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Characterization of Citrus Honey by Deuterium NMR

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Israeli honey samples were characterized with respect to the isotope ratio parameters $\delta^{13}C$, measured by mass spectrometry, and $D/H(CH_3)$ of the ethanols produced by alcoholic fermentation, measured by deuterium NMR. Ethanols obtained from fermentation of citrus honeys have $D/H(CH_3)$ values similar to ethanols from citrus juice and that exceed the values obtained from other honeys by 5 ppm. This difference in $D/H(CH_3)$ can be used to confirm the authenticity of citrus honeys. The $\delta^{13}C$ values of all honeys tested were similar and typical to C3 plants. Measurements of $D/H(CH_3)$ of ethanols derived from honey and of $\delta^{13}C$ of the honey sugars are useful for detection of adulterations of citrus honey.

Keywords: *Honey; citrus; ethanols; deuterium; NMR; adulteration*

INTRODUCTION

Citrus honey is the main honey produced in Israel. Questions concerning its purity arise not only from adulteration caused by feeding the bees with sucrose in seasons when they usually collect nectars, but also from contamination of pure citrus honey by foreign pollen left in the beehive combs from previous seasons. Stable isotope ratio analysis is a powerful tool to determine the origin of sugars in fruit commodities and in wines (Dunbar, 1982; Bricout and Koziet, 1984; Donner, 1991). In the last decade, advances in NMR technology made feasible measurements of molecular site specific isotope fractionation at natural abundance (SNIF-NMR) of deuterium (Martin et al., 1983, 1986). SNIF-NMR is currently used for the authenticity control of fruit juices and wines (Martin et al., 1988; Martin and Martin, 1988). In present work, Israeli honey samples were characterized with respect to the D/H ratios of the methyl group of the ethanols produced from them by alcoholic fermentation [$D/H(CH_3)$], and by the $\delta^{13}C$ values of their sugars. The results indicate that these measurements can be used to assess the purity of citrus honey.

EXPERIMENTAL PROCEDURES

Honey samples were diluted sixfold with tap water to obtain a final total soluble solid content of 13.5 g/kg, as measured by a refractometer. To 850 mL of diluted honey solution were added 0.75 g of $(NH_4)_2SO_4$, 4 g of yeast extract, and 0.75 g of dry yeast (*Saccharomyces bayanos*). Fermentation was conducted at 20 °C for 72–96 h. The fermented liquid was distilled in a stationary column (700 × 18 mm) filled with glass beads (diameter = 5 mm). Careful distillation yielded a distillate of 91–93% by weight from a fermented liquid of 5% ethanol, with a recovery of 93–95%. The water content of the distillate was determined by Karl Fischer titration with the reagent Titrant U (Merck 9233) and a pyridine-free methanolic solvent (Merck 9241).

Samples for NMR measurements were prepared by mixing 10 g of the distillate and 4.6 g of *N,N*-tetramethylurea (TMU) of known deuterium content (natural abundance), which was used as an intensity reference. Deuterium NMR measurements (61.4 MHz) were carried out on Bruker AM400 spec-

trometer using a 20-mm probe at 30 °C. The spectral settings were as follows: pulse width, $\pi/2$; sweep width, 1200 Hz; acquisition time, 3.998 s; recycle time, 4.0 s; data points, 4800; transients, 120; proton decoupling mode, CPD. Eight spectra with signal-to-noise ratios of >150 were recorded for each sample, and the isotope ratio on the methyl group [$D/H(CH_3)$] was calculated from the peak heights of the methyl signals of the ethanol and the TMU with the known D/H value of the TMU. This calculation is justified because the similarity of the line width of both signals. The $\delta^{13}C$ of honey samples was measured by standard methods practiced at the Geological Institute at the Hebrew University, Jerusalem (Zohary et al., 1994).

RESULTS AND DISCUSSION

The isotope ratio $D/H(CH_3)$ of ethanols obtained by fermentation of Israeli honey samples and $\delta^{13}C$ values of some Israeli honey samples are shown in Table 1. From these results it is clear that ethanols obtained by fermentation of citrus honeys have $D/H(CH_3)$ values ~4−6 ppm higher than ethanols produced from other honeys. In Table 2, typical values of $\delta^{13}C$ and of the isotopic ratios $D/H(CH_3)$ of ethanols obtained by alcoholic fermentation of a few commodities are shown.

The $\delta^{13}C$ and $D/H(CH_3)$ parameters are useful for discerning among sugars produced by the C3 and the C4 metabolic pathways (Goodwin and Mercer, 1983; Martin and Martin, 1986). Sugars from C4 plants have a higher content of heavy isotopes than C3 sugars. This fact is reflected by higher values of $\delta^{13}C$, $D/H(CH_3)$ of ethanols (see Table 2), and D/H of nonexchangeable hydrogens in cane sugar compared with values obtained from beet sugar (Bricout and Koziet, 1984; Martin et al., 1986). The $\delta^{13}C$ values of the honeys tested were typical of sugars produced by C3 metabolism and are similar to the values of beet sugar. Similar results were obtained by White and Winter (1989) who examined 50 certified samples of honey. The accepted values of $\delta^{13}C$ for natural honeys is in the range −23.5 to −27.0‰, which is typical for C3 sugars (White and Winter, 1989; AOAC, 1984).

Cactus, which is common in Israel, synthesizes sugars by the crassulacean acid metabolic (CAM) pathway (Goodwin and Mercer, 1983; O'Leary, 1988). Because the cactus does not flower at the same season as citrus plants, its nectar does not mix and affect the isotope ratio parameters of citrus honey. As expected, citrus honey and citrus juice have similar values of isotope ratio parameters (Table 2).

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Table 1. Isotope Ratio Parameters in Various Samples of Honey

source	D/H(CH$_3$)[a]	δ^{13}C%[b]
citrus	105.4	−23.7
citrus	105.2	−24.1
citrus	105.2	
citrus	104.9	−24.0
citrus	104.8	−24.4
citrus	104.7	−23.3
citrus	104.6	−23.9
citrus	104.1	−24.3
field flowers[c]	100.0	−25.7
field flowers[c]	100.0	−24.1
field flowers[c]	99.6	−23.6
field flowers[c]	99.2	−24.4
field flowers[c]	98.9	−25.0
field flowers[c]	97.8	
onion	100.3	−24.1
cotton	99.1	−25.1
eucalyptus	98.9	−24.1
zaatar[d]	98.2	−24.1
thorns[e]	97.8	−24.1
beet sugar[f]	95.3	

[a] ppm D/H of methyl group of ethanols obtained by alcoholic fermentation. [b] δ^{13}C = [[(^{13}C/^{12}C)$_{sample}$/(^{13}C/^{12}C)$_{standard}$] − 1] × 1000. [c] Mainly Cruciferea, Compositae, Labiatae, and Leguminosae. [d] *Majorana syriaca*. [e] *Centaurea verutum* L. [f] The bees were fed with beet sugar solutions.

Table 2. Isotope Ratio Parameters in Various Commodities

commodity	sugar type	D/H(CH$_3$)[a]	δ^{13}C%[a]
cane sugar	C4	111.5−112.5	−11.2 to −12.0
citrus juice	C3	104.5−108.0	−23.8 to −26.0
grape must	C3	101.7−96.2[b]	—[c]
beet sugar	C3	92.0−94.0	−24.5 to −26.7

[a] See Table 1. [b] Martin et al. (1988). [c] Not determined.

The morphology and amount of pollen observed by microscopic examination of honey is an important tool to verify its authenticity (Talpay, 1985). Israeli citrus honey that is produced from nectar collected in the Spring is often contaminated by pollen from other species remaining in the combs from the last autumn. For this reason, this honey is often disqualified and cannot be marketed as pure citrus honey. The results (Table 1) suggest that SNIF-NMR is a useful method to characterize citrus honey that can be used by the producers of citrus honey to prove the authenticity of their natural product.

Adulteration of honey is easily carried out by feeding the bees with sucrose solution. This avoids the inconvenience of preparing concentrated solutions of invert sugar and mixing this viscous product with honey. From the results in Table 2 it is seen that adulteration of honey by direct addition or feeding the bees with cane sugar solutions will increase both the D/H(CH$_3$) value of the ethanol produced from it and the δ^{13}C values. The increase in δ^{13}C values can be detected by mass spectrometry at the 10% level, by comparing the δ^{13}C values of the sugars and the protein in the tested samples (White and Winter 1989; Rossmann et al., 1992). The same is true for syrups derived from any C4 plants, like syrups derived from corn starch.

Besides evaporation of water, inversion of sugar is the main process occurring when flower nectars are transformed to honey by the bees. These processes do not affect the hydrogens covalently bound to the carbon skeleton of sugars, and it was shown by Martin et al. (1983) that sugar inversion has no effect on the SNIF-NMR parameters. Adulteration of citrus honey by excessive feeding with beet sugar will lower the D/H(CH$_3$) value of the ethanol produced from it, and this can be detected by SNIF-NMR. Thus, the lowest D/H(CH$_3$) value reported in Table 1, 95.3 ppm, is for ethanol from honey produced by bees fed with beet sugar solutions. This value is slightly higher than the values for ethanols from pure beet sugar solutions (i.e., 92.0−94.0 ppm; (Table 2). It seems that although the bees had free access to sucrose, they still collected some nectar from flowers.

For detection of adulteration by a mixture of C3 and C4 sugars, both δ^{13}C values for sugar and D/H(CH$_3$) values for ethanols have to be determined. Addition of beet sucrose syrups to honey, directly as invert sugar syrup or indirectly by feeding the bees, always lowers the D/H(CH$_3$) values of the ethanol derived from its, so citrus honey adulterated by moderate amounts of beet sugar syrup may appear to the analyst as an authentic noncitrus honey (see Table 1). Although the SNIF-NMR method is useful for verification of the purity of citrus honey, it cannot detect moderate adulterations by beet sucrose if the producer is not declaring his product to be a pure citrus honey.

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