

INSIGHTS INTO THE INFRARED AND RAMAN SPECTRA OF FRESH AND LYOPHILIZED ROYAL JELLY AND PROTEIN DEGRADATION IR SPECTROSCOPY STUDY DURING HEATING[♦]

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In terms of chemical composition, a honeybee secretion known as royal jelly (RJ) is very complex product containing water, proteins, carbohydrates, lipids, mineral salts and small amounts of polyphenols, vitamins and enzymes. Despite its chemical diversity, the bands originating from vibrational modes of the present proteins were successfully assigned in 1800–1200 cm^{-1} (Raman and IR) region where the interference of bands from other vibrational species is not substantial. The protein bands were attributed to amide I, amide II and amide III modes and their intensities, additionally, enabled to determine the protein secondary structures. The remaining bands up to 4000 cm^{-1} were attributed to other group vibrations whereas the region below 1200 cm^{-1} comprises bands from complex interacting modes within the major RJ components that can not be unequivocally attributed to distinct modes. The work also represents a pioneering effort to collect and interpret the Raman spectrum of fresh and lyophilized RJ samples and to correlate and describe the observed similarities/differences between IR and Raman spectra.

Keywords: royal jelly; infrared; Raman spectra; band assignments; protein degradation

УВИД ВО ИНФРАЦРВЕНИТЕ И РАМАНСКИТЕ СПЕКТРИ НА СВЕЖ И ЛИОФИЛИЗИРАН МАТИЧЕН МЛЕЧ И СТУДИЈА ЗА ПРОТЕИНСКАТА ДЕГРАДАЦИЈА ПРИ ЗАГРЕВАЊЕ СЛЕДЕНА СО ИНФРАЦРВЕНА СПЕКТРОСКОПИЈА

Матичниот млеч (ММ), кој претставува пчелин секрет, има комплексен хемиски состав и содржи: вода, протеини, јаглехидрати, масти, минерални соли и мало количество полифеноли, витамини и ензими. И покрај разновидноста во хемискиот состав, инфрацрвените и раманските ленти кои потекнуваат од присутните протеини беа успешно асигнирани во подрачјето од 1800 до 1200 cm^{-1} . Тоа се должи на фактот што во оваа област интерференцијата, изразена преку преклопување на ленти од другите хемиски ентитети, е незначителна. Протеинските ленти беа припишани на модовите амид I, амид II и амид III, а интензитетите на лентите дополнително овозможуваат определување на секундарните структури на протеините. Другите спектрални ленти регистрирани до 4000 cm^{-1} беа асигнирани на други групови вибрации, додека во областа под 1200 cm^{-1} не може да се направи еднозначна интерпретација на спектрите поради силното преклопување помеѓу вибрационите ленти од многуте присутни хемиски конституенти во ММ. Оваа студија претставува пионерски обид да се добијат и интерпретираат раманските спектри на свеж и лиофилизиран ММ како и да се корелираат и објаснат детектираните спектрални сличности/разлики помеѓу инфрацрвените и раманските спектри.

Клучни зборови: матичен млеч; инфрацрвен; рамански спектар; асигнација на ленти; протеинска деградација

[♦] Dedicated to Academician Gligor Jovanovski on the occasion of his 70th birthday.

1. INTRODUCTION

Royal jelly (RJ) represents the sole food provided to female larvae, destined to become queens, by young workers or nurse bees of the common honey bee species *Apis mellifica* [1]. It keeps queen bees alive for 4 to 5 years whereas the worker bees, feeding on worker jelly, survive for a mere 3–4 weeks.

RJ is characterized by plethora of health beneficial properties showing antimetastatic action [2], antioxidative [3–7], hypoglycemic and immunological activities [8], cholesterol-lowering effects as well as estrogenic [9, 10] and anti-fatigue features [11].

This nourishing product is the secretion of a highly specialized set of hypopharyngeal and mandibular glands located in the head of worker bees aged between 5 and 15 days [12, 13]. It has been found that hypopharyngeal glands produced most of the components, whereas the mandibular glands enrich the RJ with fatty acids [14] of which, the most important active ingredient to fight bacteria, viruses and fungus is unsaturated fatty acid known as 10-hydroxy-2-decenoic acid (10-HDA) [15]. The RJ is homogeneous, creamy, milky-white colored fluid with slightly acid flavor (pH 3.5–4.5) and strong and pungent odor [13].

A numerous investigations dedicated to evaluate the composition of RJ have been carried out aimed to identify the main building components. Chemically speaking, fresh RJ mainly comprises water (60–78%), proteins (9–18%), carbohydrates (7–18%), lipids (3–8%), 10-HDA (>1.4%), mineral salts (0.8–3%) and small amounts of polyphenols, vitamins and enzymes [16, 17]. On one hand, according to the complex composition comprising of major classes of biological macromolecules (proteins, lipids, carbohydrates), RJ is beneficial for human physiological functions but, on the other hand, the complex composition and dominant water content makes it very susceptible to adulteration and prone to crumbling. Adding the relatively high-price to the latter claim, it is of vital importance to provide an effective protocol for RJ temperature storage in order to retain its components unaltered. Beekeepers and apitherapists agree that fresh RJ placed under the tongue is the best way of consuming, whereas pharmaceutical companies suggest use in the form of powder. The method of converting the RJ to powder is called freeze-drying (lyophilization) and such product contains: water (<5%), proteins (27–41%), carbohydrates (21–31%), lipids (8–19%), 10-HDA (>3.5%) [3, 16].

Several studies are aimed to optimize the conditions for temperature storage and time duration before degradation of RJ components takes place. Chen and Chen [18], monitoring the content of carbohydrates, observed increase of glucose and fructose as a function of time, as well as simultaneous decrease of sucrose content due to inversion of the latter compound into specified simple sugars. The lipid class consists primarily of organic acids (80–90%), most of which mono- and dihydroxy acids and dicarboxylic acids with 8 and 10 carbon atoms [13]. The recent work [11] has reported negligible 0.4 to 0.6% reduction in 10-HDA acid in two RJ samples stored at room temperature for 12 months. It also revealed that compounds in this group are prone to conditions of storage and temperature and, although present in large amounts in RJ, are difficult to serve as indicator markers for RJ freshness. Third important major group comprises proteins and amino acids. The major present amino acids are proline, lysine, glutamic acid, β -alanine, phenylalanine, aspartate and serine [19]. No significant changes were observed in the overall concentration of free amino acids in RJ stored at 4 °C for 10 months [19]. However, proline and lysine content in the same samples stored at room temperature showed increase in the first three months and after 6–10 months decreased to levels slightly lower than those in the control samples. This suggests that, at favorable temperature conditions, a proteolytic enzymatic activity continues to occur over time [19]. However, the latest results published recently [20] signified that fresh RJ can be stored after harvest for much shorter period – only seven weeks at 4 °C and up to 21 weeks at –20 °C, whereas storage at room temperature for 3 days keeps protein stability in the product.

Having in mind that protein degradation in fresh and lyophilized RJ as a function of the (gradual) temperature increase is not reported, here, we conducted *in situ* ATR-IR spectroscopy work to elucidate this issue. In addition, the presentation and interpretation of bands in the Raman spectra of RJ has been carried out which, to the best of our knowledge, lacks for both fresh and lyophilized RJ.

2. EXPERIMENTAL SECTION

The fresh RJ was produced by *Apis mellifera macedonica* honey bees and was used immediately after harvesting. Due to its thick and sticky consistency, this sample was termed as fresh-viscous RJ, but when left at room temperature and ambient air for 5 minutes it solidified. The latter sample was denoted as fresh-solidified RJ. On the other

hand, lyophilized RJ was supplied from China as first grade pure lyophilized RJ (10-HDA $\geq 6\%$) in the form of light-yellow fine powder, processed directly from fresh royal jelly.

Attenuated total reflectance infrared (ATR-IR) spectra on fresh and lyophilized RJ were collected on FT-IR PerkinElmer 2000 interferometer using a complete heatable Golden Gate ATR Mk II system (SpecacTM) which consists of optics unit with ZnSe lenses, heatable diamond ATR top-plate, baseplate, and an automatic temperature controller with RS232 control. Sapphire anvil was mounted to the micrometer clamp. The spectra were collected in the 4000–600 cm^{-1} region (resolution 4 cm^{-1} , 16 scans per spectrum) from 20 to 100 $^{\circ}\text{C}$ with 5 $^{\circ}\text{C}$ step increments. The ATR-IR spectra in the 1750–1490 cm^{-1} were deconvoluted using the Gaussian/Lorentzian algorithm in Grams/32 Spectral NotebookTM (version 4.1) software (Galactic Industries Corporation). The ratio of peak area of 1545 and 1645 cm^{-1} bands [20] were used to follow the degradation of the protein structure as a function of the temperature.

The room temperature (20 $^{\circ}\text{C}$) Raman spectra were recorded on micro-Raman multichannel spectrometer Horiba JobinYvon LabRam 300 Infinity. An Olympus MPlanN confocal microscope with $\times 50$ (long distance) objective for magnification was selected. In order to focus the laser beam, a confocal hole of about 2 μm was used and the position on the sample surface was adjusted using motorized x - y stage. The Raman effect was obtained using 632.8 nm line from a He:Ne laser with a power of 1.9 mW. The backscattered radiation (180° configuration) was analyzed with an 1800 lines/mm grating monochromator. Raman intensities were collected with a thermo-electrically cooled CCD array detector. The resolution of the system (“apparatus function”) was 3 cm^{-1} and the wavenumber accuracy was $\pm 1 \text{ cm}^{-1}$ (both calibrated with the Rayleigh line and the 520.5 cm^{-1} line of a Si standard). The acquisition time and the accumulation for each collected spectrum were set to 20 s and 20 scans, respectively.

3. RESULTS AND DISCUSSION

The bands in the ATR-IR spectra of fresh-viscous RJ, fresh-solidified RJ (RJ left at air-conditions for several minutes) and lyophilized RJ (LRJ) occur in two well-separated regions: 3600–2800 cm^{-1} and 1750–950 cm^{-1} (Fig. 1). The higher-wavenumber region could not be used for sample discrimination because all spectra show great similarity. Here, the spectra consist of the strongest,

wide and complex band with the maximum at 3280 cm^{-1} resulting as an overlap of the water stretching vibrations and N–H stretchings from primary and secondary amines and the much weaker bands at 2930 and 2858 cm^{-1} from the C–H stretching vibrations within the $-\text{CH}_2-$ groups and secondary amines [20–22].

The second part of the spectrum from 1750–1200 cm^{-1} is of particular interest for evaluation of the freshness of the RJ sample because of the presence of bands related to the vibrational modes arising from the protein molecular structure [20, 23]. Thus, infrared (and Raman) vibrational spectra can be used to estimate the secondary structure of proteins (which is defined by patterns of hydrogen bonds between backbone amide and carboxyl groups) by inspection of the frequencies at which the amide bonds absorb radiation [24–28].

The most significant features for identification of different protein backbone conformations are amide I (stretching vibration of C=O), amide II and amide III bands (Table 1). The latter two modes are associated with the coupled C–N stretching and N–H bending vibrations of the peptide bonds [29–32].

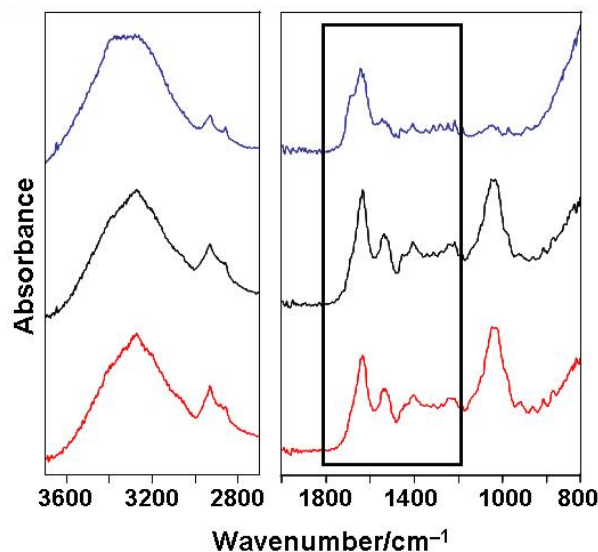


Fig. 1. ATR-IR spectra of fresh-viscous RJ (top), fresh-solidified RJ (middle) and lyophilized RJ (bottom spectra). The protein bands are marked in the black rectangular.

The strongest bands occur in the 1700–1600 cm^{-1} region and are ascribed to amide I modes (Table 1) of the proteins present in the RJ [20]. Here, the spectrum of fresh-viscous RJ shows distinct differences from the fresh-solidified and lyophilized RJ spectra (Fig. 1). The spectral discrepancy is manifested by the appearance of weak band around 1690 cm^{-1} (which either becomes a shoul-

der in the solidified RJ sample or disappears in the spectrum of LRJ sample). Furthermore, latter two samples exhibit sole band at 1640 cm^{-1} that, in the spectrum of the fresh-viscous RJ sample, is split into two components at 1646 and 1632 cm^{-1} (Fig. 1, Table 1). Although the IR spectra of RJ samples stored at different temperatures are reported [20, 23], such differences were neither registered nor discussed (Table 1). In the attempt to explain the spectral disparity in this region we would draw attention that such behavior is connected with the percentage variations among the α -helical (spiral) conformation and β -sheets which describe the protein secondary structure [31]. Thus, the appearance

of the band around 1690 cm^{-1} in the spectrum of fresh-viscous RJ implied larger percentage of β -sheets being lower in the fresh-solidified RJ and LRJ where the percentage of α -helices is higher (Fig. 1 and Table 1). Namely, human serum albumine and bovine protein studies observed a progressive decrease of main contribution at 1650 cm^{-1} and the progressive increase of the two shoulders at 1620 and 1680 cm^{-1} , typically attributed to intermolecular β -sheet structures [29–31]. The growth of these shoulders is correlated to the partial loss of the native α -helix secondary structure suggesting a transition between these two secondary structures.

Table 1

Characteristic IR bands in RJ arising from the protein secondary structure. The assignment is carried out in accordance with the literature for corresponding IR bands in various protein sources.

Fresh-viscous RJ	Fresh-solidified RJ and LRJ	Ref. [20]	Ref. [23]	Ref. [29]	Ref. [30]	Ref. [31]
1693 w ^a	1692 sh ^b	1692			1690 (amide I) β -turn	1660–1700 (amide I) β -turn
1646 vs	1640 vs	1640	1647	1655 (amide I)	1650 (amide I) α -helix structure	1650–1658 (amide I) α -helix structure
1632 w	–	1622	–	1610 (amide I)	1610 (amide I) β -helix structure	1610–1640 (amide I) β -sheet
1542 w	1542 m	1545	1541	1540 (amide II)	1540 (amide II)	1548 (amide II)
1460 w	1460 w	~1460	–	–	~1455 (amide II)	
1410 w	1410 w	~1410	1409	–	~1410 (amide II)	
1318 w	1318 vw	–	–	1330 (amide III)		
1286 w	1286 vw	–	–	1280 (amide III)		
1251 w	1251 vw	–	1247	1254 (amide III)		
1219 w	1219 w	–	–			
1187 w	1187 w	–	–			
1048 w	1048 vs	1048 vs	1054			
978 w	978 sh					

^aIntensity codes: w – weak, m – medium, s – strong, v – very, sh – shoulder.

^bAbsent in the spectrum of LRJ.

Going towards lower wavenumbers, the bands in the 1600 – 1400 cm^{-1} arise from the amide II bands [29]. In this region, the spectra depict much similar pattern confirming that these bands are less susceptible to treatment of RJ (Fig. 1). An exception is the 1540 cm^{-1} band which intensity increases with aging of the RJ sample being the weakest in the spectrum of fresh-viscous RJ sample. The decrease of amide I band at approximately 1650 cm^{-1} and the increase of amide II band at approximately 1450 cm^{-1} are strictly related, suggesting that an exchange occur in our sample, indicative of a conformational changes at a tertiary structural level. Therefore, the intensity ratio between the strongest amide I band at 1650 cm^{-1} and the 1540 cm^{-1} band from amide II vibrations is used to determine the protein degradation as a function of

the temperature for both fresh and lyophilized RJ sample (see forthcoming discussion).

The bands in the 1400 – 1200 cm^{-1} region are ascribed to amide III modes and show the lowest degree of difference among the presented spectra (Fig. 1). The most significant disparity between the IR spectra of fresh-viscous RJ is the strong intensity of the band around 1050 cm^{-1} being considerably weaker in the spectra of solidified RJ and LRJ. The origin of the band is likely to be connected to C–O stretchings arising from oxidation of the initial fresh sample left on air atmosphere. One probable suggestion is that ambient conditions trigger oxidation of the present carbohydrates in the RJ, i.e. oxidation of the free aldehyde carbonyl group (in simple sugars) to C–O group (in alcohols) takes place.

The reason for absence of the Raman spectrum of royal jelly in the literature is probably due to its weak scattering character making its collection a complex and elusive task. However, after careful adjustment of the instrumental conditions (given in the Experimental section), representative Raman spectra were collected for fresh-viscous RJ, fresh-solidified RJ and LRJ (Fig. 2). Here, an attempt was made to conduct tentative band assignment (Table 2) in accordance with the IR discussion. The Raman band around 3285 cm^{-1} arising from the overlap of the water stretching vibrations and N–H stretchings from primary and secondary amines is significantly lower in comparison with the IR analogues which is expected having in mind that water molecules are very weak scatterers. The remaining bands at 2930 , 2895 and 2855 cm^{-1} appear from the C–H stretching vibrations within the $-\text{CH}_2-$ groups and secondary amines (the corresponding IR bands are reported in Ref. [20–22]), but the absence of the latter band in the solidified and LRJ spectra suggested that it probably appears from the aldehyde C–H stretching vibration (Fig. 2, right panel). Namely, when fresh-viscous RJ is left on ambient conditions, oxidation of the free aldehyde carbonyl group (from the simple sugars) occurred diminishing the corresponding C–H stretching band.

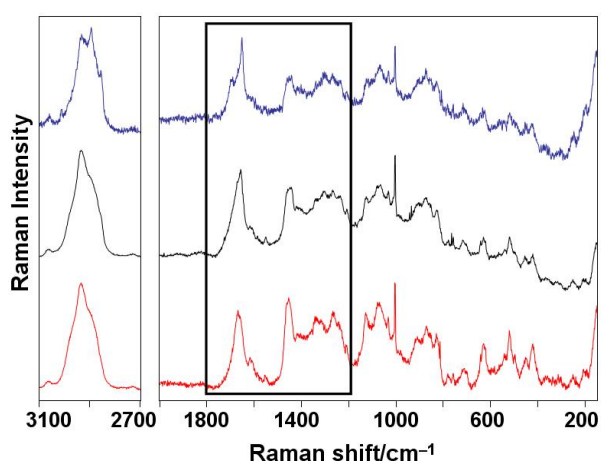


Fig. 2. Raman spectra of fresh-viscous RJ (top), fresh-solidified RJ (middle) and lyophilized RJ (bottom spectra). The protein bands are marked in the black rectangular.

The only difference between the Raman spectrum of fresh-viscous RJ and the remaining two spectra (Fig. 2, Table 2) is reflected in the appearance of weak amide I band at 1695 cm^{-1} . Its presence implies larger percentage of β -sheets (in the secondary structure of the proteins) being low-

er in the fresh-solidified RJ and LRJ where the percentage of α -helices is higher. The strongest band at $1650\text{--}1660\text{ cm}^{-1}$ and much weaker absorption around 1610 cm^{-1} originate from amide I modes representing dominant presence of α -helices over the β -helix secondary structure of proteins (Table 2 and Fig. 2). The assignment of the bands at 1552 , 1454 and 1414 cm^{-1} , as well as the bands at 1340 , 1303 and 1269 cm^{-1} , is made according to the IR considerations and the bands are ascribed to amide II and amide III modes, respectively (Table 2). It is obvious that the treatment of RJ sample does not involve changes in the Raman spectra, and therefore these bands can not be considered for discriminative purposes in the protein degradation studies.

The region below 1200 cm^{-1} presents band absorptions that can not be unequivocally attributed to any distinct vibrations because they correspond to complex interacting vibrational modes within the major components present in the RJ.

Table 2

Characteristic Raman bands in RJ arising from the secondary structure of proteins and their tentative assignment

Fresh-viscous RJ	Fresh-solidified RJ and LRJ	Assignment
1695 w^a		Amide I, β -turn
1651 s	1660 s	Amide I, α -helix structure
1610 vw	1611 vw	Amide I, β -helix structure
1552 vw	1552 w	Amide II
1454 m	1454 s	Amide II
1414 vw	1416 vw	Amide II
1340 vw	1342 vw	Amide III
1303 w	1303 vw	Amide III
1269 w	1268 vw	Amide III
1236 w	1236 w	
1209 vw	1209 vw	
1126 vw	1125 w	
1068 w	1067 vw	
1033 vw	1033 vw	
1004 vw, sh	1002 vw	

^aIntensity codes: w – weak, m – medium, s – strong, v – very, sh – shoulder.

As reported above, the intensity ratio (R) between the amide II band at 1540 cm^{-1} and the 1640 cm^{-1} band from amide I vibrations [$R = \text{Area}(1545\text{ cm}^{-1})/\text{Area}(1640\text{ cm}^{-1})$], from the ATR-IR spectral region (Fig. 3) is used to determine the protein degradation as a function of the temperature for both fresh and lyophilized RJ samples. Similar study on protein degradation during storage of fresh

RJ sample at temperatures of -20 , $+4$ and $+25$ °C showed maintenance of the protein stability up to 21 weeks, seven, and three days, respectively [20].

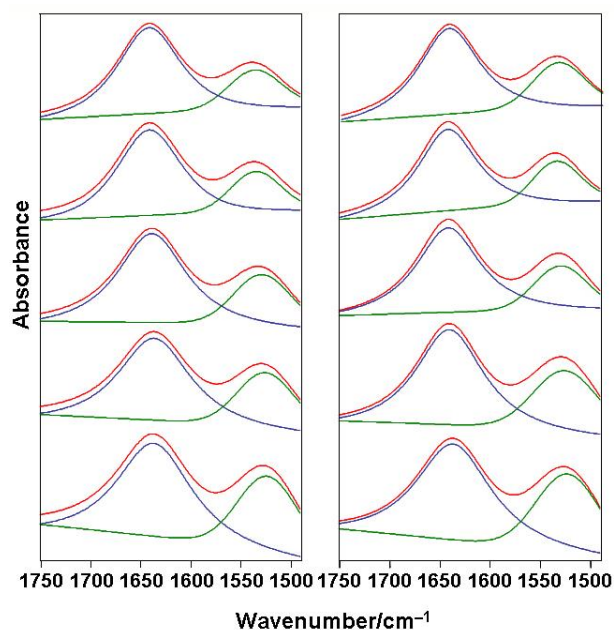


Fig. 3. Fitted ATR-IR spectra ($1750\text{--}1490\text{ cm}^{-1}$ region) of fresh RJ (left panel) and lyophilized RJ (right panel) on selected temperatures: $+20$, $+40$, $+60$, $+80$, and $+100$ °C (top-down presentation)

Table 3 presents the calculated ratio of the above areas for fresh and lyophilized RJ plotted as a function of the temperature. As seen, the ratio for fresh RJ increases from 0.266 ± 0.003 (20 °C) to 0.359 ± 0.002 (at 100 °C) whereas similar trend was obtained for LRJ sample starting from 0.288 ± 0.002 (20 °C) to 0.403 ± 0.001 (at 100 °C). The overall increase of the band area of the ascertained amide II band is 34.96% in the fresh RJ series whereas the lyophilized RJ series showed somewhat higher percentage rise (39.59%).

Additional similarity among the two series is reflected in the increase of the ratio obtained above 30 °C, showing slow, but notable rise that lasts until $65\text{--}70$ °C after which the ratio increased more dramatically. Fig. 4 depicts graphical dependence of ratio against temperature (T) fitted, using polynomial function of degree 4 (drawn using OriginPro®8, v8.0951). The corresponding equation is $R = -2 \cdot 10^{-8}T^4 + 4 \cdot 10^{-6}T^3 - 0.0003T^2 + 0.0104T + 0.1497$ ($R^2 = 0.9739$) for fresh RJ and $R = -3 \cdot 10^{-8}T^4 + 6 \cdot 10^{-6}T^3 - 0.0005T^2 + 0.0149T + 0.124$ ($R^2 = 0.9743$) for lyophilized RJ sample. Thus, one can conclude that, from protein non-disintegration point of view, safe temperatures are those below 30 °C.

Table 3

Ratio (R) of the 1545 cm^{-1} band area and 1640 cm^{-1} band area (indicative for protein degradation) obtained from in situ temperature-dependent ATR-IR spectra of fresh and lyophilized RJ

Temp. °C	R for fresh-RJ	R for LRJ	Temp. °C	R for fresh-RJ	R for LRJ
20	0.266	0.288	65	0.295	0.328
25	0.265	0.289	70	0.296	0.367
30	0.264	0.290	75	0.318	0.379
35	0.277	0.309	80	0.323	0.395
40	0.280	0.309	85	0.349	0.403
45	0.279	0.319	90	0.359	0.405
50	0.277	0.321	95	0.358	0.403
55	0.290	0.319	100	0.359	0.402
60	0.293	0.326			

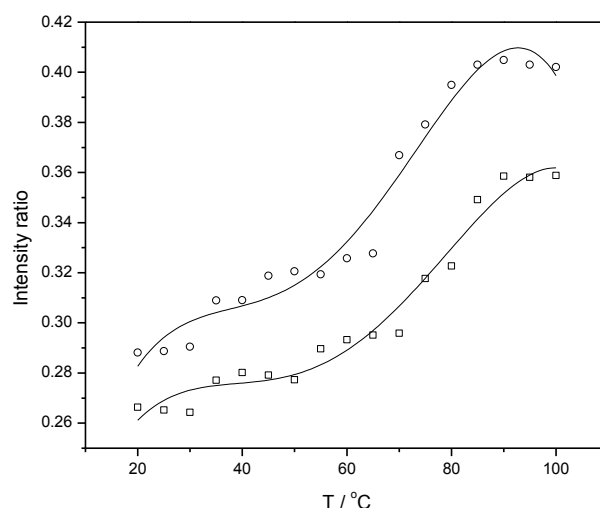


Fig. 4. Change of the ratio ($1540\text{ cm}^{-1}/1640\text{ cm}^{-1}$ band area) and added trend line as a function of the temperature (squares – fresh RJ series, circles – lyophilized RJ series)

4. CONCLUSIONS

The conducted temperature-induced ATR-IR study revealed that temperature increase speed up protein degradation, manifested by the increase of the ratio between the intensity of amide II (1542 cm^{-1}) and amide I (1640 cm^{-1}) band. ATR-IR spectra collected by gradient temperature increase from RT to 100 °C (step 5 °C) have shown that protein degradation in both fresh and lyophilized RJ samples starts around 30 °C being significantly accelerated around $65\text{--}70$ °C. Additionally, we succeeded in collecting the Raman spectra of pure fresh and lyophilized RJ samples (not found in the literature) and, as in the case of ATR-IR spectra, tentatively assigned the bands from 4000 to 1200 cm^{-1} . Therefore, Raman and ATR-IR spectroscopy are

considered as complementary and powerful techniques for screening of the molecular structure of the proteins present in RJ.

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