# Fast differentiation of floral and honeydew honeys using gas chromatography-mass spectrometry

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**Summary**

Distinguishing floral and honeydew honey using chemical analysis is generally problematic. This work solved this problem by determining the trisaccharide melezitose in honeys, which was identified as a potential marker for identification of the type of honey. The newly developed method for determination of melezitose reduces the analysis time by approximately 10 times and significantly simplifies sample preparation during derivatization of saccharides. A two-step procedure of silylation with hexamethyldisilazane followed by *N,O-bis*(trimethylsilyl)trifluoroacetamide was developed for derivatization without the formation of degradation products. The limit of detection of the method for melezitose was 4.9 mg·kg$^{-1}$ based on the selected ion *m/z* of 361 with relative standard deviation for real samples of ≤ 15 %. The simplicity and speed of the method enables its use in screening tests. The method was successful at determination of a significant difference in the melezitose content of floral (103–2 880 mg·kg$^{-1}$) and honeydew honeys (10 600–26 100 mg·kg$^{-1}$), which allowed differentiation between the types of honey.

**Keywords**

floral honey; honeydew honey; melezitose, rapid analysis; gas chromatography-mass spectrometry

Honeybees (*Apis mellifera* L.) produce honey from the saccharide-rich nectar produced by plants. Some components of honey come from plants, others are formed by biochemical reactions during honey maturation and some are added by bees [1]. Honey is therefore a complex mixture of substances composed mainly of saccharides (70–80 % w/w), water (10–20 % w/w) and a large number of components at lower percentages. The main saccharides are the monosaccharides glucose (approximately 31 % w/w) and fructose (approximately 38 % w/w). So far, approximately 200 types of components have been identified in honey in small contents, such as phenolic compounds, organic acids, vitamins, minerals or enzymes, which varied mainly due to the nectar source or weather conditions [2].

Honey is valued not only for its taste, but also for its high nutritional value and benefits to human health. A wide range of therapeutic activities have been attributed to the use of honey, such as antibacterial and anti-inflammatory properties, useful in stimulating the healing of wounds and burns or treating gastric ulcers and gastritis [3]. Currently,

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there are few studies available that scientifically prove the effectiveness of honey in medicinal use [4]. However, honey has been shown to be effective against viral infections for which there are very few effective drugs. These effects are attributed to its potential antioxidant and anti-inflammatory activities that mitigate oxidative damage caused by pathogens and by improving the immune system.

Depending on the original raw plant, honey can have two different botanical origins, which are classified as floral honey or honeydew honey. Floral honey is produced by bees from the nectar contained in the flowers of flowering plants. Honeydew honey is obtained from the secretions of certain trees and other plants (among other genera *Pinus*, *Abies*, *Castanea* and *Quercus*) or the secretions of plant-sucking insects, especially from the *Aphididae* family, living on plant parts.

The chemical composition, biochemical and physico-chemical parameters of honeydew honey are significantly different from floral honey. Honeydew honey is dark brown and viscous, has a more intense flavour and has a very low tendency to crystallize [5], which is probably due to a higher content of minor substances. Compared to floral honey, honeydew honey has a higher proportion of oligosaccharides and a smaller proportion of monosaccharides. Due to the fact that it is produced in forested areas, often far from human habitation and environmental contamination, honeydew honey is recognized as being of superior quality. Therefore, honeydew honey and floral honey have different levels of consumer appeal and market price.

In many European countries, the market for honeydew honey is growing, so an analytical method to distinguish between the two types of honey is required to prevent adulteration and fraud. Reliable identification of honeydew and floral honey is still generally difficult. Finding a marker that would clearly characterize individual types of honey is the subject of various scientific studies. Çobanoğlu et al. [6] found that phenolic compounds such as gallic, trans-cinnamic, protocatechuic or 4-hydroxybenzoic acids, as well as oxalic and benzoic acids, can be used to distinguish between honeydew and floral honey using a rather complex chemical analysis. 2-Oxooctanoic acid, 4-oxapentanoic acids, allyl-acetic acid and methyl ester of 2,6-dihydroxybenzoic acid were considered markers for Slovakian honeydew honey [7]. These compounds are characteristic and unique compounds for honeydew honey, as they were not identified in most common Slovakian single floral honeys [8].

Another option for distinguishing individual types of honey is differentiation using saccharide analysis. Honeydew honeys contain higher amounts of oligosaccharides than floral honeys, especially trisaccharides such as melezitose or raffinose, which are practically absent in floral honey [9]. Some authors [10], however, reported the presence of melezitose in floral honey, although in a lower content than in honeydew honey. Nevertheless, the trisaccharide melezitose is considered by some authors [11, 12] to be a characteristic component of honeydew honey.

The content of melezitose in honey is 0–30 $g \cdot kg^{-1}$, while in melezitose solid honeys it can reach up to 200 $g \cdot kg^{-1}$. In addition to melezitose, other trisaccharides are usually present in honey, which makes it impossible to use the flow injection analysis system in combination with tandem mass spectrometry (FIA MS-MS) for analysis. For this reason, chromatographic separation of individual components with high separation efficiency is necessary. The high-performance liquid chromatography (HPLC) method is used as standard for the analysis, with various detection means. The use of HPLC with a differential refractive index (DRI) detector was published by Manzanares et al. [13]. However, high-performance anion exchange chromatography with highly sensitive pulse amperometric detection (HPAEC-PAD) is more frequently used for analysis [14, 15]. An evaporative light scattering detector (ELSD) detector has also been used, which provides high sensitivity and is applicable in a gradient chromatography system [16, 17].

Despite the simplicity of sample preparation for HPLC analysis and the available high sensitivity of detection, the use of this method encounters a relatively low separation efficiency, which can lead to distorted values of the determined contents of individual di- and trisaccharides due to overlapping peaks. This problem can be solved by using gas chromatographic separation, which, however, is very rarely used for determination of melezitose in honey. Gas chromatographic analysis requires derivatization, which makes the saccharide compounds volatile. However, the derivatization process is time-consuming and expensive, often resulting in multitude of derivatization products for some single saccharides. A two-step procedure (oximation and trimethylsilylation) is used for the derivatization of saccharides in honey [18]. Chromatographic conditions of separation on a methyl-silicon column were published by Sanz et al. [19]. A detailed analysis of monofloral honeys from Spain using gas chromatography (GC) was published by de la Fuente et al. [20]. The use of GC has indisputable advantages over

the HPLC method in terms of separation efficiency and the possibility of using mass spectrometry (MS) for detection with electron ionization (EI). The main disadvantage of GC is the necessity of derivatization, where by-products can be formed, as well as the relatively long analysis time, which is often over 1 h.

The main goal of this work was to develop a new, fast and sensitive GC method with MS detection for the determination of melezitose in honeys in order to distinguish floral and honeydew honeys. The newly developed method includes a new rapid derivatization procedure with the elimination of by-products that could interfere with the determination of melezitose.

## MATERIALS AND METHODS

### Honey samples and chemicals

Samples of honey were obtained from small local farmers from Slovakia and Austria. Some samples from large-scale producers were purchased in a local market in Bratislava, Slovakia. The melezitose standard was from SynthCluster (Modra, Slovakia). Acetonitrile, trifluoroacetic acid (TFA), sucralose, derivatizing agents hexamethyldisilazane (HMDS) and *N,O-bis*(trimethylsilyl) trifluoroacetamide (BSTFA) were obtained from Sigma Aldrich (St. Louis, Missouri, USA).

### Derivatization procedure

An amount of 30 mg of honey sample was added in a 2 ml vial with 400 $\mu$l of acetonitrile with an internal standard of sucralose with a content of 5 000 mg·kg$^{-1}$. Subsequently, 300 $\mu$l of HMDS and 2 $\mu$l of TFA were added. The sample was silylated at a temperature of 50 °C for 30 min with stirring speed of 7 Hz in a thermostatic shaker. After the completion of the first silylation step, 300 $\mu$l of BSTFA was added to the sample and a second silylation step was performed at 80 °C for 30 min and with stirring speed of 7 Hz. After silylation, the sample was ready for direct injection into the GC system.

### GC-MS analysis

GC-MS analyses were performed on a 6890 gas chromatograph with a 7683 Series Autosampler and a 5973 MS system from Agilent Technologies (Santa Clara, California, USA). Chromatographic separations were performed on a 5 m × 0.25 mm × 0.25 $\mu$m DB-5MS capillary column (Agilent Technologies). The oven temperature was set at 220 °C and gradually increased to 300 °C at a rate of 10 °C·min$^{-1}$, then increased to 340 °C at a rate of 40 °C·min$^{-1}$. The total separation time was 9 min. The injector temperature was set to 300 °C. Samples with an injection volume of 1 $\mu$l were dosed in split mode with a split ratio of 40 : 1. Helium with a constant flow rate of 0.5 ml·min$^{-1}$ was used as a carrier gas. The transferline temperature was 320 °C and the ion source temperature was 230 °C. Data acquisition and processing were performed using MSD ChemStation software (Agilent Technologies). In selective ion monitoring (SIM) mode with electron ionization at 70 eV, 5 ions were monitored, *m/z* of 361, 362 and 207 for melezitose and *m/z* of 308 and 310 for sucralose, with a dwell time of 20 ms of each ion. The limit of detection (*LOD*) and limit of quantification (*LOQ*) were estimated from the standard deviation of 10 analytical blank solutions as recommended by the International Union of Pure and Applied Chemistry (IUPAC), *LOD* as 3σ and *LOQ* as 10σ of 10 replicates of blank analytical solutions [21].

## RESULTS AND DISCUSSION

### Derivatization

A two-step derivatization procedure with HMDS and BSTFA was optimized for the derivatization of di- and tri-saccharides in honey. Acetonitrile with sucralose as internal standard was added to the honey sample. After partial dissolution of the sample, HMDS and a catalytic amount of TFA (which does not change the alkaline environment of the reaction mixture) was added and the solution was allowed to silanize at 50 °C. During this reaction, the water present in the honey is preferably silylated to form nonpolar hexamethyldisiloxane and the hydroxyl groups on the saccharides are partially silylated. During mixing at 50 °C, gaseous ammonia escapes, shifting the reaction balance in favour of silylated saccharides. The first silylation step, which is gentle to the saccharides and does not decompose them, is followed by a second step of silylation with BSTFA, where the acidic hydrogens on the unreacted hydroxyl groups of saccharides are completely replaced. In this way, it is possible to obtain quantitative silylation of the saccharides of interest, without the formation of degradation products.

### Method validation

Since part of the solvent and derivatization agent escapes during silylation, the quantification was performed after correcting the melezitose peak area for the internal standard of the synthetic

Tab. 1. Validation parameters for melezitose determination.

| *m/z* | *a* | *b* | $R^2$ | *LOD* [mg·kg$^{-1}$] | *LOQ* [mg·kg$^{-1}$] |
|---|---|---|---|---|---|
| 361 | $5.37 \times 10^{-5}$ | $7.46 \times 10^{-4}$ | 0.999 | 4.9 | 16.7 |
| 362 | $1.94 \times 10^{-5}$ | $3.71 \times 10^{-4}$ | 0.999 | 11.5 | 38.0 |
| 217 | $1.68 \times 10^{-5}$ | $2.20 \times 10^{-4}$ | 0.999 | 14.7 | 48.5 |

*m/z* – mass to charge ratio for measured ions, *a* – slope of calibration curve, *b* – intercept of calibration curve, $R^2$ – coefficient of determination, *LOD* – limit of detection, *LOQ* – limit of quantification.

saccharide sucralose, which does not occur naturally in honey and does not elute with the present saccharides. This procedure eliminates any content changes that may occur during silylation and sample dosing, which increases the accuracy of the analysis. Tab. 1 shows the calibration curve, *LOD* and *LOQ* values for melezitose. It is clear from Tab. 1 that the signal response to the content had a linear dependence over the entire measurement range (from *LOQ* to 40 000 mg·kg$^{-1}$). Also, *LOQ* and *LOD* for individual *m/z* changed in accordance with the ratio of monitored fragments in the mass spectrum of trimethylsilyl derivative of melezitose.

**Chromatographic separation**

Since a partial goal was the development of a fast method, individual separation parameters were optimized in order to speed up the analysis, while maintaining sufficient separation efficiency. For this reason, the length of the chromatographic column was minimized to 5 m, which allowed the analysis to be shortened several times compared to the previously published separations. The next optimization step consisted in adjusting the separation temperature program, where the use of the initial temperature of 220 °C allowed it to be significantly accelerated, mainly due to the reduction of the time required for cooling down the chromatograph oven. Because the proposed analysis procedure had a high sensitivity, we proposed dosing by the split method, thus saving approximately 1 min otherwise needed for focusing the analytes during splitless injection commonly used in this type of analysis. Fig. 1 shows the selected-ion-mode (SIM) GC-MS chromatogram for *m/z* of 361 obtained from the analysis of honeydew and floral honey.

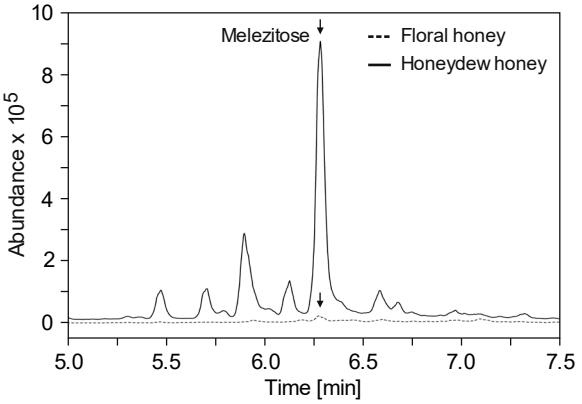


**Fig. 1.** Selected-ion-mode GC-MS chromatogram for *m/z* of 361 of honeydew and floral honeys.

From Fig. 1, it can be seen that the used separation system enabled the analysis of melezitose in honeydew and floral honey samples. The separation was efficient enough to separate co-eluting substances and sensitive enough to detect melezitose in floral honey. The time of the analysis was 9 min, which is almost 10 times shorter than the methods of GC analysis of melezitose in honey published to date.

**Analysis of honey samples**

The newly developed method was used to analyse real samples of honey obtained from small-scale farmers or purchased at a local market. The measured contents of melezitose in individual honey samples are shown in Tab. 2. From Tab. 2 it is clear that the newly developed method was sufficiently sensitive, which means that it can be used to determine the melezitose content in floral honeys as well. The relative standard deviation (*RSD*) of the determination ranged from 4.6 % to 14.1 %, which is a sufficient precision to distinguish individual honeys. In floral honeys (samples 1 to 9), melezitose content was in the range of 103 mg·kg$^{-1}$ to 2 880 mg·kg$^{-1}$. The content of melezitose in honeydew honeys ranged from 10 600 mg·kg$^{-1}$ to 26 100 mg·kg$^{-1}$. In honey samples obtained from local market, where the origin is not precisely identified, levels ranging from 96 mg·kg$^{-1}$ to 420 mg·kg$^{-1}$ were determined. It is clear from the obtained data that honeydew honeys always have a significantly higher content of melezitose than floral honeys, while the proportion between the lowest content in honeydew honey and the highest content in floral honey can

**Tab. 2.** Content of melezitose in samples of floral and honeydew honeys from various regions.

| No. | Honey type | Origin | | | Melezitose [mg·kg$^{-1}$] | *RSD* [%] |
|---|---|---|---|---|---|---|
| | | Village | Region | Country | | |
| 1 | Monofloral - rapeseed | Liptovský Ján | Liptovský Mikuláš | Slovakia | 376 | 4.6 |
| 2 | Floral | Spišská Nová Ves | Spišská Nová Ves | Slovakia | 402 | 6.5 |
| 3 | Floral | Divín | Lučenec | Slovakia | 103 | 7.0 |
| 4 | Floral | Chocholna-Velčice | Trenčín | Slovakia | 274 | 10.8 |
| 5 | Floral | Liptovský Ján | Liptovský Mikuláš | Slovakia | 1 030 | 11.6 |
| 6 | Floral | Spišská Nová Ves | Spišská Nová Ves | Slovakia | 2 880 | 14.1 |
| 7 | Floral | Ipeľský Sokolec | Levice | Slovakia | 1 200 | 9.8 |
| 8 | Floral - forest | Krásna Lúka | Sabinov | Slovakia | 441 | 11.5 |
| 9 | Floral - forest | Levice | Levice | Slovakia | 2 340 | 13.5 |
| 10 | Honeydew | Veľký Biel | Senec | Slovakia | 11 100 | 8.5 |
| 11 | Honeydew - fir tree | Bardejov | Bardejov | Slovakia | 24 100 | 7.2 |
| 12 | Honeydew - fir tree | Krásna Lúka | Sabinov | Slovakia | 18 600 | 8.6 |
| 13 | Honeydew | Rajčany | Topoľčany | Slovakia | 26 100 | 7.6 |
| 14 | Honeydew | Rajčany | Topoľčany | Slovakia | 24 800 | 9.2 |
| 15 | Honeydew | Alterilz | Styria | Austria | 10 600 | 10.4 |
| 16 | Honeydew | Linz | Linz | Austria | 25 900 | 11.4 |
| 17 | Floral | Local market | Not determined | Slovakia | 96 | 13.2 |
| 18 | Floral - forest | Local market | Not determined | Slovakia | 420 | 6.8 |

*RSD* – relative standard deviation.

be almost 4-fold. Thanks to this, it is possible to distinguish honeydew from floral honey with high degree of probability based on this marker.

## CONCLUSIONS

Reliable differentiation of floral and honeydew honeys by chemical analysis is still a serious problem. The presented newly developed method solved this problem by analysing melezitose, a trisaccharide, which has been identified as a potential marker in some publications. As part of the work, we developed a sufficiently sensitive method that allows determination of melezitose also in floral honeys. The method is very fast, the analysis itself takes approximately 9 min. Sample preparation is simple, which allows the use of automatization. For this reason, the newly developed method is especially suitable for screening the content of melezitose in honey. From the determined contents of melezitose in real samples from Slovakia and Austria, it was possible to clearly distinguish floral and honeydew honey.

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