

CORVINUS UNIVERSITY OF BUDAPEST
DEPARTMENT OF FOOD CHEMISTRY AND NUTRITION

PhD School of Food Science



**CHEMICAL CHARACTERISTICS OF MILKWEED HONEY AND
ITS COMPARISON WITH ROBINIA HONEY**

Kasperné Szél Zsuzsanna

Theses of PhD

Budapest
2006

PhD School/Program

Name: PhD School of Food Science

Field: Food Science

Head: Prof. András Fekete
CORVINUS UNIVERSITY OF BUDAPEST

Supervisor: Dr. habil. Kornél Korány
CORVINUS UNIVERSITY OF BUDAPEST

The applicant met the requirement of the PhD regulations of the Corvinus University of Budapest and the thesis is accepted for the defence process.



.....
Head of PhD School



.....
Supervisor

I. PREMISES OF THE WORK, AIMS TO BE REACHED

Milkweed honey is not a well known honey in Europe, the plant itself – despite its American origin - is widely spread only in Hungary, thus its honey may well become a Hungaricum. IT composes 1-3 % of the Hungarian honey production. Its honey is light, colorless or slightly yellowish, similar to robinia honey in appearance. Milkweed honey, because of the special structure of the flower does not contain its pollen. So it is impossible to identify it via pollen analysis. Producers might mix it to robinia honey in the hope of a higher profit. The sugar composition of milkweed honey is different from that of robinia honey (it will crystallize sooner), that is why robinia honey mixed with milkweed honey is of less value, because it will keep its fluidity for only a few months. Thus it would be important to specify certain characteristics of milkweed honey that would make it possible to differentiate it from other unifloral honeys.

In Hungary, research dealing with the chemical composition of honeys is scarce. Research abroad has generally been done on honeys of plant species that are not grown in Hungary at all.

Below the results of former research done on milkweed and robinia honey is summarized. FÖLDHÁZINÉ et al. (1996a,b) examined the chemical composition of several unifloral honeys. They measured the electrical conductivity of milkweed honey (n=3) the lowest, $1,77 \cdot 10^{-4}$ S/cm on the average, followed by robinia honey (n=9) $1,86 \cdot 10^{-4}$ S/cm. Electrical conductivity mainly depends on the mineral material content of honeys, the higher the mineral material content, the higher the conductivity. The high mineral content is also in correlation with the dark color of the honeys, and/or the origin of the honey. The honeydew honeys are always darker than floral honeys.

During their examinations, the prolin content of ten unifloral honeys was also measured. Prolin constitutes the largest amount of free amino acids present in honeys, which is mostly added to the honey by the bees. The prolin content of robinia honeys (n=9) was the lowest, 199 mg/kg, followed by milkweed honey with 305 mg/kg.

The micro- and macro element content of eight kinds of unifloral honeys were determined. The element composition of robinia and milkweed honeys were similar, which was supported by the results of GULYÁS (1993) and SZÉL (2000) also. The examinations also showed that the mineral content of the linden and chestnut honeys is higher in general, sunflower, rape, facelia honeys have lower mineral content than robinia and milkweed honeys.

Sugar composition of the honeys were also measured. The fructose, glucose, saccharose, erlose and melecitose contents of 14 kinds of honeys were compared. The fructose/glucose ratio of milkweed honeys was 1.44, that of robinia honey was 1.63. Both honeys contained 1% saccharose and 3% erlose. Sugar content was also determined by other researchers.

FÖLDHÁZI (1994) studied the sugar composition of honeys of different botanical origin, among them that of robinia and milkweed honeys. The fructose/glucose ratio of milkweed honey was also lower here (1,4, n=3), than that of robinia honeys (1,7, n=7). The average saccharose, turanose, isomaltose and erlose content was higher in robinia honeys than in milkweed honeys. The maltose content of milkweed honeys was higher, than robinia honeys, its maltotriose content was the highest among all studied unifloral honeys.

SZABÓ (2001) analyzed the sugar composition of ten Hungarian unifloral honeys. According to his results, robinia honey also had a greater fructose/glucose ratio (1.4, n=5), than milkweed honey (1.36, n=3). The turanose and isomaltose content of robinia honey was higher than that of milkweed honey, but at the same time he measured more maltose, melecitose and erlose in milkweed honey.

Research dealing with the enzyme content of honeys is relatively rare. Most of the enzymes are added to honey by the bees during honey ripening. Invertase (α -glycosidase) is responsible for decomposing saccharose, diastase is a starch decomposing enzyme. Besides glucosoxidase has a role in the antibacterial effect of honey. Generally, the above three enzymes are examined, but primarily in foreign studies. There are several methods for determining the enzyme content of honeys. Hungarian results can be compared only to results gained by the same methods. PERSANO et al. (1990) determined the diastase number of 76 honey samples 8.4 (± 2.9) by the Schade-White-Hadorn method, which is applied also in Hungary. DÁVID (1994) measured the diastase activity of five unifloral honeys- including robinia and milkweed honeys. With robinia honey (n=8), he determined an average of 13 (± 2) diastase number, with milkweed honey (n=3) he determined 15 (± 4) diastase number. KERÉKES and SITKEI (1996) performed diastase measurements on robinia honeys (n=11) and gained an average of 17.83 (± 2.92) diastase number. In 2000, the Central Food Research Institute and the Department of Honeybee Breeding and Bee Biology of the Institute for Small Animal Research (ISR) measured the diastase and glucoseoxydase activity of robinia and milkweed honeys. According to their results milkweed honeys (n=8) had a diastase number of 24.5, in average, while robinia honeys (n=10) had 16.3. The milkweed honeys had a higher glucoseoxydase activity (8.28 nmol/unit) than robinia honeys (3.67 nmol/unit). The differences were statistically significant in both cases. (SZÉL et al.2002). No studies have been found on the invertase content of milkweed honeys.

The aroma material composition of milkweed honeys has not been determined in Hungary before. The determining of several chemical compounds (amino acids, proteins, coloring agent etc.) cannot be found in Hungarian studies.

The main aim of my PhD studies was the analysis of the chemical compounds of milkweed honey and its comparison to robinia honey, which is very similar to it and is easily mistaken for it.

My goal was to determine some parameters that have a role in the qualification of honey: pH, invertase and diastase enzyme activity. In order to compare them with robinia honey the same examinations were planned to be carried out on robinia honey as well. The sugar content of honeys has long been analyzed. My aims also included the comparison of the above mentioned two honeys based on eight of their sugar components.

Many people say that milkweed honey has a characteristic odor and taste. Sensory qualification are based on the above. This solely is a good basis to differentiate it from other unifloral honeys. But for the sensory qualification, etalons are needed that are difficult to purchase. I also set it up as a question, whether the organoleptically sensible specificity in odor and taste can be supported by instrumental analysis. In order to observe that, both unifloral honeys were planned to be analyzed by gas chromatography. Some samples were planned to be examined by the steam distillation aroma extraction method as well as the Likens-Nickerson aroma extraction method.

Chemical compounds implying the botanical origin of honeys supposedly originate from the odor agents of the flowers, that is why the examination of the aroma materials of robinia and milkweed honeys were also taken into consideration.

Flower nectars are ripened into honey by the bees and are stored in self-made cells wax cells. honey is thus enriched by these substances added by the bees and the chemical compounds seeping from the wax. The analysis of the compounds of the bees' wax was also set as an aim. A relatively new analytic method is measuring by the electric nose instrument. It seemed expedient to compare the two unifloral honeys by this method as well.

II. MATERIAL AND METHOD

1. **Samples included in the study and the premises of examination**

In my examination robinia and milkweed flowers, robinia (8) and milkweed (11) honey samples and bees' wax, collected in 2001 and 2002 were included. The analysis of aroma materials were executed at the Department of Food Chemistry and Nutrition of Corvinus University of Budapest, the other measurements were carried out in the Laboratory of Honey of ISR and the Central Food Research Institute.

2. **Measuring pH**

The pH of the samples was measured according to Hungarian standard No. 6943/3-80 (HUNGARIAN STANDARD 1980b)

3. **Determining diastase activity**

The diastase activity of the honey samples were determined according to the Schade-White-Hadorn method described in the Hungarian Standard No.6943/6-81 (HUNGARIAN STANDARD 1981)

4. **Determining invertase activity**

Invertase activity was determined by Siegenthaler method. (SIEGENTHALER 1977)

5. **Determining sugar composition**

Sugar composition was determined by HPLC. The applied method was basically the same as described in the thesis of K. Szabó (SZABÓ 2001), but modified at some points (solution of samples, column and detector temperature). A Jasco type HPLC instrument was applied, connected with a PU-980 pump, a Rheodyne injector and an ERC-7515 RI detector. The chromatographical column was type Supelcosil LC-NH₂, 250*4.6 mm, and of 5 µm grain size. 75:25 acetonitril:water mixture was used as an eluent at 1ml/min rate of flow. To evaluate the results, a Jasco-Borwin software was available. In my studies the following eight sugar standards were included: fructose, glucose, saccharose, turanose, maltose, isomaltose, melecitose, erlose with which outer calibration was executed. The amount of sugars was computed in per cent on dry material. The dry material content of the honeys was determined by a Zeiss-Abbe refract meter.

6. **Examination of aroma materials**

6.1 Flower extracts gained by steam distillation

Robinia and milkweed flowers were cut into 0.5 cm pieces in order to extract their odor components. Three times, 200 g flowers were boiled for 1.5 hours with 180 g salt and 900 ml distilled water. The gained odor components were absorbed in 4 cm³ high purity hexane. As an inner standard, 0.1 g benzyl alcohol was used in 2001, in 2002 9 mg undecanol was added to it. The hexane solution was distilled to 0.3 cm³, out of which 1 µl was injected.

6.2 Honey extracts gained by steam distillation

600 g of each honey sample was dissolved in 1000 cm³ distilled water, then divided into three parts. 50 cm³ 96% ethanol was added to each 330 cm³ honey solution and then filled up to 500 cm³ with distilled water. As an inner standard 0.1 g benzyl alcohol was used in 2001, in 2002 9 mg undecanol was added to it. All of the three honey solutions were boiled in a 500 cm³ flask, during the distillation 3 x 80 cm³ condensed solution was collected. The condensed samples – after their unification – were extracted by 3 x 80 cm³ high purity pentane, with 20 g NaCl added to them. The pentane phase was dehydrated for one night by sodium-sulphate (NaSO₄), then distillation and concentration followed.

6.3 Honey extracts gained by Likens-Nickerson Method

600 g of honey dissolved in 1000 cm³ distilled water was boiled in a 2000 cm³ flask in a way that on the other side of the instrument 200 cm³ high purity pentane was being boiled. Water was defrosted from the gained pentane, then distilled and concentrated like the pentane gained by steam distillation.

6.4 Gaining bees' wax extracts

Empty honeycombs were soaked in isosugar and the extract was gained by the Likens-Nickerson Method described in 6.3. Twice 100 g honeycombs were each soaked in 600 cm³ isosugar, then in the first case, the extract was made after 4 weeks, then in the second case after 4 months.

6.5 Separating the aroma materials

Aroma components were separated by a gas chromatograph equipped with a mass spectrometer detector. In order to keep the experimental conditions under control, before running each sample, a mixture was run containing C₁₀, C₁₂, C₁₄, C₁₆, C₁₈, C₂₀, normal hydrocarbons and inner standard. Each sample extract was measured twice parallels. The identification of the components the was done based on the WILEY138.L spectrum library.

Instruments and equipments used for the measurements:

-Hewlett Packard 5890/II gas chromatograph- mass selective detector 5971 A

-Capillary column:

60 m x 0.25 mm Supelcowax 10 (fused silica) 0.25 film thickness (in 2001 in the case of 4 samples 30 m x 0.25 mm Supelcowax 10 (fused silica) 0.25 film thickness was used, which was taken into consideration at the evaluation.)

-10 µl gas chromatography injector

-Software: G 1034C Version C.03.00 HP 19891994

7. Analysis of odor agents with electric nose

The odor of honeys run in 2002 were analyzed with an AppliedSensor 3320 electric nose. The instrument consists of 10 MOS FET (Metal oxide Semiconductor Field Effect Transistor) type, 12 MOS type and 1 moisture detectors.

2 x 10 ml was taken from the robinia and milkweed honey samples. The taken amount was placed in glass containers with special sealing. During the measuring the honey samples were heated to 40 °C, then after 5 minutes samples were taken with a two-needle automatic sampler.

III. RESULTS

1. **Invertase and diastase enzyme activity, pH of milkweed and robinia honey samples**

The invertase activity of the honey samples was determined by the Siegenthaler method. In the first year the average invertase number of milkweed honeys was higher (15) than that of the robinia honeys (7), but in the second year this difference disappeared, the average invertase number of both unifloral honeys was 10.

The average diastase number of milkweed honeys was higher (28) than that of the robinia honeys in 2001, but the difference reduced to a minimum in 2002. (26,25).

Analyzing the pH of unifloral honeys, the pH of milkweed honeys proved to be lower in both years, than that of the robinia honeys and the difference was statistically significant ($p < 0.001$). In the average of two years the pH of milkweed honeys was 3.5, the pH of robinia honeys was 3.9.

2. **The sugar composition of milkweed and robinia honeys**

In the average of the two-year measurements, the fructose content of the milkweed honeys was lower (45.99 %, $p < 0.001$), their glucose content was higher (37.64 %, $p < 0.05$), than that of the robinia honeys (fructose: 51.16 %, glucose 32.71 %) and these differences were statistically significant. My results have also been supported by the data in other studies (FÖLDHAZI 1994, SZABÓ 2001). The turanose and isomaltose content of robinia honeys was higher, their erlose+melecitose content was lower than that of the milkweed honeys. Considering the data of the two years, the difference is significant in the case of turanose ($p < 0.05$) and erlose+melecitose ($p < 0.05$).

3. **Analysis of odor agents of milkweed and robinia flowers**

In the cases of both the milkweed and the robinia flowers 5 compounds probably of floral origin were determined. The common components of the robinia flower and the robinia honey were: beta-ionone, cyclododecane, cyclohexadecane, 10-methyl-eicosane, and the 1-alpha-terpineol. The common components of the milkweed flower and the milkweed honey: 1-nonanol, 2-nonenal, 2,6,10,14,-tetramethyl-heptadecane, 2-metoxi-3-(1-propenyl)-phenol and the tetradecanal.

4. **Analysis of the aroma materials of milkweed and robinia honeys**

4.1 The analysis of aroma materials of honey samples by steam distillation

Analyzing the composition of aroma materials of milkweed honeys is a new scientific study. Based on the two-year series of measurements the 2,4,5-trimethyl-1,3-dioxalane, the ethyl-benzol, the 1,4-dimethyl-benzol, the 2-decenal, the 1-nonanol, the 2-isopropyl-4,5,6,-trimethyl-3-nitroalanin, and the 2-metoxi-3-(1-propenil)-phenol components were present in statistically significantly greater amount in milkweed honeys than in robinia honeys. The 1-nonanol and the 2-metoxi-3-(1-propenil)-phenol present in milkweed honeys were also detectable in milkweed flowers, which indicates the floral origin of these components. There was significantly greater amount of hexestrol, cyclohexadecane and 11,14,17-eicosanetric acid methyl ester in robinia honeys than in milkweed honeys. The cyclohexadecane present in robinia honeys was also among the aroma materials of robinia flowers.

The components present in both milkweed and robinia honeys, milkweed honeys contained a significantly greater amount of nonanal, beta-maaliene, ethyl-9-hexadecenoate and pentacosane, robinia honeys contained significantly more linalool.

During aroma extraction by steam distillation, the following six main components appeared at the end of the chromatograms both in milkweed and robinia honeys: hexadecane acid, ethyl ester, tricosane, ethyl-oleate, pentacosane, cis- and trans-ethyl-linoleate. The relative intensity of these components was mostly higher in milkweed honeys.

To sum up the analysis of aroma examinations by steam distillation done in 2001-2002, it can be concluded that milkweed honeys contained almost twice as many unique components, than robinia honeys. Among the components present in both unifloral honeys, in forty-one cases of the fifty-four, the relative intensity was higher with milkweed honeys. These findings might explain the richness of odors in milkweed honeys also sensible organoleptically.

4.2 Analysis of aroma materials of honey samples by Likens-Nickerson simultaneous distillation

Four milkweed and four robinia honey samples were submitted to Likens-Nickerson simultaneous distillation and extraction beside the steam distillation method. Applying the Likens-Nickerson method, new components appeared on the chromatograms characteristic of milkweed and robinia honeys. One of them, the 1- α -terpineol might be of floral origin (robinia).

The difference between the classical steam distillation and the Likens-Nickerson method can be described based on the chromatograms. The compounds, solely present by the steam distillation, independently of the type of the honey are the ethyl esters of chemically different fatty acids. The components, independent of the type of the honey, present at the end of the chromatograms by the Likens-Nickerson method are saturated and unsaturated hydrocarbons. The Likens-Nickerson method proved to be more effective in extracting the components appearing in the first half of the chromatogram.

2-furancarboxialdehyde (furfural), 1-(2-furanyl)-ethanon, 5-metoxy-furancarbaldehyde and nonacosane were present only in the chromatograms gained by the Likens-Nickerson method. These components though were present also on gas chromatographic runnings made with pure isosugar. We may conclude that these mostly furan compounds appear only with Likens-Nickerson method, independently of the unifloral honeys during the decomposition of sugar molecules.

5. Analyzing the odor agents of beeswax

In order to identify the components of beeswax origin, beeswax pieces soaked in isosugar for different lengths of time were analyzed by Likens-Nickerson method and gas chromatography. Based on my results the honey components of beeswax origin are the following: heneicosane, docosane, tricosane, pentacosane, heptacosane.

6. Results of the experiments with electronic nose

To detect the differences between the odor of the unifloral honeys the electronic nose instrument was also tried. In 2002, in the cases of 4 milkweed and 4 robinia honeys, after the main component analysis and discriminancy analysis, dominant difference in odor was

detectable between the two unifloral honeys. This new method – requiring no sample preparation- offers great opportunities in qualifying unifloral honeys.

7. New scientific results

1. The invertase number of milkweed honeys was 12 (± 4.74) according to the Siegenthaler method, based on the series of measurements during the two years. In case of the robinia honeys the invertase number was 8 (± 4.69) in average of the year-measurement.
2. The common components of milkweed and robinia honeys were: beta-ionon, cyclododecane, cyclohexadecane, 10-methyl-eicosane and the 1-alpha-terpineol. The common components of the milkweed honey and the milkweed flower were: 1-nonanol, 2-nonanal, 2,6,10,14,-tetramethyl-heptadecane, 2-metoxi-3-(1-propenyl)-phenol and tetradecanal.
3. The following results were observed by the gas chromatography of the aroma materials of milkweed honeys (n=8) gained by stem distillation. Based on the two-year series of measurements the 2,4,5-trimethyl-1,3-dioxalane, the ethyl-benzol, the 1,4-dimethyl-benzol, the 2-decenal, the 1-nonanol, the 2-isopropyl-4,5,6,-trimethyl-3-nitroalanin, and the 2-metoxi-3-(1-propenil)-phenol components were systematically present only in milkweed honeys. . The 1-nonanol and the 2-metoxi-3-(1-propenil)-phenol present in milkweed honeys were also detectable in milkweed flowers, which indicates the floral origin of these components.
4. According to the results of the examinations of the aroma materials of robinia honeys hexestrol, cyclohexadecane and 11,14,17-eicosanetricienic acid methyl ester were present only in these honeys. The cyclohexadecane present in robinia honeys was also among the aroma materials of robinia flowers.
5. The components present in both milkweed and robinia honeys, milkweed honeys contained a significantly greater amount of nonanal, beta-maaliene, ethyl-9-hexadecenoate and pentacosane, robinia honeys contained significantly more linalool.
6. During aroma extraction by steam distillation, the following six main components appeared at the end of the chromatograms both in milkweed and robinia honeys: hexadecane acid ethyl ester, tricosane, ethyl-octadec-9-enoate (ethyl-oleate), pentacosane, cis- and trans-ethyl-linoleate. The relative intensity of these components was mostly higher in milkweed honeys.
7. To sum up the analysis of aroma examinations by steam distillation done in 2001-2002, it can be concluded that milkweed honeys contained almost twice as many unique components, than robinia honeys. Among the components present in both unifloral honeys, in forty-one cases of the fifty-four, the relative intensity was higher with milkweed honeys. These findings and the observations under point 6 might explain the richness of odors in milkweed honeys also sensible organoleptically.
8. When applying the Likens-Nickerson simultaneous distillation and extraction method in case of the samples analyzed by the traditional steam distillation method, new components were detected characteristic of milkweed and robinia honeys.
9. The compounds, solely present by the steam distillation, independently of the type of the honey are the ethyl esters of chemically different fatty acids. The components, independent of the type of the honey, present at the end of the chromatograms by the Likens-Nickerson method are saturated and unsaturated hydrocarbons. The Likens-Nickerson method proved to be more effective in extracting the components appearing in the first half of the chromatogram.
10. Furane compounds were gained only in case of the Likens-Nickerson method, independently of the unifloral honeys.

11. The main difference between the classical steam distillation method and the Likens-Nickerson method was that the main component of honeys in case of the steam distillation method was the ethyl-octadec-9-enoate, while in case of Likens-Nickerson method it was tricosane.
12. According to my studies, honey components probably of beeswax origin were the following: heneicosane, docosane, tricosane, pentacosane, heptacosane.
13. Processing the results of the electronic nose examinations by main component analysis and discriminancy analysis, a dominant difference in odor was detectable between the two unifloral honeys.

IV. CONCLUSIONS AND SUGGESTIONS

The results of the chemical analysis of milkweed honeys have contributed to the description of the less known unifloral honey. It can be appointed, that the diastase activity values of milkweed honeys in our measurements meet the requirements of the HUNGARIAN FOOD CODE 2001/110. The pH and the enzyme activity values of the milkweed were compared to that of the robinia honey. The higher enzyme activity values of milkweed honeys, subject to alteration cannot be considered characteristic of the unifloral honey, because the differences between the annual values exceed the differences between the unifloral honeys. The differences between the pH values measured have not been supported by other authors.

The two unifloral honeys were compared based on their eight sugar components. Based on the results there is a significant difference between the fructose, turanose, isomaltose, and the erlose+melecitose contents of the honeys. Part of our results have been supported by other authors as well. In practice if a honey has to be identified as pure robinia honey or if it is mixed with milkweed honey, sugar measurements definitely will contribute to the correct decision even if they are not sufficient in themselves.

A remarkable proportion of my studies deal with the analysis of the aroma materials gained by gas chromatography. Several of the components (7) were characteristic of the milkweed honeys only, others (3) featured robinia honeys. Among these components, the ones that proved to be of floral origin are especially valuable. Several components have been dealt with which were present in both honeys but they were present characteristically in greater amount in one of the unifloral honeys. These findings provide an opportunity to tell the difference between the unifloral honeys in disputable cases.

There were almost twice as many unique aroma components in milkweed honeys than in robinia honeys, and among the common components four fifth showed a higher relative intensity in the milkweed honeys. These results might explain the fact why milkweed honeys are richer in odor. The richness of taste and odor of milkweed honeys sensible also organoleptically can be supported by instrumental analysis as well.

The extraction of aroma materials were done by the classical steam distillation as well as the Likens-Nickerson simultaneous distillation and extraction method- with some of the samples. With the help of the Likens-Nickerson method new components appeared on the chromatograms characteristic of the unifloral honeys. The greater efficiency of the Likens-Nickerson method vs. the classical distillation method is verifiable in case of the components appearing in the first half of the chromatogram. It is suggestible to try the method on greater sample number. Some furane components appeared on the chromatograms only when applying the former method, which have to be neglected when evaluating the honey aromas, because they originate from the decomposition of sugar.

Part of the honey aroma materials come from the honeycombs made of beeswax. The analysis by gas chromatography of honeycombs soaked in isosugar suggest that 5 alkanes present in honeys are of beeswax origin. These components have to be neglected when qualifying unifloral honeys.

The differentiation of honeys by electronic nose seems to be promising. It can be suggestible to make measurements in greater numbers by electronic nose and to include other unifloral honeys as well. The advantage of the method is that it is simple and quick, and some time it may substitute the human nose during the sensory qualification process.

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