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# Gas chromatographic–mass spectrometric investigation of volatile and extractable compounds of crude royal jelly

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ABSTRACT

Using headspace solid-phase microextraction (HS-SPME) followed by diethyl ether and methanol extraction, it was possible to isolate as many as 185 organic compounds out of 17 samples of crude royal jelly (RJ). Of the above compound number, 169 compounds were positively identified by means of gas chromatography–mass spectrometry. The volatile fraction of RJ consists of 25 different compounds where approximately 47% of the total ion current (TIC) of volatile compound chromatograms were composed of substances characterized by bactericidal (phenols) and repelling (octanoic acid and 2-heptanone) activities. Preliminary investigations have shown that RJ stored for 10 months at −18 °C and 4 °C keeps its composition of volatile compounds unchanged, however, at the same time at room temperature RJ phenol contents is decreased twice, whereas the fraction of aliphatic acids is increased 2.8 times due to the presence of both acetic and butyric acids. The chromatogram of RJ ether extracts showed 85 different compounds, however about 88% of TIC consisted exclusively of 8 compounds, i.e. 10-hydroxy-2-decenoic, 10-hydroxydecanoic, 3,10-dihydroxydecanoic, 8-hydroxyoctanoic, 2-decene-1,10-dioc and (Z)-9-hydroxy-2-decenoic acids. Nine aliphatic acids, which were detected for the first time, are the homologues of hydroxy- and oxo-acids identified earlier in RJ. In the RJ methanol extracts 82 compounds were identified, mainly carbohydrates and their derivatives. Approximately 87% of TIC consisted of fructose, glucose and sucrose. Special attention was paid to discrepancies between obtained and literature data concerning the presence of free amino acids in RJ. It was suggested that these inconsistencies can be explained by the differences in the methods of RJ collection and/or sample preparation.


## 1. Introduction

Royal jelly (RJ) is a slightly acidic (pH 3.5–4.5) secretion from the hypopharyngeal and mandibular glands of young worker bees (*Apis mellifera* L.) and is used to feed queen larvae [1]. In the colony of honeybees, RJ is fed temporarily (for 3 days) to the brood of workers and drones, but it is the only food of the honeybee queen throughout its life span. For commercial purposes, it is collected from queen cells because these are the only cells where relatively large amounts (up to 500–600 mg) of RJ are deposited: the cells of queen larvae are "stocked" with RJ much faster than the larvae can consume it. A well-managed hive can produce approximately 500 g of royal jelly during summer season.

RJ is characterized by its antimetastatic action [2], antioxidative activity [3–7], as well as hypoglycemic and immunological activities [8], cholesterol-lowering effects, estrogenic [9,10] and anti-fatigue [11] properties. Therefore, in many countries, RJ has become a commercial product and is sold either as pure lyophilized jelly or in the form of products containing some amounts of crude royal jelly supplemented with other ingredients.

A unique feature of RJ is that it is composed of a set of short-chained hydroxy fatty acids [12,13]. It was Lercker [13] who originally discovered that major RJ fatty acids consist of 10-hydroxy-2-decenoic (10-HDA) and 10-hydroxydecanoic acids (10-HDDA) constituting between them at least 60–80% of the total amount of organic acids found in RJ. 10-HDA is known to possess various pharmacological effects, including antibiotic activity [14,15] and antitumoral action [16–18]. It has also been reported to stimulate the growth of glial and neural stem cells in the brain [19,20].

There have been numerous investigations dedicated to the composition of RJ resulting in the identification of its main components. Chemically, fresh royal jelly comprises water (50–65%), proteins (11–18%), carbohydrates (10–15%), fatty acids and lipids (4–8%), mineral salts (ca. 1.5%), and small amounts of polyphenols and vitamins. The lyophilized product contains 33–42% of proteins, 22–31% of carbohydrates and 15–30% of fats [3]. To know the composition of RJ is essential for the evaluation of its freshness and

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genuineness, as well as the quality of commercial products [21]. Royal jelly has been studied by several authors [8–15,22–28] however, on the whole, the composition of the volatile and extractive compounds has not been investigated sufficiently up to now.

In our previous work [29] 35 aliphatic acids were identified from the ether extract of commercial preparations containing lyophilized royal jelly. These acids were characterized by their GC retention parameters and mass spectra. Among them, 9 acids were reported for the first time as natural products. The main purpose of the present study was to investigate the composition of volatile and extractable components of freshly harvested RJ. In order to accomplish the task high-performance capillary gas chromatography coupled with mass spectrometry (GC–MS) was used. The isolation of analytes from complex matrix was carried out by making use of HS-SPME (volatile compounds), as well as successive extraction of RJ with solvents of different polarity. As there are some inconsistencies in the results concerning the contents of free amino acids in royal jelly described in the literature [24,28] and those obtained in our research [29], we paid particular attention to possible causes of the discrepancy.

## 2. Experimental

### 2.1. Materials

Pyridine and bis(trimethylsilyl)trifluoroacetamide (BSTFA) with addition of 1% trimethylchlorosilane were purchased from Sigma–Aldrich (Poznań, Poland). Extractions were carried out by diethyl ether and methanol (POCH SA, Gliwice, Poland). SPME Holder 57330-U with fused silica fibers with different stationary phases were purchased from Supelco Inc. (Bellefonte, PA, USA).

The 17 samples of royal jelly used in our experiments came from two *Apis mellifera carnica* Poll. colonies maintained in Białystok region (north-eastern Poland) and two other colonies maintained in Małopolska region (south-western Poland). RJ was gathered in June–July 2009 and 2010 from queen cells containing young larvae (less than 5 days of larval life). The queen cells were transported into laboratory for direct treatment and analysis described as follows.

One of the RJ samples from Małopolska region was divided in 5 aliquot parts (2 g for aliquot). Each aliquot was placed in a glass vial of 16 mL in volume and sealed. The samples from two vials were used without delay to determine volatile and extractive compounds. One of the remaining vials was stored at −18 °C. The fourth and fifth vials were stored at 4 °C and room temperature (in the dark), respectively.

### 2.2. Sample preparation and analysis

#### 2.2.1. HS-SPME determination of volatiles from RJ

Freshly harvested RJ (1.5–2.0 g) was transferred into a headspace vial of 16 mL in volume and immersed into temperature-controlled water bath (40 °C). The septum of screw-cap was picked by the needle protecting the SPME fiber, and the fiber coating was exposed to a headspace gas phase for 50 min. The volatiles collected on the fiber were desorbed by introducing SPME fiber for 10 min into the injection port of the GC–MS apparatus. Analytes were separated and analyzed by GC–MS on a HP 6890 gas chromatograph with mass selective detector MSD 5973 (Agilent Technologies, USA). This device was fitted with a HP-5MS fused silica column (30 m × 0.25 mm i.d., 0.25 μm film thickness), with electronic pressure control and split/splitless injector. Helium flow rate through the column was 1 mL/min in constant flow mode. The injector (250 °C) worked in splitless mode. The initial column temperature was 40 °C rising to 220 °C at 3 °C/min. The MSD 5973 detector acquisition parameters were as follows: transfer line temperature equaled 250 °C and the detector was held at 270 °C. The electron-ionization mass spectra were obtained at 70 eV of ionization energy. Detection was performed in a full scan mode from 29 to 600 a.m.u. After integration, the fraction of each component in the total ion current was calculated.

To determine of the retention times of reference compounds and to calculate linear temperature programmed retention indices (LTPRI) of RJ volatiles, a SPME fiber was inserted for 2–3 s into the vial with mixture of $C_5$–$C_{18}$ *n*-alkanes. The separation of alkanes was performed under the above conditions.

#### 2.2.2. Determination of extractable compounds from RJ and queen bee larvae

500–700 mg of RJ was transferred into a retort of 25 mL in volume and extracted for 15 min, constantly stirred, by three portions of 10 mL of diethyl ether. Next, the non-soluble material was extracted by three portions of 10 mL of methanol. The duration of each extraction cycle at room temperature was 15 min. The joint extracts were filtered through a paper filter and the solvent was removed on a rotor evaporator at 50 °C. After the mass of oil-like residue left on the walls was determined, it was washed out by 2 mL of diethyl ether or methanol, and 0.5 mL of this solution was put into a vial of 2 mL in volume. After evaporation of the solvent, 220 μL of pyridine and 80 μL of BSTFA were added into the vial. The reaction mixture was sealed and heated during 0.5 h at 60 °C to obtain trimethylsilyl (TMS) derivatives.

Carefully extracted from queen cells, 3 larvae (less than 5 days of larval life) were washed twice with methanol and then thoroughly homogenized in a fresh portion (2 mL) of the solvent. After filtration through a paper filter, methanol was removed and dry residue was silanized as above.

Obtained solutions of TMS derivatives were separated and analyzed with the aid of the GC–MS apparatus and capillary column HP-5MS. Injection of 1 μL of the sample was performed with the aid of HP 7673 autosampler. The injector (250 °C) worked in splitless mode for 5 min. The initial column temperature was 50 °C rising to 300 °C at 5 °C/min. Detection was performed in the full scan mode from 41 to 600 a.m.u.

Hexane solution of $C_{10}$–$C_{40}$ *n*-alkanes were separated under the above conditions. Retention indices were calculated from the results of the separation of this solution and RJ extracts.

For identification mass spectral data and gas chromatographic retention parameters were used. Registered mass spectra and LTPRI values were compared with literature data [30–32] and with our previously published data [29,33].

## 3. Results and discussion

In this investigation, the sample procedure includes head-space SPME (HS-SPME) concentration of volatile compounds (VOCs) and successive extraction of RJ with diethyl ether and methanol.

### 3.1. Volatile compounds of RJ

Before the analysis, preliminary studies were performed to select of SPME fiber stationary phase. The types of fiber coating examined were as follows: polydimethylsiloxane 100 μm (PDMS 100), carboxen/polydimethylsiloxane 85 μm (Car/PDMS), and divinylbenzene/carboxen/polydimethylsiloxane 50/30 μm (DVB/CAR/PDMS). The comparison of fiber extraction efficiency for RJ volatiles was performed at extraction time of 50 min, and extraction temperature of 40 °C. According to the obtained results, the best effectiveness of extraction–desorption cycle was obtained by the DVB/CAR/PDMS fiber. The PDMS 100 fiber sorbs well from gas phase relatively high boiling compounds.

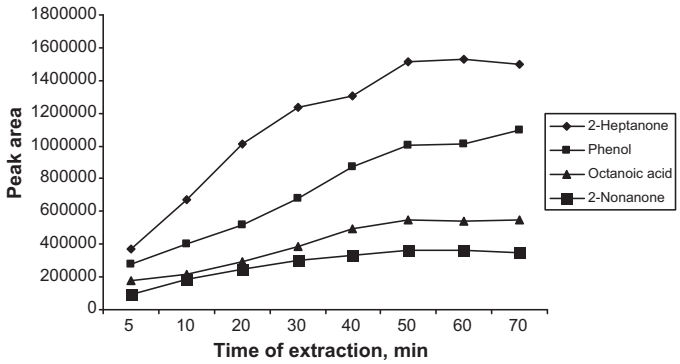


**Fig. 1.** Effect of exposition time on the extraction efficiency some of the principal RJ volatiles with the DVB/CAR/PDMS fiber at 40 °C.

Nevertheless, the chromatograms demonstrated hardly any peaks of compounds with low boiling temperature. Lower alcohols, carbonyl compounds and esters were registered with the use of fiber CAR/PDMS. On the other hand, this fiber does not completely return (during desorption stage) of the components with high boiling temperature.

For the selected DVB/CAR/PDMS fiber was determined the extraction time profile for volatile analytes of RJ. Fig. 1 shows this profile for the four principal volatiles. The results demonstrated that an extraction time of 50 min was sufficient to reach equilibrium at 40 °C.

To the best of our knowledge the composition of volatile RJ parts has not been fully investigated. These data, however, are not only essential from the scientific point of view but they are also important for practical purposes in order to easily and efficiently assess the freshness and authenticity of this commercial product.

Table 1 presents a list of 25 volatile organic compounds determined by HS-SPME/GC–MS technique in two samples of freshly harvested RJ. Additionally, in order to assess of possible changes in the VOCs composition, one of these samples was stored for 10 month at room temperature as well as at 4 °C and −18 °C. Sample 1 was harvested in August of 2009 and came from a bee colony maintained in Małopolska region. Sample 2 was harvested in Białystok region almost 1 year later (July 2010).

In spite of the differences concerning the time and sampling site of the two fresh RJ samples, their VOCs composition was very similar. Among the identified compounds the most abundant ones in fresh RJ were carbonyls: 2-heptanone, acetone, 2-nonanone and benzaldehyde. The first one, which is produced by bees' mandibular glands [34], shows a particularly high content and acts as a mild anxiety pheromone in the guard and foraging bees [35]. This compound has also a repellent effect and it was supposed that it is used to deter potential enemies. The repelling activity of 2-heptanone in RJ from queen cells can be directed against a dangerous ectoparasite of honeybees *Apis mellifera* L., the mite *Varroa destructor* Anderson & Trueman [36]. It can also enhance a repellent action of octanoic acid, whose relative content in the volatile RJ emissions amounts to 7% (Table 1). According to the laboratory and field studies [27], octanoic acid prevents invasion of queen cells by the mite.Another significant group (27–28%) of VOCs found in fresh RJ are both volatile phenolic compounds (phenol, *o*-guiacol and methyl salicylate) as well as benzoic acid and its methyl ester. As it is well known, all these compounds have clear antibacterial properties. Thus we can conclude that the main part of volatile organic compounds found in fresh RJ (ca. 47% of TIC) are components which are protective in nature whose activities are directed against parasites as well as microorganisms.RJ antibacterial properties protect jelly against its rapid quality deterioration, nonetheless its quality will be spoilt by improper storage [3,11]. The data showing the changes in the composition of VOCs in RJ during storage at different

**Table 1**
Relative composition (%) of volatile compounds in samples of fresh RJ and their changes during storage at different temperature.

| Compound | LTPRI | Fresh RJ | | Sample 1 after 10 months of storage | | |
|---|---|---|---|---|---|---|
| | | Sample 1 | Sample 2 | −18 °C | 4 °C | r.t.[a] |
| Ethanol | <500 | 5.4 | 5.1 | 5.2 | 5.4 | 5.7 |
| Acetone | 500 | 6.2 | 5.3 | 6.4 | 6.0 | 5.9 |
| Methyl acetate | 510 | 0.2 | Trace[b] | 0.3 | 0.3 | 2.9 |
| 2-Butanone | 591 | 1.6 | 1.4 | 1.6 | 1.5 | 1.6 |
| Ethyl acetate | 604 | 2.7 | 0.4 | 2.4 | 2.9 | 4.7 |
| Acetic acid | 647 | Trace | n.r[c] | n.r. | 0.2 | 6.9 |
| 3-Methylbutanal | 656 | n.r. | n.r. | n.r. | n.r. | 0.8 |
| 2-Methylbutanal | 658 | Trace | Trace | Trace | Trace | 1.1 |
| 2-Pentanone | 688 | 1.4 | 1.1 | 1.4 | 1.3 | 1.9 |
| 2-Methyl-2-butenal | 758 | n.r. | n.r. | n.r. | Trace | 1.6 |
| Toluene | 763 | 1.6 | 0.7 | 1.6 | 1.6 | 1.5 |
| Butyric acid | 800 | n.r. | n.r. | n.r. | Trace | 4.6 |
| Hexanal | 804 | 0.4 | 0.5 | 0.5 | 0.5 | 1.1 |
| 2-Heptanone | 890 | 19.8 | 20.0 | 20.1 | 20.2 | 21.7 |
| Benzaldehyde | 959 | 4.2 | 4.1 | 4.1 | 4.0 | 2.5 |
| Phenol | 985 | 12.0 | 11.1 | 11.9 | 11.5 | 7.2 |
| 2-Methoxyphenol (*o*-guaiacol) | 1089 | 1.1 | 1.6 | 1.2 | 1.3 | 0.9 |
| 2-Nonanone | 1091 | 5.0 | 4.8 | 4.9 | 5.0 | 5.3 |
| Methyl benzoate | 1094 | 3.9 | 4.5 | 3.9 | 4.0 | 4.2 |
| Octanoic acid | 1178 | 6.9 | 7.4 | 6.7 | 6.8 | 7.2 |
| Benzoic acid | 1180 | 2.8 | 3.6 | 2.8 | 2.5 | n.r. |
| 2-Methoxy-*p*-cresol | 1191 | 0.3 | Trace | 0.4 | 0.1 | n.r. |
| Methyl salicylate | 1193 | 6.9 | 7.2 | 7.1 | 6.6 | 2.5 |
| NN (*m*/*z* 136,93,121,77) | 1305 | 0.7 | Trace | 0.7 | 0.8 | 0.9 |
| 1-Pentadecene | 1491 | 16.9 | 21.2 | 16.8 | 20.0 | 7.3 |
| Carbonyls | | 38.6 | 37.2 | 39.0 | 38.5 | 43.3 |
| Phenols | | 20.3 | 19.9 | 20.6 | 19.5 | 10.6 |
| Aliphatic acids | | 6.9 | 7.4 | 6.7 | 7.0 | 19.5 |

[a] Room temperature.
[b] Trace – below 0.01% of TIC.
[c] Not registered.

**Table 2**
Composition of ether extracts from RJ samples.

| Compound, TMS | RI | N[a] | Relative composition (%) | | |
|---|---|---|---|---|---|
| | | | Min | Max | Median (*n*)[b] |
| 2,3-Butanediol, *levo*- | 1046 | 3 | 0.01 | 0.1 | – |
| 2,3-Butanediol, *meso*- | 1054 | 3 | Trace[c] | 0.1 | – |
| *Lactic acid* | 1074 | 17 | 0.01 | 0.1 | 0.08 ± 0.03 (17) |
| Benzoic acid | 1250 | 6 | Trace | 0.06 | – |
| Octanoic acid | 1270 | 17 | Trace | 0.1 | 0.05 ± 0.03 (15) |
| Nicotinic acid | 1295 | 3 | Trace | 0.05 | – |
| Glycerol | 1297 | 17 | Trace | 0.05 | 0.03 ± 0.01 (14) |
| *Succinic acid* | 1325 | 15 | 0.01 | 0.1 | 0.07 ± 0.05 (15) |
| Pyrocatechol | 1330 | 3 | Trace | – | – |
| Hydroquinone | 1409 | 11 | Trace | 0.03 | 0.02 ± 0.01 (9) |
| *2-Hydroxyoctanoic acid* | 1470 | 16 | 0.02 | 0.1 | 0.07 ± 0.02 (16) |
| *7-Oxooctanoic acid* | 1475 | 11 | Trace | 0.1 | 0.06 ± 0.04 (6) |
| 3-Hydroxyoctanoic acid | 1490 | 17 | 0.01 | 0.4 | 0.2 ± 0.1 (17) |
| 4-Hydroxybenzoic acid, methyl ester (HOB) | 1494 | 9 | Trace | 0.05 | – |
| NN (*m*/*z* 293,73,251,252,237,75) | 1514 | 10 | 0.02 | 0.1 | 0.05 ± 0.04 (10) |
| 7-Hydroxyoctanoic acid | 1558 | 17 | 0.05 | 0.9 | 0.6 ± 0.4 (17) |
| 7-Hydroxy-2-octenoic acid | 1608 | 8 | Trace | 0.02 | – |
| *3-Methyl-3-hydroxyglutaric acid* | 1624 | 5 | Trace | 0.03 | – |
| 8-Hydroxyoctanoic acid | 1630 | 17 | 3.1 | 6.5 | 4.9 ± 1.0 (17) |
| 4-Hydroxybenzoic acid | 1634 | 17 | 0.01 | 0.3 | 0.2 ± 0.1 (17) |
| 3-Hydroxydecanoic acid, 3-HDA | 1670 | 17 | 0.9 | 1.5 | 1.2 ± 0.2 (17) |
| 8-Hydroxy-2-octenoic acid | 1680 | 17 | Trace | 0.1 | 0.07 ± 0.04 (11) |
| Suberic acid | 1710 | 17 | Trace | 0.1 | 0.06 ± 0.04 (8) |
| *9-Oxo-2-decenoic acid (9-ODA)* | 1734 | 4 | Trace | 0.03 | – |
| 9-Hydroxydecanoic acid | 1752 | 17 | 0.2 | 0.5 | 0.3 ± 0.1 (17) |
| 2-Octene-1,8-dioic acid | 1760 | 17 | Trace | 0.1 | 0.06 ± 0.04 (8) |
| 4-Hydroxyhydrocinnamic acid | 1766 | 6 | Trace | 0.01 | – |
| 4-Hydroxy-3-methoxyphenylethanol, HVA | 1781 | 6 | Trace | 0.02 | – |
| 8-Hydroxy-2-decenoic acid | 1788 | 7 | Trace | 0.02 | – |
| (Z)-9-Hydroxy-2-decenoic acid, (Z)-9-HDA | 1803 | 17 | 1.2 | 2.5 | 1.7 ± 0.4 (17) |
| *(E)-9-Hydroxy-2-decenoic acid, (E)-9-HDA* | 1819 | 8 | Trace | 0.02 | – |
| 10-Hydroxydecanoic acid, 10-HDAA | 1827 | 17 | 13.0 | 17.6 | 15.9 ± 1.4 (17) |
| NN (*m*/*z* 73,289,147,199,81,95) | 1837 | 4 | Trace | 0.01 | – |
| α-Fructofuranose | 1845 | 17 | Trace | 0.1 | 0.07 ± 0.04 (10) |
| β-Fructofuranose | 1855 | 17 | Trace | 0.3 | 0.20 ± 0.07 (12) |
| (E)-10-Hydroxy-2-decenoic acid, 10-HDA | 1883 | 17 | 50.3 | 66.7 | 54.8 ± 14.5 (17) |
| Sebacic acid | 1905 | 17 | 2.5 | 4.1 | 3.3 ± 0.6 (17) |
| α-Glucopyranose | 1932 | 12 | Trace | 0.7 | 0.4 ± 0.2 (8) |
| 10-Hydroxydodecanoic | 1932 | 17 | 0.1 | 0.6 | 0.3 ± 0.2 (17) |
| 3,9-Dihydroxydecanoic acid | 1940 | 17 | 0.1 | 0.4 | 0.2 ± 0.1 (17) |
| 11-Hydroxydodecanoic acid | 1945 | 17 | 0.3 | 0.8 | 0.5 ± 0.2 (17) |
| *p*-Coumaric acid | 1947 | 7 | n.r.[d] | Trace | – |
| 2-Decene-1,10-dioic acid | 1959 | 17 | 3.1 | 5.6 | 4.1 ± 0.8 (17) |
| 1-Hexadecanol | 1963 | 4 | Trace | 0.06 | – |
| 10-Hydroxy-2-dodecenoic acid | 1982 | 17 | Trace | 0.1 | 0.05 ± 0.03 (11) |
| 11-Hydroxy-2-dodecenoic acid | 1997 | 17 | Trace | 0.3 | 0.10 ± 0.08 (14) |
| 3,10-Dihydroxydecanoic acid, 3,10-DDA | 2014 | 17 | 4.4 | 7.8 | 5.9 ± 1.3 (17) |
| 12-Hydroxydodecanoic acid | 2016 | 17 | 0.1 | 0.4 | 0.3 ± 0.1 (17) |
| 8,9-Dihydroxydecanoic acid | 2026 | 17 | Trace | 0.2 | 0.14 ± 0.06 (15) |
| β-Glucopyranose | 2034 | 15 | n.r. | 0.2 | 0.09 ± 0.01 (12) |
| Hexadecanoic acid | 2050 | 17 | Trace | 0.1 | 0.03 ± 0.01 (14) |
| 12-Hydroxy-2-dodecenoic acid | 2069 | 17 | Trace | 0.2 | 0.09 ± 0.05 (13) |
| 9,10-Dihydroxy-2-decenoic acid | 2073 | 17 | 0.03 | 0.5 | 0.22 ± 0.16 (17) |
| 3-Hydroxydecanedioic acid | 2091 | 14 | 0.01 | 0.1 | 0.06 ± 0.04 (14) |
| 1,12-Dodecanedioic acid | 2100 | 17 | 0.01 | 0.1 | 0.07 ± 0.03 (17) |
| 3,10-Dihydroxydodecanoic acid | 2112 | 17 | Trace | 0.03 | 0.02 ± 0.01 (7) |
| 3,11-Dihydroxydodecanoic acid | 2127 | 17 | 0.1 | 0.4 | 0.2 ± 0.1 (17) |
| *9,10-Dihydroxydodecanoic acid* | 2135 | 17 | Trace | 0.1 | 0.04 ± 0.03 (11) |
| 13-Hydroxytetradecanoic acid | 2138 | 17 | Trace | 0.1 | 0.07 ± 0.03 (13) |
| 10,11-Dihydroxydodecanoic acid | 2153 | 17 | 0.01 | 0.2 | 0.11 ± 0.06 (17) |
| 2-Dodecene-1,12-dioic (traumatic) acid | 2155 | 17 | Trace | 0.03 | 0.02 ± 0.01 (8) |
| Caffeic acid | 2157 | 3 | Trace | – | – |
| 11,12-Dihydroxydodecanoic acid | 2174 | 17 | Trace | 0.1 | 0.05 ± 0.04 (8) |
| *13-Hydroxy-2-tetradecenoic acid* | 2194 | 6 | Trace | 0.02 | – |
| 3,12-Dihydroxydodecanoic acid | 2200 | 17 | Trace | 0.1 | 0.07 ± 0.03 (14) |
| NN (331,103,73,81,219) | 2210 | 4 | Trace | 0.02 | – |
| *14-Hydroxytetradecanoic acid* | 2215 | 9 | 0.02 | 0.04 | 0.03 ± 0.01 (9) |
| 10,12-Dihydroxydodecanoic acid | 2220 | 17 | Trace | 0.1 | 0.07 ± 0.03 (11) |
| Oleic acid | 2222 | 11 | Trace | 0.02 | – |
| Octadecanoic acid | 2250 | 11 | 0.02 | 0.1 | 0.06 ± 0.04 (11) |
| *11,12-Dihydroxy-2-dodecenoic acid* | 2267 | 14 | 0.01 | 0.05 | 0.02 ± 0.01 (14) |
| 3-Hydroxydodecanedioic acid | 2288 | 4 | Trace | 0.03 | – |
| 3,13-Dihydroxytetradecenoic acid | 2316 | 8 | Trace | 0.02 | – |
| *Eicosanoic acid* | 2446 | 5 | Trace | 0.1 | – |

Table 2 (*Continued*)

| Compound, TMS | RI | N[a] | Relative composition (%) | | |
|---|---|---|---|---|---|
| | | | Min | Max | Median (*n*)[b] |
| *n*-Pentacosane | 2500 | 11 | 0.01 | 0.05 | 0.03 ± 0.01 (11) |
| Unsaturated acid (*m*/*z* 73,75,164,136,81,371) | 2611 | 4 | Trace | 0.01 | – |
| *n*-Heptacosane | 2700 | 16 | 0.05 | 0.4 | 0.13 ± 0.12 (16) |
| 1-Tetracosanol | 2755 | 5 | Trace | 0.02 | – |
| *Tetracosanoic acid* | 2844 | 7 | Trace | 0.02 | – |
| *n*-Nonacosane | 2900 | 11 | 0.11 | 0.3 | 0.06 ± 0.05 (11) |
| 9-Hentriacontene | 3074 | 7 | Trace | 0.03 | – |
| 7-Hentriacontene | 3077 | 5 | Trace | 0.02 | – |
| *n*-Hentriacontane | 3100 | 11 | Trace | 0.2 | 0.07 ± 0.06 (11) |
| Cholesterol | 3149 | 7 | Trace | 0.03 | – |
| 9-Tritriacontene | 3275 | 6 | 0.02 | 0.1 | 0.04 ± 0.04 (6) |

[a] Number of the RJ samples containing of the component.
[b] *n*, number of samples with concentration of component ≥0.01% of TIC.
[c] Trace – below 0.01% of TIC.
[d] Compound was not registered.

temperatures are presented in the last three columns of Table 1 The relative content of VOCs in samples stored for 10 months at −18 and 4 °C did not change significantly. At same time, in the sample stored at room temperature, twofold decrease of phenols and substantial increase of relative concentration of volatile $C_2$ and $C_4$ acids and $C_5$ aldehydes was observed. It can be assumed that the increase in RJ acidity noted by the authors in [3,39] can be partly explained by the formation of acetic and butyric acids.The data presented in this chapter are preliminary in nature. However, they agree with the reports of other authors, who used different chemical markers to assess the freshness and quality deterioration of RJ during storage at different temperatures. In paper [3], the total acidity of RJ was used as such a marker. On the other hand, the authors in [37] measured time-depending changes of the content of water soluble proteins and browning reaction. In [38] furosine (Amadori product) was used as a suitable index for assessing the freshness of RJ. According to all the above authors, RJ retains its good quality during prolonged storage at low temperature ranges from 0 °C to −20 °C, but quickly loses its high quality at room temperature. The authors of the cited publications [3,37,38] concluded that the deterioration of RJ during storage was due to Maillard reaction sensitive to ambient temperature.

### 3.2. Extractable compounds of RJ

The fractional composition of ether and methanol extracts from 17 investigated RJ samples was determined. From 6.1 to 10.2% of the mass (average 8.5 ± 4.0%) was extracted by ether. As can be seen from presented below data, this fraction contains presumably fatty acids and lipids. In methanol extract was transferred 12.6–24.2% (average 14.0 ± 5.7%) of the RJ mass. This extract consists from more polar compounds: carbohydrates, carbohydrate acids and alcohols. These data are in accordance with literature information on the relative concentration of fatty acids and lipids, and carbohydrates in fresh RJ [3].

Table 2 presents the list of 85 organic compounds extracted by diethyl ether from RJ samples under the investigation. Here we present the intervals of relative content of the identified compounds as well as average values for the *n*-samples, in which the content of the compound was not less than 0.01% of TIC. More than half of extracted with diethyl ether compounds (47 substances) belong to hydroxy and dicarboxylic fatty acids. Thirty five of listed in Table 2 acids were previously reported as RJ constituents by authors [13,15,25,29,40] and nine of them were identified also in extracts of mandibular glands taken from adult queen bees *A. mellifera carnica* [41]. Nine out of the 13 components, which we detected in RJ for the first time (all these compounds are printed in Table 2 in italics), belong to hydroxy and oxo-carboxylic acids.

In accordance with literature data [13,15], among the compounds listed in Table 2 the most abundant ones were 10-HDA, 10-HDAA, sebacic acid, 3,10-DDA, and additionally, 2-decene-1,10-dioic acid. In addition to these aliphatic acids, small amounts of aromatic substances: 4-hydroxybenzoic acid, 4-hydroxy methyl benzoate (HOB) and 4-hydroxy-3-methoxyphenylethanol (HVA) were detected in some of ether extracts. It is noteworthy that HOB, HVA, as well as two isomers of 9-hydroxy-2-decenoic acid (9-HDA), are ranked among the signal components (pheromone) of honey bee queen [42,43]. With an exception of HOB, all these compounds were identified in RJ for the first time.

Among the identified compounds, only 40 substances were registered in all 17 samples, however they comprise ca. 96% of total ion current (TIC). At the moment it is difficult to explain such a variation of the relative content of different substances in the RJ and the presence of some of them only in a few selected samples (usually it refers to minor compounds). These differences may be due as much to differences in nutrition in individual families, even in the same apiary, or are incidental, for example, can be explained by varying degrees of purity of the cells where the queen lays eggs. Accidental pollution, evidently, can be attributed to both *p*-coumaric and caffeic acids, since these substances are typical components of propolis, which bees use for "polishing" their cells.

Table 3 contains the results of the identification and semi-quantitative determination of the most polar compounds extracted from RJ with methanol. In total, 82 compounds were detected in the extracts but only 39 of them were present in all the 17 samples. Fructose, glucose (each of these sugars represented by four anomers) and sucrose constituted the highest relative content (ca. 76% of TIC). In different samples the contents of trehalose ranged from 1.7 to 3.7%. The participation of 19 other disaccharides, as well as nine tri- and tetrasaccharides were in total approximately 6% of TIC. Overall, these data remain in good agreement with reports on the content of sugars in RJ available in the literature [21,26].

The main differences between the results obtained here and other published data concern the content of free amino acids in RJ. Boselli et al. [24] published results of the investigation on 10 RJ samples using GC–MS, in which they found nine free amino acids (FAAs) the sum total of which amounted on average to 7.3 mg/g RJ. Recently, the researchers from China [28] found 26 FAAs in fresh RJ, the average total content of which amounted to 9.21 mg/g RJ. Meanwhile, from among all FAAs found in [24,28], only one amino acid proline was identified in our research in the samples from Polish RJ (Table 3). In addition, in our investigations, FAAs were identified only in one out of six commercial preparations containing lyophilized RJ and produced in three different countries [29].We

**Table 3**
Relative composition (%) of carbohydrates and other polar compounds in methanol extracts from RJ and bee queen larvae.

| Compound, TMS | LTPRI | Royal jelly | | | | Larvae |
|---|---|---|---|---|---|---|
| | | N[a] | Min | Max | Median (n)[b] | |
| Lactic acid | 1071 | 3 | Trace[c] | – | – | 0.2 |
| Glycolic acid | 1083 | 4 | Trace | 0.03 | – | – |
| Alanine, N,O-bis-TMS | 1115 | n.r.[d] | – | – | – | 0.6 |
| Glycine, N,O-bis-TMS | 1128 | n.r. | – | – | – | 0.5 |
| Sarcosine, N,O-bis-TMS | 1148 | n.r. | – | – | – | 0.1 |
| β-Alanine, N,O-bis-TMS | 1194 | n.r. | – | – | – | 0.1 |
| Valine, N,O-bis-TMS | 1226 | n.r. | – | – | – | 0.5 |
| Urea | 1260 | 3 | Trace | 0.04 | – | – |
| Leucine, N,O-bis-TMS | 1284 | n.r. | – | – | – | 0.2 |
| Phosphoric acid | 1291 | 17 | 0.2 | 1.1 | 0.5 ± 0.3 (17) | 1.3 |
| Glycerol | 1294 | 16 | Trace | 0.1 | – | 2.0 |
| Proline, N,O-bis-TMS | 1300 | 15 | 0.01 | 0.3 | 0.11 ± 0.09 (15) | 1.9 |
| Glycine, N,N,O-tris-TMS | 1304 | n.r. | – | – | – | 0.1 |
| Isoleucine or norleucine, N,O-bis-TMS | 1309 | n.r. | – | – | – | 0.8 |
| β-Aminoisobutyric acid, N,O-bis-TMS | 1316 | n.r. | – | – | – | 0.2 |
| Succinic acid | 1320 | n.r. | – | – | – | 0.1 |
| Glyceric acid | 1346 | 5 | Trace | 0.02 | – | Trace |
| Serine, N,N,O-tris-TMS | 1378 | n.r. | – | – | – | 0.7 |
| Threonine, N,O,O-tris-TMS | 1406 | n.r. | – | – | – | 0.2 |
| Homoserine, N,O,O-tris-TMS | 1488 | n.r. | – | – | – | 0.04 |
| Malic acid | 1513 | 5 | Trace | 0.03 | – | Trace |
| Hexanedioic (adipic) acid | 1517 | n.r. | – | – | – | 0.03 |
| Pyroglutamic acid, N,O-bis-TMS | 1527 | n.r. | – | – | – | 0.3 |
| Threitol | 1530 | n.r. | – | – | – | 0.01 |
| Aspartic acid, N,O,O-tris-TMS | 1536 | n.r. | – | – | – | 0.04 |
| Erythritol | 1540 | 5 | Trace | 0.02 | – | – |
| Isothreonic acid | 1579 | 7 | Trace | 0.1 | – | – |
| Phosphoric acid derivative (*m*/*z* 299,73,315) | 1587 | n.r. | – | – | – | 0.1 |
| Tiglylglycine, O-TMS | 1594 | n.r. | – | – | – | 0.1 |
| Threonic acid | 1598 | 5 | Trace | 0.04 | – | – |
| Phenylalanine, N,O-bis-TMS | 1636 | n.r. | – | – | – | 0.2 |
| Glutamine, N,O,O-tris-TMS | 1642 | n.r. | – | – | – | 1.7 |
| Arabinoic acid, γ-lactone | 1654 | 17 | Trace | 0.09 | 0.05 ± 0.06 (13) | – |
| Arabinofuranose | 1681 | 4 | Trace | 0.1 | – | – |
| Asparagine, N,O,O-tris-TMS | 1691 | n.r. | – | – | – | 0.05 |
| Ribonic acid, γ-lactone | 1693 | 17 | Trace | 0.4 | 0.23 ± 0.17 (15) | – |
| Lysine, N,N,O-tris-TMS | 1720 | n.r. | – | – | – | 0.1 |
| Xylitol | 1731 | n.r. | – | – | – | 0.05 |
| Arabinitol | 1762 | 10 | Trace | 0.03 | – | 0.1 |
| Ribitol | 1767 | 5 | Trace | 0.1 | – | Trace |
| α-Glyceryl phosphate | 1799 | 17 | 0.03 | 0.2 | 0.10 ± 0.06 (17) | 0.5 |
| Methyl α-fructopyranoside | 1815 | 17 | 0.01 | 0.3 | 0.16 ± 0.11 (17) | – |
| α-Sorbofuranose | 1831 | 14 | 0.11 | 0.6 | 0.40 ± 0.27 (14) | – |
| Methyl β-fructofuranoside | 1832 | 17 | 0.03 | 0.5 | 0.22 ± 0.10 (17) | – |
| Pinitol | 1835 | 4 | Trace | 0.04 | – | – |
| α-Fructofuranose | 1846 | 17 | 3.7 | 12.7 | 7.4 ± 2.6 (17) | 0.8 |
| β-Fructofuranose | 1850 | 17 | 14.3 | 22.9 | 18.6 ± 2.8 (17) | 0.8 |
| Tetradecanoic acid + carbohydrate | 1855 | n.r. | – | – | – | 0.1 |
| Inositol, penta-OTMS | 1874 | 11 | 0.02 | 0.7 | 0.3 ± 0.2 (11) | – |
| β-Fructopyranose | 1887 | 17 | 0.4 | 2.4 | 0.9 ± 0.6 (17) | – |
| Tyrosine, O,O-bis-TMS | 1890 | n.r. | – | – | – | 0.1 |
| β-Glucofuranose | 1893 | 17 | 1.1 | 3.1 | 2.0 ± 0.6 (17) | – |
| Quinic acid | 1905 | 4 | Trace | 0.3 | – | – |
| Gluconic acid, γ-lactone | 1927 | 17 | 0.7 | 1.4 | 1.2 ± 0.3 (17) | – |
| α-Glucopyranose | 1932 | 17 | 16.9 | 23.6 | 22.5 ± 3.3 (17) | 0.5 |
| Histidine, N,N,O-tris-TMS | 1942 | n.r. | – | – | – | 0.2 |
| α-Galactopyranose | 1940 | 8 | Trace | 2.7 | 2.2 ± 1.4 (6) | – |
| β-Galactopyranose | 1946 | 6 | Trace | 0.2 | – | – |
| Tyrosine, N,O,O-tris-TMS | 1957 | n.r. | – | – | – | 1.4 |
| Carbohydrate acid | 1968 | 17 | Trace | 1.0 | 0.7 ± 0.3 (8) | – |
| Mannitol | 1974 | 17 | 0.1 | 1.3 | 0.3 ± 0.3 (17) | 0.2 |
| Sorbitol | 1980 | 5 | Trace | 0.3 | – | – |
| Glucitol | 1982 | 17 | Trace | 0.2 | 0.09 ± 0.03 (16) | 1.3 |
| *chiro*-Inositol | 1992 | 4 | Trace | 0.1 | – | 0.1 |
| NN (*m*/*z* 71,73,147,299,387) | 1996 | 6 | Trace | 0.1 | – | – |
| *myo*-Inositol, penta-TMS | 2000 | n.r. | – | – | – | 0.3 |
| Hexitol | 2023 | n.r. | – | – | – | 0.1 |
| β-Glucopyranose | 2030 | 17 | 17.9 | 31.8 | 23.5 ± 4.7 (17) | 0.7 |
| Inositol isomere (*neo*-?) | 2039 | n.r. | – | – | – | 0.04 |
| Gluconic acid | 2044 | 17 | 1.6 | 5.8 | 4.3 ± 2.3 (17) | 0.5 |
| Hexadecanoic acid | 2050 | n.r. | – | – | – | 0.5 |
| *scyllo*-Inositol | 2069 | n.r. | – | – | – | 0.1 |
| 2-(Acetamino)-2-deoxy galactopyranose | 2117 | 9 | Trace | 0.02 | – | – |
| *myo*-Inositol | 2129 | 17 | 0.1 | 0.3 | 0.2 ± 0.1 (17) | 2.1 |

Table 3 (*Continued*)

| Compound, TMS | LTPRI | Royal jelly | | | | Larvae |
|---|---|---|---|---|---|---|
| | | *N*[a] | Min | Max | Median (*n*)[b] | |
| 2-(Acetamino)-2-deoxy α-glucopyranose | 2131 | 8 | Trace | 0.04 | – | – |
| 2-(Acetamino)-2-deoxy β-glucopyranose | 2141 | 8 | Trace | 0.01 | – | – |
| Oleic acid | 2222 | n.r. | – | – | – | 2.9 |
| Tryptophan, N,N,O-tris-TMS | 2238 | n.r. | – | – | – | 0.2 |
| Octadecanoic acid | 2251 | n.r. | – | – | – | 0.3 |
| α-Glucopyronosyl phosphate | 2260 | 4 | Trace | 0.04 | – | 0.4 |
| Glucoside (*m*/*z* 73,437,200,210) | 2296 | 17 | 0.04 | 0.5 | 0.3 ± 0.3 (17) | – |
| NN (*m*/*z* 240,72,212) | 2302 | 4 | Trace | 0.01 | – | – |
| Aspartic acid derivative (*m*/*z* 232,73,494) | 2331 | n.r. | – | – | – | 0.1 |
| Glucoside (*m*/*z* 73,437,210,200,520) | 2346 | 17 | 0.02 | 0.1 | 0.06 ± 0.05 (17) | – |
| Glucoside (*m*/*z* 73,437,200) | 2410 | 13 | Trace | 0.1 | 0.06 ± 0.05 (11) | – |
| Uridine (uracil riboside) | 2463 | n.r. | – | – | – | 0.2 |
| Adenosine (adenine riboside) | 2670 | 17 | 0.02 | 0.4 | 0.10 ± 0.10 (17) | 0.7 |
| α-Lactulose | 2696 | 17 | 0.02 | 0.1 | 0.07 ± 0.06 (17) | 2.5 |
| Sucrose | 2715 | 17 | 5.3 | 13.9 | 10.8 ± 3.1 (17) | 0.2 |
| α-Maltose | 2748 | 17 | 0.04 | 0.3 | 0.2 ± 0.1 (17) | 0.1 |
| α-Cellobiose | 2760 | 17 | 0.1 | 0.3 | 0.2 ± 0.11 (17) | – |
| α-Maltulose | 2777 | 17 | 0.01 | 0.1 | 0.07 ± 0.07 (17) | – |
| β-Maltulose | 2781 | 17 | trace | 0.1 | 0.05 ± 0.04 (15) | – |
| 1-Monooleyl glycerol | 2784 | n.r. | – | – | – | 0.1 |
| Turanose | 2791 | 17 | 0.1 | 0.3 | 0.2 ± 0.1 (17) | 1.5 |
| Disaccharide | 2796 | 9 | Trace | 0.2 | 0.08 ± 0.06 (9) | – |
| β-Maltose | 2801 | 17 | 0.2 | 0.8 | 0.6 ± 0.4 (17) | – |
| Trehalose | 2816 | 17 | 1.7 | 3.7 | 2.1 ± 1.1 (17) | 65.8 |
| Guanosine | 2830 | 5 | Trace | 0.3 | – | – |
| β-Palatinose | 2835 | 11 | Trace | 0.2 | 0.11 ± 0.08 (11) | – |
| Leucrose | 2849 | 12 | 0.04 | 0.2 | 0.08 ± 0.06 (12) | 0.1 |
| Disaccharide | 2861 | 17 | 0.04 | 0.1 | 0.06 ± 0.04 (17) | – |
| β-Cellobiose | 2871 | 17 | 0.04 | 0.2 | 0.10 ± 0.08 (17 | – |
| Disaccharide | 2880 | 17 | 0.02 | 0.1 | 0.06 ± 0.06 (17) | – |
| Disaccharide | 2930 | 4 | Trace | 0.07 | – | – |
| α-Isomaltose | 2956 | 17 | 0.03 | 0.2 | 0.10 ± 0.08 (17) | – |
| Gentibiose | 2990 | 17 | 0.2 | 0.8 | 0.6 ± 0.4(17) | – |
| β-Isomaltose | 3006 | 17 | 0.02 | 0.2 | 0.11 ± 0.08 (17) | – |
| Disaccharide | 3106 | 13 | 0.01 | 0.1 | 0.05 ± 0.04 (13) | – |
| Disaccharide | 3116 | 8 | Trace | 0.04 | – | – |
| 25-Hydroxy-24-methylcholesterol | 3250 | 7 | Trace | 0.04 | – | 0.5 |
| β-Sitosterol | 3345 | 14 | Trace | 0.05 | – | 0.6 |
| 3-Hydroxystigma-5,24(28)-diene | 3355 | n.r. | – | – | – | 0.1 |
| Trisaccharide | 3417 | 14 | Trace | 1.0 | 0.7 ± 0.3 (12) | – |
| Raffinose | 3505 | 17 | 0.02 | 0.4 | 0.33 ± 0.27 (17) | – |
| 1-Kestose | 3517 | 17 | 0.2 | 1.2 | 0.9 ± 0.4 (17) | 0.2 |
| Erlose | 3550 | 17 | 0.3 | 1.1 | 1.0 ± 0.4 (17) | 0.3 |
| Melizitose | 3585 | 8 | 0.01 | 0.9 | 0.6 ± 0.5 (8) | – |
| Trisaccharide | 3619 | 4 | Trace | 0.02 | – | – |
| Maltotriose | 3630 | 14 | 0.02 | 0.2 | 0.08 ± 0.07 (14) | – |
| Tri- or tetrasaccharide | 3770 | n.r. | – | – | – | 0.2 |
| Tetrasaccharide | >4000 | 3 | Trace | 0.1 | – | – |
| Tetrasaccharide | >4000 | 4 | Trace | 0.4 | – | – |
| 1,2-Diglyceride (*m*/*z* 129,385,313) | >4000 | n.r. | – | – | – | 0.4 |
| Glycerol, 1-palmitate-3-oleate- | >4000 | n.r. | – | – | – | 0.2 |
| 1,2-Diglyceride (*m*/*z* 129,413) | >4000 | n.r. | – | – | – | 0.3 |
| Glycerol, 1,3-dioleate- | >4000 | n.r. | – | – | – | 0.2 |

[a] Number of the RJ samples containing of the component.
[b] *n*, number of samples with concentration of component ≥0.01% of TIC.
[c] Trace – below 0.01% of TIC.
[d] Compound was not registered.

can assume that the apparent differences are associated with the specificity of RJ sample collection and subsequent preparation for analysis. First of all, it is doubtful whether the fact of finding "free" amino acids can be acknowledged in [24], because on the sample preparation stage RJ was mixed with a solution of ethanol (95%) and 1 N HCl (70:25, v/v) and the slurry was heated to the boiling point. Under these conditions, it is impossible to rule out acid hydrolysis of compounds comprising amino acid moieties. Secondly, the results of analysis could have been influenced by the way in which RJ was collected from queen larvae cells: automatic squeezing by compressing the cells, or even careless handling of the larvae (and their damage) can lead to the release of "contaminants" in the form of free amino acids from their bodies into raw RJ. This can be proven by the data given in the last column of Table 3 which presents the composition of methanol extract of homogenate of 3- to 4-day-old queen larvae. As can be seen, in this extract, 21 FAAs were identified comprising over 9% of TIC. In their work, the authors [28] fail to indicate how RJ was harvested at 10 different apiaries and transported to their laboratory. If FAAs found in their samples had been indeed acquired from bee larvae, then their finding must be an analytical artifact. However, this fact does not only decrease the utility value of the product, on the contrary, it increases its value because the homogenizate from bee larvae, *Apis mellifera*, together with a complex of essential amino acids, contains many other valuable nutrients, such as adenosine, uridine, unsaturated fatty acids and their glycerides (Table 3).

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