JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

J. Agric. Food Chem. **2005,** *53,* 207−210 **207**

FT-Raman Spectroscopic Simultaneous Determination of Fructose and Glucose in Honey

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A new method for mass percentage determination of fructose and glucose based on FT-Raman spectroscopy is evaluated with a standard HPLC-based method. FT-Raman spectra manipulation is done via the spectrometer software, and a PLS (partial least squares) method is developed with the TQ Analyst software (Ver 1. 1a). The simultaneous quantitative determination uses an input range from 1700 to 700 cm^{-1} without correction or baseline factors. The standards used in the PLS method are honey samples previously analyzed by HPLC to obtain their mass percentage concentrations in fructose and glucose. The returned results are statistically tested with those of the HPLC method. Both methods appear to score equally in terms of reproducibility. The honey content of the two sugars in total was found up to 40−74%. The honey samples content in fructose and glucose was determined by HPLC (24.1−42.9% and 16.2−33.1%, respectively) and FT-Raman (24.0−40.8% and 21.1−32.2%, respectively).

KEYWORDS: FT-Raman; honey; fructose; glucose; quantitative determination

INTRODUCTION

Honey is an important agricultural product for Greece (*1*−*3*), known for its dietary and medicinal properties for centuries. A large proportion of honey's components consists of sugars, mainly fructose, glucose, sucrose, maltose, and melesitose and other mono- to oligo-saccharides. The quantitative determination of these sugars provides data connected to its floral origin. Unifloral honeys of different botanical origin vary in their sugar composition. Fructose content in Greek honeys varies between 27% and 44% for blossom honeys and 29−38% for honeydew honeys, whereas glucose varies between 22% and 40% and 19−32% for blossom and honeydew honeys, respectively. Therefore, the need to provide fast and accurate quantification of the sugars is important.

Techniques used so far for the determination of the percentage of each of the most abundant sugars in honey include the following: high performance liquid chromatography (HPLC) (*4*−*7*, *10*), gas chromatography−mass spectrometry (GC−MS) (*8*, *9*), nuclear magnetic resonance (NMR) (*10*), Fourier transform infrared spectroscopy (FT-IR) (*10*, *11*), and dispersive Raman spectroscopy (*12*). The application of FT-Raman spectroscopy for the detection of honey adulteration has been proposed (*13*).

In our work, FT-Raman spectroscopy is used to provide simultaneously the mass percentage concentration of fructose and glucose in honey. An HPLC classical method is used as a reference (*5*).

MATERIALS AND METHODS

Honey Samples. A total of 21 unifloral honey samples were analyzed three times with the HPLC method to obtain mass percentage concentrations of fructose and glucose. Their FT-Raman spectra were also recorded in triplets. Those honey samples were then divided into two groups: the first group of 11 honey samples was used to develop the PLS method library, therefore they were named as "standards" (St1−11), and the second group was used to evaluate the proposed against the standard method, therefore they were named as "unknown" (S1−S10).

Chemicals. Pure fructose and glucose were purchased from BDH, Poole England. Standard solutions of these two sugars were prepared as described by Bogdanov et al. (*5*) to be used as external reference for the HPLC method. All solvents were HPLC grade purchased from BDH. CHROMASIL filters of 45/25 pore size were also used.

Sample Preparation for HPLC Analysis. The determination of honey sugars via HPLC is described in detail by Bogdanov et al. (*5*). In our case, this is considered to be the reference method and is followed as described without any modification. Five (*5*) g of honey sample was diluted in 40 mL of water. Twenty-five (25) mL of methanol was transferred into a 100 mL volumetric flask, and the honey solution was added. The flask was then filled with water. The sample was poured through a membrane filter into sample vials and stored properly.

An Agilent 1100 series HPLC system consisting of a binary pump and a refractive index temperature-regulated detector was used. Analysis

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10.1021/jf048793m CCC: $30.25
Published on Web 12/17/2004

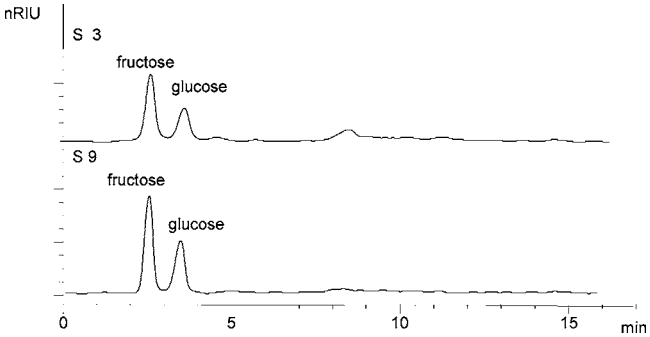

Figure 1. Typical HPLC chromatograms of two honey samples S3 (fructose 24.0%–glucose 21.1%) and S9 (fructose 39.4%–glucose 32.1%).

Table 1. Standards Content in Fructose and Glucose As Determined by HPLC

standard	fructose (%)	glucose (%)
St1	26.7 ± 0.07	17.3 ± 0.8
St2	35.0 ± 1	30.3 ± 0.9
St3	40.8 ± 1	32.9 ± 0.3
St4	36.0 ± 1	17.5 ± 0.2
St5	32.5 ± 0.6	14.8 ± 0.7
St6	30.6 ± 1	22.1 ± 0.8
St7	40.4 ± 1	35.3 ± 0.4
St8	40.1 ± 0.2	30.7 ± 1
St9	44.9 ± 1	24.6 ± 0.3
St10	29.0 ± 1	19.5 ± 0.2
St11	31.2 ± 0.3	22.6 ± 0.6

was held at 30 °C for the column and the detector. An analytical amino-modified column of 5 μm particle size 250 × 4.6 mm was set at a flow rate of 1.3 mL/min with a mobile phase of acetonitrile/water 80:20 (v/v). A sample volume of 20 μL was injected.

The mass percentage of the sugars was calculated via a single external standard procedure, based on the comparison of peak areas of the sugars to those of the standard solution according to the formula $w = (A_1 \times V_1 \times m_1 \times 100)/(A_2 \times V_2 \times m_o)$, where A_1 is the peak area of the given sugar compound, A_2 is the peak area of the given sugar compound in the standard solution, V_1 is the total volume of the sample solution in milliliters, V_2 is the total volume of the standard solution in milliliters, m_1 is the mass amount of the sugar in grams in the total volume of the standard (V_2), and m_o is the sample weight in grams.

To ensure reliability of the procedure, the standard solution was injected prior to each batch of measurements. Each sample was measured with the above procedure three times to obtain the standard deviation for statistical treatment.

FT-Raman Spectroscopy. FT-Raman spectra were recorded using a Nicolet 750 FT-Raman spectrometer, equipped with a Nd:YAG laser source that emits at 1064 nm. In addition, a CaF_2 beam splitter, an indium−gallium−arsenide (InGaAs) detector, and 180° backscattering geometry were used in the spectrometer. Raman laser power at sample was set at 1.5 W. Routine procedures such as bench alignment and fine-tuning of the spectrometer were held before each batch of measurements. Sample cells were Wimad WG-SM NMR tubes of 4.97 mm outer diameter and 0.38 mm wall thickness. Spectra were accumulated from 200 scans collected at a resolution of 8 cm^{-1}. Spectra of each sample were collected in triplicate. Each sample was manipulated with the built-in "automatic smooth" and "automatic baseline correct" functions of the software.

RESULTS AND DISCUSSION

Figure 1 shows two typical chromatograms of S3 and S9 honey samples as they were analyzed by HPLC. The retention time for fructose was approximately 6 min, while for glucose it was 6.8 min (**Table 2**). The mass percentage content for the 11 "standards" fluctuated between 26.7% and 44.9% and 14.8−35.3% for fructose and glucose, respectively. The 10 "unknown" samples were quantified at 24.1−42.9% and 16.2−33% for fructose and glucose, respectively. The standard deviation values were between 0.3 and 3, and 0.2−4 for fructose and glucose, respectively.

The method chosen for the manipulation of spectra with the TQ Analyst software was PLS because according to the manual it is proposed for quantification of components whose peaks shift or overlap in their mixtures. The only restriction is that the standards number must be ≥3 × the number of components. This restriction is met because we use 11 standards to quantify two components. The software used to develop the PLS method requires the input of spectra, path length, and the concentrations of the targeted components. The path length option was chosen to be constant because the beam crosses the same amount of sample in each measurement. This is the same as the internal diameter of the NMR tube used for sample acquisition. To obtain input spectra of known glucose and fructose content, either standard mixtures of pure fructose and glucose, or honey of known fructose and glucose content spectra, must be recorded. As it is shown in **Figure 2**, the spectra of fructose and glucose show major differences in spectrum texture and significant band shifts from the honey spectra. Therefore, the proposed method was developed of honey spectra and their content in fructose and glucose as determined by HPLC.

The most important spectral region lies between 1700 and 700 cm^{-1}, because characteristic groups and sugars emit at this region (**Figure 2**). In this region, neither fructose nor glucose show characteristic peaks to correlate to their concentration in honey. The region used for the TQ Analyst software spectra library between 1700 and 700 cm^{-1} (**Figure 2**) has nine characteristic peaks, and fructose and glucose show peaks throughout this region.

The peaks shown in the honey spectra are discussed below. The first peak at 1460 cm^{-1} corresponds to the $-CH_2-$ bending

Table 2. "Unknown" Honey Samples Content in Fructose and Glucose As Determined by HPLC and FT-Raman

unknown sample	RT[a] fructose	HPLC fructose (%)	FT-Raman fructose (%)	*F* test	*t* test	RT[a] glucose	HPLC glucose (%)	FT-Raman glucose (%)	*F* test	*t* test
S1	6.079 ± 0.003	38 ± 1	37 ± 2	4	1	6.971 ± 0.002	22.9 ± 0.8	24 ± 2	4	1
S2	6.11 ± 0.01	29.3 ± 0.3	31 ± 3	9	1	7.02 ± 0.01	24.2 ± 0.2	23 ± 4	16	1
S3	6.103 ± 0.004	24.1 ± 0.8	24.0 ± 0.4	0.6	0.2	7.005 ± 0.005	16.2 ± 0.2	21.1 ± 0.9	0.8	9.2
S4	6.036 ± 0.005	40 ± 3	36 ± 3	1	2	6.94 ± 0.01	33 ± 2	28.1 ± 0.8	4	4
S5	6.047 ± 0.004	33 ± 1	34 ± 4	16	0	6.940 ± 0.006	24.3 ± 0.2	26 ± 4	16	1
S6	6.06 ± 0.03	37.3 ± 0.8	37.1 ± 0.7	0.6	0.3	6.97 ± 0.04	32 ± 4	31 ± 2	16	0
S7	5.991 ± 0.005	37.6 ± 0.9	41 ± 2	4	3	6.874 ± 0.004	31.0 ± 0.9	32 ± 3	9	1
S8	6.09 ± 0.01	38.3 ± 0.6	41 ± 3	9	2	7.01 ± 0.01	32.4 ± 0.9	31.3 ± 0.9	1	1.5
S9	6.000 ± 0.006	42.9 ± 0.9	39 ± 1	1	5	6.88 ± 0.01	31 ± 3	32 ± 1	9	1
S10	6.03 ± 0.04	41.2 ± 0.9	39.3 ± 0.8	0.8	2.7	6.92 ± 0.06	29 ± 1	32.1 ± 0.9	1	4

[a] Retention time.

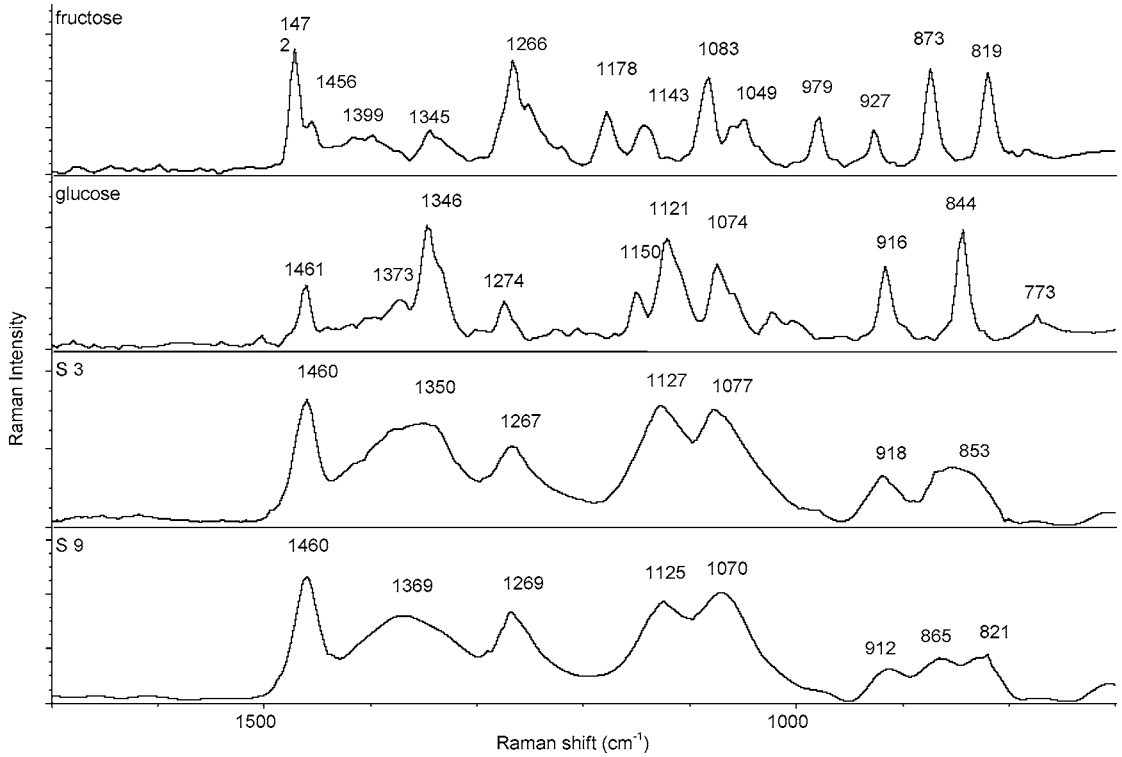

Figure 2. FT-Raman spectra of solid fructose and glucose and two honey samples S3 (fructose 24.0%–glucose 21.1%) and S9 (fructose 39.4%–glucose 32.1%).

(*12*). The region between 1430 and 1300 shows two peaks, one at 1372 cm^{-1} reported for the bending of CH and OH (*12*) and another at 1349 cm^{-1} assigned to CH_2 wagging vibration (*14*, *15*). A strong and sharp peak at 1268−1267 cm^{-1} was reported for the vibration of C(6)−OH and C(1)−OH (*13*). C−O stretching vibration may result at 1126−1124 cm^{-1} (*15*). A strong peak at 1077−1070 cm^{-1} could arise due to a major contribution by the bending vibration of C(1)−H and COH (*13*, *14*). A minor peak at 922−918 cm^{-1} was attributed to the bending vibration of C(1)H and COH (*13*, *16*). Two moderate peaks at 865 and 821 cm^{-1} were found to be due to the vibration of CH (12) and C(1)H, CH_2 (*16*) shown as one broad band on the spectrum of S3. A moderate peak at 707−706 cm^{-1} corresponds to the stretching of CO and CCO, OCO bending (*16*). The above peaks are common in the pure sugars spectra but are either overlapped or shifted in the honey spectra.

In our case, 11 honey samples were chosen as standards to include a wide range of concentrations of the sugars in question. Those 11 samples were analyzed by HPLC, and their content in fructose (26.7−44.9%) and glucose (14.8−35.3%) (**Table 1**) was used as input for the TQ analyst PLS method.

The 10 "unknown" honey samples content in fructose and glucose as determined by FT-Raman (24−41% and 21.1−32.1%, respectively) in triplets are shown in **Table 2**. The standard deviation values were between 0.4 and 4, and 0.8−4 for fructose and glucose, respectively.

To compare the two methods reproducibility and accuracy, the *F*-test and *t*-test statistical tools were used. In all measurements, the two methods score statistically below the theoretical value of 19.00 for the *F*-test, and in 4 out of 20 cases they score above the theoretical value of 2.776 for the *t*-test (**Table 2**). In addition, the coefficient of variation or relative standard deviations was also calculated for each unknown sample (**Table 3**). Thus, the two methods are statistically equivalent in terms of accuracy and reproducibility.

Table 3. Unknown Sample Relative Standard Deviations

unknown sample	RSD[a] fructose[b]	RSD fructose[c]	RSD glucose[b]	RSD glucose[c]
S1	3.850	5.226	3.474	8.318
S2	0.926	11.078	0.923	16.100
S3	3.214	1.725	1.266	4.314
S4	6.357	8.805	4.612	2.918
S5	2.913	10.385	0.968	16.414
S6	2.126	1.743	10.787	6.855
S7	3.051	5.224	2.757	10.012
S8	1.515	7.153	2.771	2.889
S9	2.037	3.852	8.964	3.799
S10	2.228	2.056	3.952	3.096

[a] Relative standard deviation. [b] HPLC method. [c] FT-Raman method.

FT-Raman spectroscopy for the mass percentage determination of fructose and glucose in honey is a good alternative to time-consuming and complicated methods. The fact that spectra are recorded without any sample preparation further simplifies the proposed method. No solvents or consumables are needed for such simple analyses, and so the cost analysis may be reduced significantly. The capacity of a setup based on the proposed method may be many times higher than standard HPLC in terms of analysis/working day.

In conclusion, the simultaneous mass percentage determination of fructose and glucose in honey has been achieved with the application of FT-Raman spectroscopy combined with the appropriate software. The proposed method further demonstrates the efficiency of transmittance spectroscopy methods for the quantitative analysis of complex mixtures. The standard HPLC method applied provided input for the development of the spectra library as well as served as a reference method to statistically evaluate the new method. Fructose was found to range between 24.0& and 42.9%, while glucose ranged between 16.2% and 33.1%. The major drawbacks for the application of other analytical methods as they have been reported so far are

long analysis times (HPLC, GC−MS) and the necessity of certain reactions to be held prior to the analysis (i.e., acetylation for GC−MS analysis). The application of NMR spectroscopy is demanding in infrastructure and is not suggested for application in complex mixtures of natural products such as honey.

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Received for review July 20, 2004. Revised manuscript received November 1, 2004. Accepted November 1, 2004. The authors thank the Greek Ministry of Agriculture and the European Union for financially supporting this research according to the Council of Regulation (EC) 1221/97.

JF048793M