourier transform mid-infrared (FTIR) spectrometer (Agilent Technologies) coupled with Golden Gate single-reflection diamond Attenuated Total Reflectance (ATR) accessory (Specac). Hemolymph samples were transferred on ATR plate directly from the glass microcapillary tubes as obtained, immediately after hemolymph extraction. The absorption IR spectra of the hemolymph samples were recorded at room temperature (24 ± 2 °C) using a spectral resolution of 4 cm^{-1} . Qualitative interpretation of hemolymph IR spectra (assignment of molecular vibrations) and corresponding spectral data analysis was performed using Origin version 8.1 (Origin Lab Corporation) based on spectral atlases/libraries and available scientific literature.

RESULTS AND DISCUSSION

As presented on Figure 1, a typical FTIR-ATR spectrum of honey bee hemolymph (presented as an average spectrum of all analyzed hemolymph spectra; n=49) has revealed characteristic spectral features (absorption bands) assigned to vibrations of functional groups that correspond to the associated macromolecules present in the hemolymph. A strong band with an absorption maximum at 3327 cm^{-1} is the most intense band in the hemolymph spectrum, and it occurs due to the stretching vibrations of hydroxyl groups of water and carbohydrates (glucose, fructose, trehalose) (Max and Chapados, 2007; Kuroiwa et al., 2015). Given the high proportion of protein-based components in the honey bee hemolymph, it is most likely that this absorption band overlaps with N-H stretching vibrations (amide A) of proteins that typically absorb in the spectral range from 3500 to 3300 cm^{-1} (Socrates, 2001; Kong and Yu, 2007). The vibration at 2927 cm^{-1} corresponds to C-H stretching of methyl and methylene groups of both proteins and lipids. The medium intensity vibration at 1636 cm^{-1} was assigned to C=O and C-N stretching vibrations (amide I) which can be associated with peptides and enzymes (globular proteins) present in the honey bee hemolymph.

The region between 1500 and 950 cm^{-1} , also known as a fingerprint region as it contains complicated series

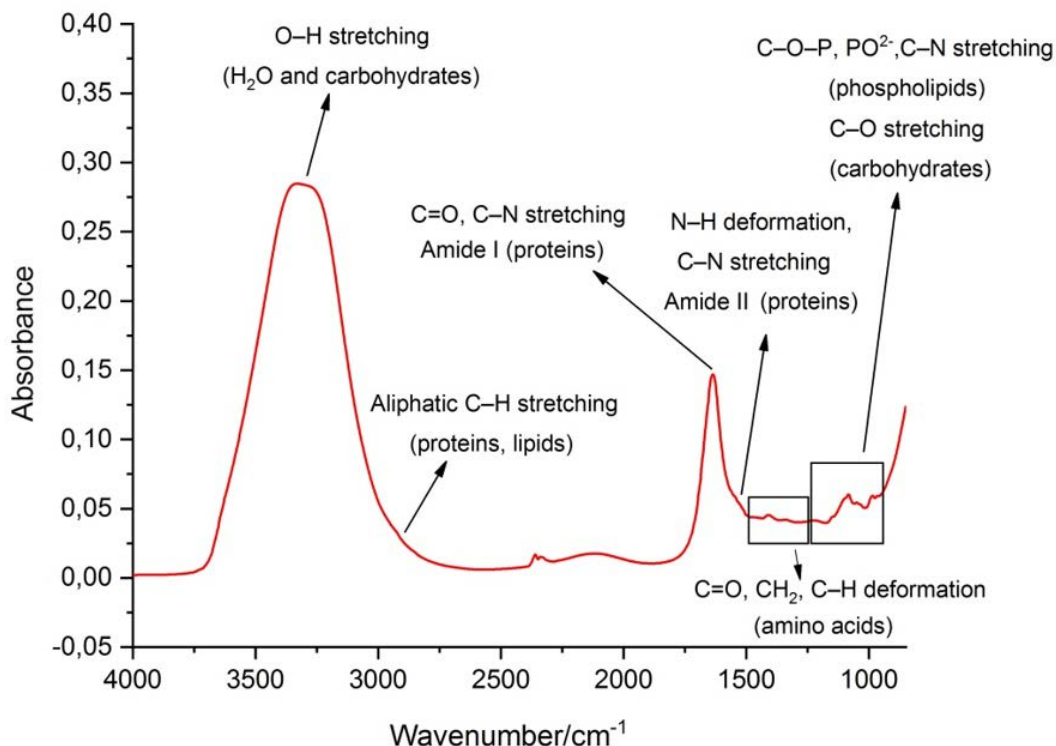


Figure 1. Characteristic FTIR-ATR spectrum of the honey bee hemolymph (spectral range: 4000-600 cm^{-1}) with assignment of major underlying molecular vibrations

of absorptions providing unique spectral patterns, is populated by a number of absorption bands related to protein components and phospholipids, as well as overlaid sugar absorptions. The most significant spectral variations within analyzed hemolymph spectra were observed in this particular region. The N-H deformation and C-N stretching vibrations (amide II) of peptides, enzymes and other hemolymph protein-based constituents occur in the spectral range between 1510 and 1544 cm^{-1} . The spectral region between 1470 and 1280 cm^{-1} is characterized by a series of weak broad signals at 1459 and 1407 cm^{-1} due to CH_2 bending and C=O asymmetric stretching (COO^-), and at 1337 due to C-H deformation vibration which overlaps with C-N stretchings (amide III). These absorption bands are attributed to hemolymph free amino acids (Chen, 1985; Socrates, 2001). It can be assumed that these signals are highly specific for proline (Wellner et al., 1996) given that proline represents a predominant free amino acid in the honey bee hemolymph (50-80% of the total free amino acid fraction) whose content changes significantly depending on the age of workers while no significant changes have been observed in the

content of other hemolymph amino acids (Crailsheim and Leonhard, 1999). These findings have been confirmed on the hemolymph sample set studied here, which is further substantiated below by FTIR spectrum presenting average age-related spectral differences (Figure 2).“

An absorption band arising at 1222 cm^{-1} is assigned to PO_2^- stretching of lipids (phospholipids). Spectral region from 1200 to 900 cm^{-1} is also represented by numerous vibrations; a weak band with an absorption maximum at 1083 cm^{-1} can be attributed to PO_2^- symmetric stretching vibrations of phospholipids. The bands occurring at 1050 cm^{-1} due to C-O-P stretching, and at 986 cm^{-1} due to CN asymmetric stretching ($\text{CH}_3)_3\text{N}^+$ also signify the presence of lipids (Socrates, 2001). However, this spectral region is also characterized by overlapping effects with sugar signals occurring at 1087 cm^{-1} (glucose and fructose), 983 cm^{-1} and 965 cm^{-1} (fructose) due to C-O stretching vibrations (Max and Chapados, 2007). It is likely that similar overlapping effects are represented by trehalose absorptions that occur at 1146 and 1050 cm^{-1} (Kuroiwa et al., 2015).

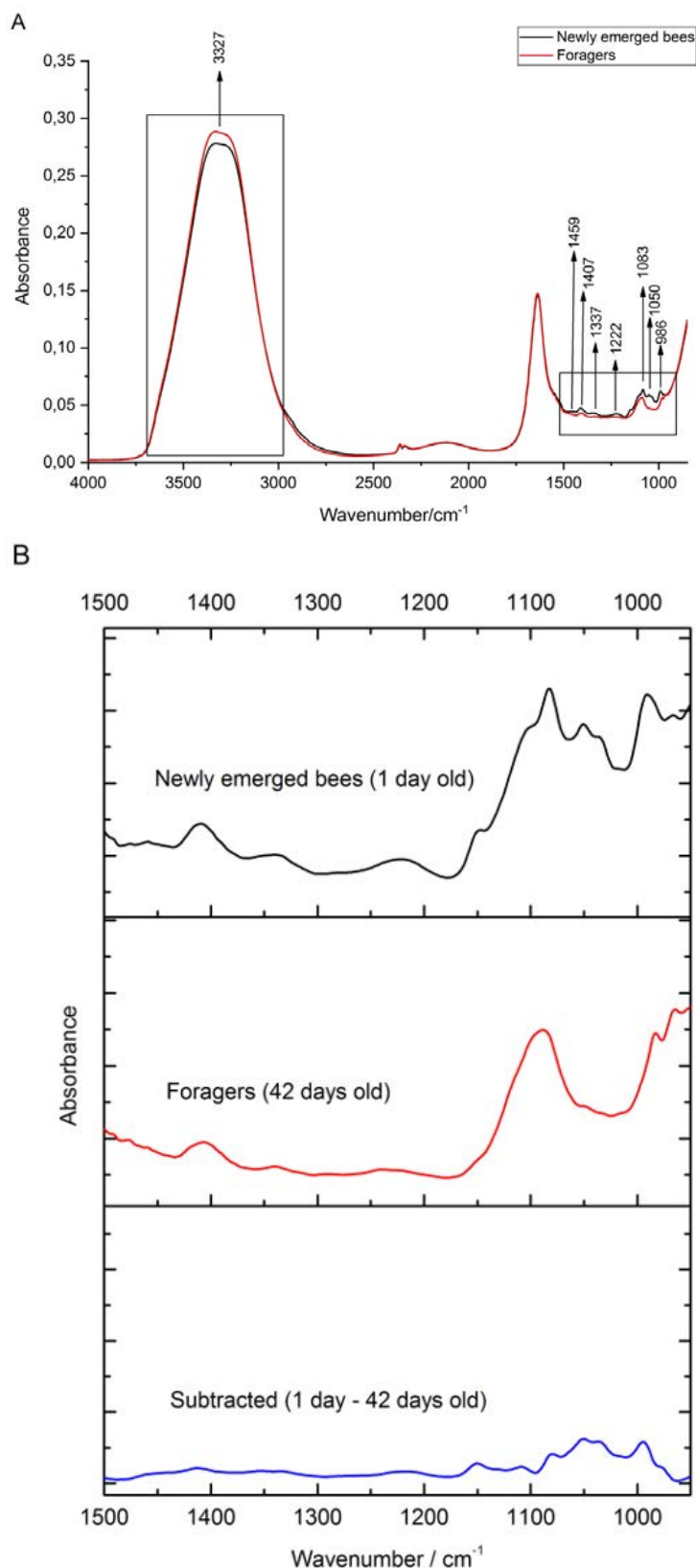


Figure 2. Spectral differences between average hemolymph spectra of newly emerged workers and foragers: the whole FTIR-ATR spectrum / spectral region between 4000 and 600 cm⁻¹ (A); three-panel presentation of a fingerprint region (1500-950 cm⁻¹) emphasizing the most indicative spectral differences (B)

Qualitative interpretation of average hemolymph spectra of newly emerged bees and foragers, as well as integral spectral features and variations of absorption intensities of particular vibrations observed (Figure 2A) indicate that newly emerged bees contain a higher proportion of proteins, amino acids and lipids (primarily phospholipids), while foragers hemolymph contain higher proportion of water and carbohydrates. As presented in Figure 2A, overall spectral variations and corresponding compositional changes observed in the hemolymph of newly emerged workers were manifested through higher absorption intensities of protein and lipid based analyte signals (at 2927, 1459, 1407, 1337, 1222, 1083, 1050 and 986 cm⁻¹) compared to average spectrum of foragers. As opposed to this observation, foragers hemolymph exhibits a simpler IR spectrum in which indicative water / carbohydrates bands have been observed at 3327, 1087, 983 and 965 cm⁻¹. The most variable and indicative age-related spectral differences have been observed in the fingerprint region (1500-950 cm⁻¹), which is emphasized in Figure 2B.

Specific IR spectral features observed in analyzed hemolymph samples indicate the possibility of using FTIR-ATR spectroscopy as a rapid, reagent-free, easy-to-use and low-cost analytical tool for honey bee hemolymph studies covering different metabolic, age-related and/or pathological research aspects. In order to confirm and upgrade these preliminary findings, further spectroscopic studies on honey bee hemolymph should be complemented by additional chemometric modelling (including spectral data pre-processing), as well as multivariate statistical analysis performed on a larger sample size. To our best knowledge, this preliminary study represents the first report on the IR spectral features of insect hemolymph, and opens the possibility for utilization of FTIR-ATR spectroscopy for research on hemolymph of other insect species.

CONCLUSIONS

The results of this study showed that honey bee hemolymph exhibits a unique IR spectrum dominated by characteristic absorption bands arising from hemolymph major constituents - water, proteins, amino acids, carbohydrates (glucose, fructose, trehalose) and lipids. Analysis of average hemolymph spectra revealed characteristic spectral features indicating quantitative compositional variations between the hemolymph of newly emerged bees and foragers. The major differences were reflected in the higher proportion of proteins, amino acids and lipid-based components in the hemolymph of newly emerged bees compared to foragers hemolymph. Contrary, it was observed that foragers hemolymph contains higher amount of water and carbohydrates which can be associated with age/task relating metabolic changes - foraging activity (nectar collection).

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