

Methods for Determining Carbohydrates, Hydroxymethylfurfural, and Proline in Honey: Collaborative Study

JONATHAN W. WHITE, JR.¹

*U.S. Department of Agriculture, Federal Research, Science and Education Administration,
Eastern Regional Research Center, Philadelphia, PA 19118*

A modification of the official selective adsorption method for honey carbohydrates, 31.124-31.133, was studied collaboratively; the determinations of sucrose, total monosaccharides, disaccharides, and higher sugars by this procedure were satisfactory and were adopted by the AOAC. High pressure liquid chromatography of glucose, fructose, and sucrose in honey showed better precision than the modified official method and gave concordant results; it was also adopted. Two methods for hydroxymethylfurfural do not qualify. A method for proline was also adopted.

The official selective adsorption (SA) method for carbohydrates in honey (1) uses copper reduction and hypiodite oxidation for determining individual sugars (fructose, glucose, sucrose, reducing disaccharides as maltose, and higher sugars) after class separation by adsorption on activated charcoal columns. Although this method led to a considerable revision of knowledge of the composition of honey (2), the somewhat cumbersome wet methods have limited its acceptance as a regulatory procedure. Comparable results have been obtained with simplified procedures (3) in the laboratory of the Associate Referee. A high pressure liquid chromatographic (HPLC) method has also been reported for determining glucose, sucrose, and maltose in honey (4).

The presence of enough hydroxymethylfurfural (HMF) in honey to respond to the aniline chloride or resorcinol test (31.138-31.139) has long been considered indicative of adulteration with commercial invert sirup. However, it has been recognized that honey may generate enough HMF for a positive test by excessive exposure to heat from improper processing or storage. A quantitative method is needed for HMF, since amounts produced by handling or storage abuse, although providing equivocal or positive color tests, are generally lower than those arising from adulteration.

A chemical procedure and an ultraviolet (UV) absorption procedure for HMF were described by Winkler (5); the former has been adopted by the Codex Alimentarius (6). Neither method has been collaboratively studied; the simplicity of the UV method would make it preferable if concordant results could be obtained.

Proline is a natural constituent of honey and has been useful in distinguishing honey and sirups. The method of Ough (7), in which the predominant free amino acid reacting with acid ninhydrin solution is proline, is specific. Interferences from other amino acids is $\leq 5\%$, which is insignificant when their relative occurrence in honey is considered. This method has been applied to honey samples (8): The distribution of proline content was surveyed in 740 samples of United States honey.

Collaborative Study

Six 10-lb containers of processed honey from different lots were obtained from a commercial packer. Two containers each represented 3 different colors.

After preliminary analysis for sucrose and proline by the methods under study, aqueous solutions of these compounds were added to 4 of the samples; 2 samples already contained the concentrations desired. This produced 3 pairs of samples, 2 each low, average, and high in sucrose and proline content. Each sample was then evaporated in a rotary vacuum evaporator to the original density.

HMF was determined spectrophotometrically in each sample; 4 were selected to represent average and high values for this constituent. Two composite samples were prepared from available unprocessed honeys to provide a pair with low HMF content. Each sample was thoroughly mixed to ensure homogeneity. The low HMF samples (Samples G and H) were not heated. Aliquots were shipped in 2 oz wide-mouth screw-cap polypropylene bottles with instructions to refrigerate the samples which were

¹ Present address: 217 Hillside Dr, Navasota, TX 77868.

Table 1. Composition of collaborative samples for determining carbohydrates, hydroxymethylfurfural, and proline in honey

Sample	Component ^a		
	Sucrose	Proline	HMF
A	average	high	—
B	average	low	—
C	high	average	average
D	low	average	average
E	high	high	high
F	low	low	high
G	—	—	low
H	—	—	low

^a No effort was made to adjust concentrations of the remaining components, which ranged as follows: glucose 29.4–33.31; fructose 35.7–41.1; maltose 7.9–9.6; monosaccharides 67.2–72.6; disaccharides 9.7–13.2; higher sugars 1.4–2.1.

the lowest in HMF. See Table 1 for composition of collaborative samples.

p-Toluidine, although not on the Occupational Safety and Health Administration list of carcinogens, carries a warning label stating that it produces cancer in animals and listing precautions in its use. This information was brought to the attention of the collaborators.

As a convenience, standardized charcoal (Darco G60) and filter aid (Dicalite 4200) (1+1), (31.125), enough for 2 adsorption columns, instructions for an alternative wet packing procedure (2), and a practice sample of known composition were sent to the collaborators. The collaborators were requested to report one result for each constituent determined by each method.

The HMF and sucrose determinations were required; the remaining carbohydrate and proline analyses were optional but were recommended because the column fractions in which the sucrose is determined would be available as a result of the sucrose determination.

A high pressure liquid chromatographic (HPLC) method (4) for glucose, fructose, and sucrose was also included as an option, to be used on the same samples by laboratories possessing adequate instrumentation, to provide comparative analyses.

Experimental

Carbohydrates

Storage precautions were unnecessary to prevent reduction of sucrose content and change of monosaccharides by action of honey invertase because the samples had been heat-processed.

Procedural differences between the official method for honey carbohydrates and the modified SA method tested in this study are summarized in Table 2. Results from the specific glucose oxidase method for glucose in honey (9), when applied to whole honey solutions, do not differ from those by the official method. Details of the HMF procedures appear elsewhere (5).

Hydroxymethylfurfural

The collaborators were instructed to determine the purity of their standard HMF by measuring the absorbance at 284 nm and using an absorptivity of 16,830 L/mol cm (10).

The calibration factor of Winkler (5) was provided for calculating the HMF content from the UV absorbance values as follows:

$$\text{HMF (mg/100 g honey)} = 43.1 \times [A_{285} - (A_{245} + A_{325})/2] / \text{light path in cm}$$

Later calculations, using data from Winkler's paper, showed the HMF which had been used by Winkler had a molar absorptivity of 15,600. Hence, the factor should have been $43.1 \times 15,600/16,830 = 39.95$. Therefore, all collaborative results reported by this method were multiplied by $15,600/16,830 = 0.927$ to compare these results with results from the chemical method.

Proline

In the procedure supplied, analysts were instructed to dilute 2.5 g honey to 100 mL with water. This results in rather low absorbance values, more useful for samples with proline content of 100 mg/100 g. Dilution to 50 mL would have been preferable as a routine.

Only the methods recommended for adoption are described below; some suggestions from the collaborators have been incorporated in the methods.

Chromatographic Separation of Sugars

Alternative Method

31.133

Principle

For use when sucrose is sugar of primary interest. Sugars are sepd by charcoal column, 31.124–31.125. Glucose is detd on disaccharide fraction 2 by glucose oxidase before and after invertase hydrolysis and calcd to sucrose. Other sugars are detd by weighing residues of sepd fractions.

Table 2. Differences between the official AOAC method and the proposed method for carbohydrates in honey

Item	Official method	Modified method
Preparation of fractions	adsorpt. on stdzd charcoal filter aid column, desorpt. by dil. alc. soln to provide 3 fractions ^a	same
Glucose (fraction 1)	hypoiodite oxidn under controlled conditions	glucose oxidase reagent
Fructose (fraction 1)	reducing value with Shaffer-Somogyi reagent after hypoiodite destruct. of glucose	by diff. betw. dry wt of fraction 1 and glucose value
Sucrose (fraction 2)	increase of reducing value with Shaffer-Somogyi reagent caused by mild acid hydrol., with correction for reducing disacch.	increase in glucose by glucose oxidase after yeast invertase hydrol.
Maltose (reducing disacch.) (fraction 2)	reducing value by Shaffer-Somogyi reagent calibr. against maltose	by diff. betw. dry wt of fraction 2 and sucrose value
Higher sugars (fraction 3)	reducing value, as glucose, after 1 hr hydrol. in boiling 1N HCl	dry wt of fraction 3

^a For example, 1% alcohol elutes monosaccharides, 7% alcohol elutes disaccharides, and 50% alcohol elutes higher sugars.

31.134**Reagents**

(a) *Column*.—Prep. as in 31.124. Alternatively, use slurry prepn: Place glass wool plug at bottom of column and add ca 1 cm dry filter aid (Dicalite 4200, or equiv.). Wet filter aid layer from below. With outlet open, add slurry of 20 g adsorbent mixt. in 200 mL H₂O from top. Let drain 5 min and apply 4 psi (27.6 kPa) pressure until surface is stabilized. Then apply 10 psi (69 kPa) pressure, release, and remove excess adsorbent beyond 17 cm depth by suction from above. Add ca 1 cm filter aid. Wash column as in 31.124.

(b) *Acetate buffer soln.*—0.1M, pH 4.5. Add 5.72 mL HOAc to 500 mL H₂O, adjust to pH 4.5 with 1M NaOH soln, and dil. to 1 L.

(c) *Tris buffer soln.*—pH 7.6. To 48.44 g tris(hydroxymethyl)aminomethane (available as Trizma base, No. T 1503, Sigma Chemical Co.) in 500 mL H₂O, add 384 mL 0.8M HCl, adjust to pH 7.6 if necessary, and dil. to 1 L.

(d) *Glucose oxidase-peroxidase reagent (GOP)*.—Dissolve 120 mg glucose oxidase (Type II: purified, 15,000–20,000 units/g; Sigma Chemical Co. G 6125, or equiv.) and 32 mg peroxidase (Type I: from horseradish, salt-free powder; Sigma Chemical Co. P 8125, or equiv.) in 400 mL tris buffer, (c). Add soln of 270 mg *o*-tolidine.2HCl (available from Fisher Scientific Co. as Fisher certified T-320) in 520 mL H₂O. Refrigerate in brown bottle. Filter before use, if necessary. Stable ≥6 weeks.

(e) *Invertase reagent*.—Dissolve 12.5 mg invertase (Grade VI, from baker's yeast, essentially melibiase-free, activity ca 200 units/mg; Sigma Chemical Co. I 5875, or equiv.) in 50 mL pH 4.5 acetate buffer soln, (b).

(f) *Glucose std soln.*—0.1 mg/mL. Dissolve 25.0 mg

glucose (SRM 41, NBS) in 25 mL H₂O in 250 mL vol. flask. Boil 2 min and dil. to vol. or dil. to vol. and hold final soln 2 hr before use.

31.135**Preparation of Fractions**

Proceed as in 31.126.

31.136**Determination of Sucrose**

Pipet 2 mL fraction 2 into each of four 18×150 mm test tubes. Prep. 2 series, one control, other inverted. For each series, arrange in rack tube with 2 mL H₂O, 2 sample tubes, tube with 2 mL glucose std, 2 sample tubes, etc., finishing with 2 mL glucose std. To all tubes in control series add 0.50 mL H₂O; to all tubes in inverted series add 0.50 mL invertase reagent (or 0.50 mL pH 4.5 acetate buffer may be added to std tubes). Hold all tubes 30 min at room temp.

At intervals appropriate to measuring system to be used (i.e., 30 or 60 sec with flow-thru cells; longer with manual cells), add 5.00 mL room temp. glucose oxidase reagent to each tube, beginning with inverted series followed by control series. After 60 min, add 0.15 mL 4N HCl to first tube and mix thoroly (vortex mixer). Continue adding 4N HCl at same intervals as previously established. One min after first addn, det. A at 530 nm.

Av. A for each pair of sample tubes and use as std A av. of stds read before and after corresponding sample tubes.

$$\mu\text{g Glucose} = (\mu\text{g glucose in std tube}) \times (A \text{ of sample tube} / A \text{ of std tube})$$

$$\% \text{ Sucrose in honey} = 0.02375 (\mu\text{g glucose in inverted tube} - \mu\text{g glucose in control tube}) / \text{g sample},$$

where $0.02375 = \mu\text{g glucose} \times 1.9 \times 10^{-6} \times (1/2) \times 250$

$\times 100$; $\mu\text{g glucose} \times 1.9 = \mu\text{g sucrose}$; $10^{-6} = \mu\text{g/g}$; $\frac{1}{2} = 2 \text{ mL analyzed}$; $250 = \text{mL diln of sample}$; $100 =$ to convert to %.

31.137 **Distribution of Sugars**

Filter fractions if filter aid is visible. Evap. to dryness, on steam bath with current of air or N, 50.0 mL fraction 1, 100 mL fraction 2, and entire fraction 3, finally transferring each fraction to sep. weighed 50 mL beakers. Dry to const wt in vac. oven at $\leq 95^\circ$.

- % Monosaccharides = g fraction 1 \times 500/ g sample
- % Disaccharides = g fraction 2 \times 250/ g sample
- % Higher sugars = g fraction 3 \times 100/ g sample

High Pressure Liquid Chromatographic Method

31.138 **Apparatus**

(a) *Chromatograph*.—Waters Associates Model ALC/GPC, or equiv., with Model 6000 solv. delivery system and Model U6K injector.

(b) *Detector*.—Waters Associates R401 refractive index detector, or equiv.

(c) *Recorder*.—Varian Aerograph Model A-25 dual pen recorder, or equiv.

(d) *Column*.—300 \times 4 (id) mm μ -Bondapak/Carbohydrate (Waters Associates, No. 84038).

(e) *Magnetic stirrer*.—Fisher Versamix stirrer No. 14-511-90 (Fisher Scientific Co.), or equiv.

(f) *Sample clarification kit*.—Available in kit form from Waters Associates (No. 26865), or equiv.; 0.45 μm filters stable in org. solvs are suitable.

(g) *Syringes*.—10 μl No. 701-N point style No. 1, 2 \times 0.020" od, 25 gage needle (Hamilton Co.).

31.139 **Reagents**

(a) *Mobile phase*.—Nonspectro acetonitrile dild with H_2O (83+17). Degas mobile phase daily by mag. stirring 15 min under vac.

(b) *Sugar std soln*.—Place 3.804 g fructose, 3.010 g glucose, and 0.602 g sucrose into 100 mL vol. flask, dissolve in 50 mL H_2O , and add CH_3CN to vol. Composition of std approximates 5 g honey dissolved in 50 mL aq. CH_3CN (1+1).

31.140 **Operating Conditions**

Fructose, glucose, and sucrose are baseline sep and quantitated in 20 min under following conditions: flow rate, 1.0 mL/min (ca 500 psig, 345 kPa); temp., ambient (ca 23 $^\circ$); detector (R401), 8 \times (fructose and glucose) and 2 \times (sucrose); attenuation, 10 mv on recorder, detector set so that 380 μg fructose gives full-scale deflection of pen; and chart speed, 0.1"/min. Mono-, di-, and trisaccharides are eluted from column in order of MW.

Received June 20, 1978. Accepted June 26, 1978.

31.141 **Preparation of Sample**

Weigh 5.000 g sample in small beaker and transfer to 50 mL vol. flask with 25 mL H_2O . Immediately dil. to vol. with CH_3CN and filter thru 0.45 μm filter, using sample clarification kit.

31.142 **Chromatography**

Inject 10 μL std soln into chromatograph. Establish retention times, measure peak hts, and check reproducibility. Repeat for sample soln. Calc. glucose, fructose, and sucrose from integrator values or from peak hts as follows:

$$\text{Wt \% sugar} = 100 \times (PH/PH') \times (V'/V) \times (W'/W)$$

where PH and PH' = peak hts (or integrator values) of sample and std, resp.; V and V' = mL sample and std (50 and 100) solns, resp.; and W and W' = g sample (5.000) and std, resp.

Reference

JAOAC 60, 838-841(1977)

Proline

31.116 **Principle**

Proline, predominant free amino acid of honey, is reacted with acid ninhydrin soln. Interference from other amino acids is negligible, $\leq 5\%$.

31.117 **Reagents and Apparatus**

(a) *Ninhydrin soln*.—3%. Dissolve 3.0 g ninhydrin in 100 mL peroxide-free ethylene glycol monomethyl ether. Store solv., not reagent, over Zn metal in amber bottle.

(b) *L-(-)-Proline*.—Eastman No. 2488; dry in vac. oven and store in desiccator. Prep std solns as follows: (1) *Stock soln*.—0.5 mg/mL H_2O . Dil. 25 mg proline to 50 mL with H_2O . Refrigerate stock soln. (2) *Working soln*.—50 $\mu\text{g}/\text{mL}$. Dil. 10 mL stock soln to 100 mL with H_2O . Prep. fresh daily.

(c) *Reaction tubes*.—18 \times 130 mm borosilicate screw-cap tubes with Teflon liners.

31.118 **Determination**

Weigh 2.500 g honey, transfer to 50 mL vol. flask, and dil. to vol. with H_2O . Pipet 0.5 mL into each of 3 reaction tubes, add 0.25 mL HCOOH and 1.00 mL ninhydrin soln. Cap tightly, shake well, and place in boiling H_2O bath 15 min. Cool 5 min in 22 $^\circ$ H_2O bath, remove cap, and pipet 5 mL aq. isopropanol (1+1) into each. Mix well and det. A at 520 nm against blank of H_2O carried thru method. Read all tubes within 35 min of cooling.

Correct for color of honey by detg A of soln contg 0.5 mL prep'd honey soln, 1.25 mL H_2O , and 5.00 mL

isopropanol (1+1). Subtract value from that of reacted sample before calcg.

Prep. calibration curve as in detn, using proline std soln instead of honey. A of 0.5 mL of soln of 50 μg proline/mL is ca 0.35 in 10 mm cell.

Calc. mg proline/100 g honey.

Reference

J. Food Sci. **34**, 228-230(1969)

J. Apicul. Res. **17**, 89-93(1978)

Results and Discussion

Results were received from 17 collaborators, about half of whom conducted the optional analyses. All data are shown in Tables 3-6, with a statistical analysis of each group. The single analyses were treated in pairs, as Youden (11) recommends, to estimate precision and accuracy. Before analysis, Youden's ranking test was applied to identify collaborators reporting results for all samples that were greatly different ($P = 0.05$) from the others and therefore showed a pronounced systematic error. These are identified in the tables and the results for all 6 samples of an identified collaborator for that analysis were eliminated from the statistical calculations. In addition, Dixon's test for outliers (12) was used once in each set to identify the few single values greatly differing ($P = 0.05$) from the remaining data. Because the unit block procedure was used, the value paired with the outlier was also eliminated. Seven of the 490 values reported for all analyses (except HPLC) were eliminated from the calculations after this test was applied, as well as the 7 values paired with them. The presence of significant ($P = 0.05$) systematic error for each pair of samples for each constituent determined is shown in the table by the F -value, calculated as recommended by Youden (11).

Carbohydrate by Modified Selective Adsorption (SA) Procedure

Although the procedures tested here have given acceptable precision in the Associate Referee's laboratory, it must be concluded that, under the conditions of the collaborative study, they do not provide an acceptable level of inter-laboratory agreement for measuring glucose and fructose in honey. The s_d for fructose is several times that for glucose. Since fructose is calculated by difference between 2 other measurements, variations in its determination derive from additive variations—those of weight of

monosaccharide fraction and the glucose analysis. This is obvious from the magnitude of the F -values for fructose, significant for 2 of the 3 pairs. Significant systematic error was not present in the glucose determinations. An alternative to the use of the official wet method for glucose and fructose analysis on the column fraction may be polarimetric analysis of the evaporated fraction, which gave values concordant with the official method in a limited study (13).

Determination of sucrose by this procedure is satisfactory; no significant F -value for systematic error was obtained. The s_d for maltose (actually nonsucrose disaccharides) is roughly double that for sucrose, again reflecting its calculation by difference. Distribution of carbohydrates in the monosaccharide, disaccharide, and higher sugar categories is useful in identifying falsified materials. The precisions reported in Table 3 for the measurements are adequate for the purpose of the analysis. A significant systematic error for the disaccharide measurement is shown by 2 significant F -values. It is possible that the systematic error and the precision of the disaccharide measurement could be improved sufficiently by evaporating 100 mL rather than the 50 mL portion of the column eluate.

Carbohydrates by High Pressure Liquid Chromatography

Five collaborators had suitable equipment for this analysis. While this number may be minimal, collaborative results (Table 4) indicate the method is promising for simplifying honey analysis. Collaborator 18 did not use the column specified in the procedure, but used Amino-Sil-X-I $\text{\textcircled{R}}$, 13 μm , instead. Although this could disqualify the results from that collaborator, in view of the small number reporting, the data were retained and subjected to Youden's ranking test and Dixon's test for outliers. These tests eliminated all the glucose results and 4 of the sucrose results reported by Collaborator 18 from the statistical calculations shown in the table.

In comparing the standard deviations paired for the HPLC and modified SA methods (Table 5), the calculated values are lower for the HPLC procedure in 7, 6, and all 9 comparisons for glucose, fructose, and sucrose, respectively. Systematic error was not significant for 8 of the 9 pair comparisons. Differences between the

Table 3. Collaborative results for determining carbohydrates in honey by a modified AOAC method

Coll.	Sample ^a					
	A	B	C	D	E	F
Glucose						
1	32.93	32.83	30.18	31.50	29.68	30.87
4	25.78 ^b	27.31 ^b	30.75	32.45	36.63 ^b	37.77 ^b
6	33.97	34.06	31.66	30.52	27.92	32.25
8	33.36	31.19	28.40 ^b	30.23 ^c	27.35	29.39
9	32.01	33.97	30.56	31.69	30.39	31.43
11	34.62	33.68	31.29	32.45	30.77	32.51
13	32.86	31.63	31.46	33.16	30.16	30.60
Mean ^d	33.29	32.89	30.98	31.96	29.38	31.18
s _d		1.168		0.786		1.542
s _r		1.001		0.755		0.962
s _b		0.426		0.153		0.852
F ^e		1.36		1.08		2.57
DF ^f		5		5		5
Fructose						
1	38.91	39.33	38.86	39.59	36.76	38.22
4	44.83	46.01	37.83	35.40	30.67	27.03
6	37.65	37.65	36.38	39.28	36.24	35.03
9	40.89	41.18	39.24	39.06	38.51	40.32
11 ^g	35.43	36.67	35.21	33.20	34.53	32.94
13	39.49	41.26	36.01	37.05	36.43	38.90
Mean ^d	40.35	41.07	37.64	38.08	35.72	35.70
s _d		4.154		1.850		5.819
s _r		0.482		1.3		1.805
s _b		2.918		0.876		3.912
F ^e		74.4**		1.81		10.39*
DF		4		4		4
Maltose						
1	8.19	9.02	9.00	9.30	10.17	10.31
4	8.06	8.81	10.80	9.20	8.70	8.58
5	8.07	8.82	8.09	9.26	8.47	10.25
6	7.18	8.45	7.39	9.17	8.64	9.57
9	8.42	9.07	8.22	9.18	9.39	8.78
11	7.32	8.64	7.96	8.76	8.60	9.65
13	8.14 ^c	9.86 ^b	9.12	9.49	10.24	10.18
Mean ^d	7.87	8.80	8.65	9.19	9.17	9.62
s _d		0.514		0.856		0.857
s _r		0.205		0.755		0.589
s _b		0.333		0.285		0.440
F ^e		6.27*		1.29		2.12
DF		5		6		6
Sucrose^h						
1	2.52	2.12	4.83	0.66	4.67	0.75
2	2.30	1.85	3.57	0.59	3.79	0.72
3 ^o	3.00	2.55	6.12	0.92	7.85	1.30
4 ^o	2.14	1.57	2.93	0.53	3.67	0.66
5	2.95	2.39	5.20	0.77	4.51	0.71
6	2.74	1.74	4.87	0.00 ^b	4.85	0.62 ^c
7	2.31	1.95	4.58	0.54	4.15	0.60
8	1.93	1.94	4.32	0.55	3.95	0.59
9	2.48	2.08	4.63	0.57	4.06	0.47
11	2.48	2.21	4.24	0.59	4.45	0.65
13	2.63	2.09	5.05	0.67	4.69	0.70
14	2.17	1.88	4.05	0.59	4.65	0.53
Mean ^d	2.45	2.03	4.53	0.614	4.47	0.636
s _d		0.297			0.544	0.107
s _r		0.180			0.306	0.055
s _b		0.167			0.306	0.065
F ^e		2.72			3.16	3.80
DF		9			9	8

Table 3. (Continued)

Coll.	Sample ^d					
	A	B	C	D	E	F
Monosaccharides						
1	71.84	72.16	68.54	71.09	66.44	69.09
4	70.61	73.32	66.15	70.28	67.20	64.80
5	72.09	72.87	69.52	71.76	69.90	71.89
6	71.62	71.62	68.04	69.80	65.96	67.28
9 ^e	72.90	75.15	69.80	70.75	69.80	71.75
11 ^e	70.05	70.35	66.50	65.65	65.30	65.45
13	72.35	72.89	67.47	70.21	66.59	69.50
Mean ^d	71.70	72.57	68.00	70.62	67.22	68.52
s _d		0.577		1.464		2.669
s _r		0.755		0.629		1.524
s _b		0 ⁱ		0.935		1.550
F ^e		0.58		5.42		3.07
DF		4		4		4
Disaccharides						
1	10.71	11.14	13.85	9.96	14.84	11.06
4	10.20	10.38	13.73	9.73	12.37	9.24
5	11.02	11.21	13.29	10.03	12.98	10.96
6	9.92	10.19	12.26	9.17	13.49	10.19
9	10.90	11.15	12.85	9.75	13.45	9.25
11	9.80	10.85	12.20	9.35	13.05	10.30
13	10.77	11.33	14.17	10.16	14.93	10.88
Mean ^d	10.47	10.89	13.19	9.73	13.59	10.27
s _d		0.626		0.794		1.104
s _r		0.220		0.349		0.546
s _b		0.414		0.505		0.629
F ^e		8.12*		5.19*		4.10
DF		6		6		6
Higher Sugars						
1	2.08	1.53	1.86	1.89	2.22	2.32
4	1.47	1.41	1.58	1.78	1.96	2.03
5 ^e	1.76	1.90	1.95	2.56	2.42	2.43
6	1.38	1.05	1.39	1.93	1.83	1.82
9	0.73	1.45	0.57 ^b	1.72 ^c	2.13	1.95
11	1.31	1.28	1.59	1.65	1.89	1.99
13	1.51	1.47	1.73	1.74	2.18	2.17
Mean ^d	1.55	1.37	1.63	1.80	2.04	2.05
s _d		0.355		0.140		0.227
s _r		0.301		0.086		0.075
s _b		0.133		0.079		0.152
F ^e		1.39		2.68		9.13*
DF		5		5		5
Solids and Water						
1	100.47	99.91	100.30	100.14	99.58	99.51
4	98.12	100.19	97.50	98.99	97.61	93.11
5	99.95	101.66	100.80	101.55	101.38	102.32
6	98.76	97.94	97.73	98.10	97.36	96.33
9	100.37	102.83	99.26	99.42	101.46	99.99
11 ^e	97.00	97.56	96.33	93.85	96.32	94.78
13	100.47	100.77	99.41	99.31	99.78	99.59
Mean ^d	99.69	100.33	99.17	99.59	99.53	98.48
s _d		1.630		1.716		3.450
s _r		0.958		0.439		1.332
s _b		0.932		1.17		2.250
F ^e		2.89		15.3**		6.71*
DF		5		5		5

^a See Table 1 for description of samples.

^b Excluded as outlier by Dixon's test (12).

^c Value not included in statistical analysis; paired with excluded value.

^d Without excluded values.

^e For presence of systematic errors.

^f Degrees of freedom.

^g Excluded from calculations by Youden's ranking test (11).

^h Paired samples for sucrose are D and F (low), A and B (average), and C and E (high).

ⁱ Negative value for s_b².

Table 4. Collaborative results for carbohydrates in honey by HPLC

Coll.	Sample ^a					
	A	B	C	D	E	F
Glucose						
6	34.95	33.39	31.40 ^b	33.07 ^c	30.23	31.15
9	33.3	33.3	31.0	30.9	29.8	31.5
10	33.62	33.53	30.67	30.78	28.00 ^b	29.92 ^c
16	31.9	33.3	29.9	30.8	27.4	31.1
18 ^d	37.1	37.1	31.5	36.2	31.3	31.6
Mean	33.44	33.38	30.52	30.83	29.14	31.25
s _d		0.921		0.724		1.160
s _r		0.855		0.377		1.013
s _b		0.242		0.437		0.399
F		1.16		3.67		5.39
DF ^e		3		2		2
Fructose						
6	40.86	39.90	37.65 ^b	40.06 ^c	36.22	37.20
9	38.1	38.3	37.1	37.2	35.7	37.6
10	39.39	39.40	37.13	37.28	33.94	36.76
16	37.7	38.9	36.4	37.8	34.4	37.3
18	38.1	37.7	35.0	40.1	35.4	37.4
Mean	38.83	38.84	36.41	38.10	35.13	37.25
s _d		1.460		0.288		0.821
s _r		0.566		1.664		0.515
s _b		0.952		0 ^f		0.452
F		6.66		0.03		2.54
DF		4		3		4
Sucrose ^g						
6	2.73	2.04	5.01	0.40	4.49	0.48
9	2.7	2.2	5.1	0.6	5.1	0.7
10	2.89	2.34	5.24	0.48	4.51	0.57
16	2.54	2.12	4.85	0.55	4.39	0.57
18	4.4 ^c	3.8 ^c	5.0	0.6 ^b	4.4	1.9 ^c
Mean	2.72	2.18	5.04	0.51	4.58	0.58
s _d		0.175			0.265	0.123
s _r		0.080			0.194	0.025
s _b		0.012			0.128	0.085
F		4.74			1.87	23.4 [*]
DF		3			4	3

^a See Table 1 for description of samples.

^b Value not included in statistical analysis; paired with excluded value.

^c Excluded as outlier by Dixon's test (12).

^d All 6 values were excluded from statistical analysis by Youden's ranking test (11).

^e Degrees of freedom.

^f Negative value for s_b².

^g Paired samples for sucrose are D and F (low), A and B (average), and C and E (high).

average values of the 6 samples for glucose, fructose, and sucrose for the 2 methods (Tables 3 and 4) were analyzed by the *t*-test. *t*-Values of 0.81, 1.08, and 1.60 for glucose, fructose, and sucrose, respectively, did not exceed *t*_{0.05}(5DF) = 2.57, indicating agreement in the results by the 2 methods for the 3 sugars. The HPLC procedure is thus suitable for the 3 analyses, al-

though additional study is needed for final acceptance.

Hydroxymethylfurfural

Collaborative results for the 2 HMF methods were disappointing. Significant (*P* = 0.05) *F*-values for systematic error resulted for 2 of the 3 comparisons for each method. The 2 methods

Table 5. Comparison of precision and systematic error for carbohydrate analysis by the SA and HPLC methods

Statistic	Pair	Glucose		Fructose		Sucrose	
		SA	HPLC	SA	HPLC	SA	HPLC
s_d	1	1.168	0.921	4.154	1.460	0.297	0.175
	2	0.786	0.724	1.850	0.288	0.544	0.265
	3	1.542	1.160	5.819	0.821	0.107	0.123
s_r	1	1.001	0.855	0.482	0.566	0.180	0.080
	2	0.755	0.377	1.375	1.664	0.306	0.194
	3	0.962	1.013	1.805	0.515	0.055	0.025
s_b	1	0.426	0.242	2.918	0.952	0.167	0.012
	2	0.153	0.437	0.876	—	0.306	0.128
	3	0.852	0.399	3.912	0.452	0.065	0.085

studied do not give comparable results, in contrast to Winkler's conclusion (5). A t -test on the first 18 values in Table 3, in Winkler's paper comparing the 2 methods yields $t = 1.96$; $t_{0.05}(17DF) = 2.11$. After rejection of data by the ranking and outlier tests, 21 pairs of values remain in Table 6 in which the same collaborators used both methods on the same samples. The same calculation with differences between totals for these 21 pairs of analyses yields $t = 9.81$; $t_{0.01}(20DF) = 2.845$, which indicates a significant between-methods difference. This difference may be accounted for by the dissimilarity in types of honey used in the 2 studies. This is explained further in another publication (14). The UV method is superior in both precision and systematic error (Table 6). Unfortunately, the values from the chemical method are probably more accurate because of the specific nature of the reaction involved and the empirical nature of the baseline correction in the UV method. The validity of Winkler's correction has been questioned by Gautier *et al.* (15) and Romann and Staub (16). A major problem with the chemical method is its use of p -toluidine. For these reasons, neither method can be considered suitable for adoption. A new method which has the precision of the UV method and the accuracy of the chemical method has been developed by the Associate Referee (14) and was subjected to collaborative study in 1978.

Proline

Collaborative results for determining proline are quite satisfactory (Table 7): the coefficients of variation for precision are 2.32, 2.56, and 3.10%, in order of increasing concentration, with an average value of 2.66%. The significant

F -value for the one pair (Samples B and F) resulted from the data reported by Collaborators 2 and 5; although the values were low, they were not rejected as outliers.

Collaborators' Comments

Collaborator 1 strongly objected to the use of p -toluidine. Collaborator 2 questioned the HMF standardization procedures for both methods, the need for analyzing a proline standard for each day, and the failure to recommend appropriate concentrations for a standard curve; he said that the sucrose procedure is a satisfactory and sensitive method but believed that the column procedure required too much time. Collaborator 4 preferred the sucrose procedure to the official titrimetric procedure. Two of the collaborators stored the ninhydrin reagent rather than the methyl Cellosolve solvent over Zn metal and reported high and erratic values for proline. The cause of the difficulty was discussed with each before this report was written and both re-analyzed the samples for proline with the proper reagent; the second set of results are given in Table 7. Collaborator 6 noted that the UV spectra of the low HMF samples did not match the spectrum for the standard; he also provided comments to clarify several method descriptions and noted that inclusion of concentrations for calibration curves would have saved time. Collaborator 7 found the timing requirements for the glucose oxidase reagent cumbersome, detracting from the value of the method, and objected to the use of reagents with short shelf-life; he requested concentration values for preparing standard curves for proline and HMF, objected to the use of p -toluidine, and noted that the 2 HMF methods did not give

Table 6. Collaborative results for hydroxymethylfurfural in honey^a

Coll.	Sample					
	C	D	E	F	G	H
UV Method ^b						
1	5.6	6.1	9.5	15.9	2.1	2.4
2	6.0	6.7	9.8	16.5	2.3	2.5
3	5.8	6.2	9.5	15.5	2.1	2.5
4	6.20	6.36	9.80	16.72	2.28	2.44
5	5.8	6.3	10.1	16.9	2.2	2.5
6	6.12	6.74	9.8	16.8	2.76	2.38
7	6.1	6.7	9.8	16.4	2.1	2.4
8	6.24	6.86	9.92	16.8	2.52	2.70
9 ^c	6.60	7.08	10.20	16.83	2.84	2.84
10	6.6	7.1	9.6	16.3	2.2	2.3
11	5.36	5.96	9.28	15.64	2.08	2.76
12 ^c	5.47	5.48	7.83	13.64	2.01	2.20
13 ^c	5.41	6.01	9.53	15.44	1.45	1.16
Mean ^d	5.98	6.50	9.71	16.35	2.26	2.49
s _d		0.500		0.516		0.182
s _r		0.156		0.217		0.189
s _b		0.336		0.331		0 ^e
F ^g		10.2**		5.66*		0.93
DF ^f		9		9		9
Chemical Method						
1 ^c	4.0	3.8	5.9	9.0	0.42	0.0
2	4.6	4.7	7.9	13.8	0.8	0.5
3	4.4	2.3	10.7	11.3	1.0	0.6
4 ^c	3.48	3.48	5.93	12.11	0.57	0.47
5	4.8	4.8	7.7	13.8	1.7	1.5
6	4.25	4.48	8.0	14.9	0.59	0.17
7	4.9	4.8	8.9	14.4	0.9	0.5
9	5.36	5.36	8.9	13.8	0.57	0.57
10	4.9	4.1	6.5	9.7	1.5	1.5
11	4.25	4.25	7.16	11.5	0.95	1.22
12	3.85	4.35	7.1	12.4	0.85	0.5
13	5.16	5.08	8.0	12.5	0.71	0.52
17	4.73	4.70	7.83	14.35	0.76	0.5
Mean ^d	4.66	4.45	8.06	12.95	0.94	0.74
s _d		0.766		1.516		0.572
s _r		0.319		1.254		0.154
s _b		0.491		0.602		0.389
F ^g		5.74**		1.46		13.64**
DF		10		10		10

^a See Table 1 for description of samples.

^b All collaborative values were multiplied by 0.928 (see text).

^c Excluded from statistical analysis by Youden's ranking test (11).

^d Without excluded values.

^e s_b² slightly negative.

^f Degrees of freedom.

^g For presence of systematic errors.

comparable results. Collaborator 9 commented on the need for concentration data for standardizations; he thought that the HMF time schedule (due to unstable color) was too demanding and requested clarification of directions for proline analysis. Collaborator 10 proposed that water and acetonitrile be degassed separately and then only briefly after being combined, contending that the degassing proce-

dures recommended would tend to strip the more volatile component. He also felt that flow rates for HPLC should be given as a range. He obtained baseline separations at 3 instead of the 1 mL/min recommended with a proportional savings in time and reported glucose and fructose values both by peak height and integrator; the latter showed a mean bias of +2.5%. (The peak height values were used for this report.) Col-

Table 7. Collaborative results for proline in honey (mg/100 g sample)

Coll.	Sample ^a					
	A	E	B	F	C	D
1	99.2	101	39.8	43.0	76.1	80.8
2	96.4	99.6	35.4	37.3	68.1 ^b	67.6 ^c
3	89	98	40	45	72	76
4	95.4	99.2	40.0	44.2	68.8	76.0
5	50.5 ^c	101.1 ^b	37.2	38.3	68.0	80.3
6	93.1	100.4	39.7	43.3	72.2	77.5
7	92.3	94.6	38.5	41.7	69.8	76.7
8 ^d	198.4	190.4	66.4	69.6	125.6	140.0
9 ^d	85.6	91.2	26.4	35.6	62.4	65.2
10	92.56	100.8	37.5	42.5	69.9	76.0
11	86.9	101.5	38.2	44.0	69.8	78.4
15	97.1	104.7	39.2	43.6	75.6	78.5
Mean ^e	93.55	99.98	38.55	42.29	71.36	77.80
s _d		3.82		2.77		2.79
s _r		2.90		1.03		1.98
s _b		1.76		1.82		1.96
F ^f		1.73		7.25**		1.98
DF ^g		8		9		8

^a See Table 1 for description of samples.

^b Value not included in statistical analysis; paired with excluded value.

^c Excluded from statistical analysis by Dixon's test (12).

^d Excluded from statistical analysis by Youden's ranking test (11).

^e Without excluded values.

^f For presence of systematic errors.

^g Degrees of freedom.

laborator 11 thought that the sucrose procedure had major advantages over the official titrimetric procedure and that the proline method had considerable merit, being easy and quick, and yielding repeatable results. He recommended standardized shaking time for the HMF chemical procedure, and observed that the results from the 2 HMF methods did not agree.

Recommendations

Based on the collaborative results, it is recommended—

(1) That the modified selective adsorption procedure for sucrose, monosaccharides, disaccharides, and higher sugars be adopted as interim official first action.

(2) That the method for proline in honey be adopted as interim official first action.

(3) That the HPLC method for glucose, fructose, and sucrose in honey be adopted as interim official first action.

(4) That the method for HMF in honey be further studied.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee D and was adopted as interim first action by the Association. The Association subsequently adopted the method as official first action at the 1978 Annual Meeting. See *J. Assoc. Off. Anal. Chem.* (1979) **62**, 412.

Acknowledgments

The Associate Referee acknowledges with appreciation the statistical counsel of John G. Phillips, consulting statistician, and statistical assistance by Sandra P. Graham, Northeastern Region, U.S. Department of Agriculture (USDA), and the cooperation of the following collaborators: Janet L. Booth, Food and Drug Administration (FDA), Seattle, WA; L. J. Burton, Agriculture Canada, Calgary, Alberta, Canada; L. W. Doner, A. P. Hoban, O. N. Rudyj, and J. Sciliano, U.S. Department of Agriculture (USDA), Philadelphia, PA; Ronald E. Draper, FDA, San Francisco, CA; Vincent Franco, FDA, New York, NY; Rosemary Botcher, Rose Ann Brannen, Walter Funderburk, Sylvia Kresel, David Lorenz, and James E. Thean, Florida Department of Agriculture, Tallahassee, FL; G. Kuhn and L. Zygmunt, Quaker Oats Co., Barrington, IL; Robert Meloy, Sioux Honey Association, Sioux City, IA; Robert Mipro, U.S. Customs Laboratory, New Orleans, LA; R. J. Reina, FDA, Boston, MA; Walter Schmidt, FDA, Philadelphia, PA; Patricia J. Schneider, FDA, Kansas City, MO; John J. Stamp, FDA, Los Angeles, CA; Donald W. Thompson, FDA, Atlanta, GA.

REFERENCES

- (1) *Official Methods of Analysis* (1975) 12th Ed., AOAC, Washington, DC, secs 31.124-31.133
- (2) White, J. W., Jr, Riethof, M. L., Subers, M. H. & Kushnir, I. (1962) *Composition of American Honey*, U.S. Department of Agriculture, Agricultural Research Service, Washington, DC, Tech. Bull. 1261
- (3) White, J. W., Jr (1979) Submitted to *J. Assoc. Off. Anal. Chem.*
- (4) Thean, J. E., & Funderburk, W. C., Jr (1977) *J. Assoc. Off. Anal. Chem.* **60**, 838-841
- (5) Winkler, O. (1955) *Z. Lebensm. Unters. Forsch.* **102**, 161-167
- (6) Codex Alimentarius Commission (1969) Recommended European Regional Standard for Honey, CAC/RS 12-1969, FAO/WHO
- (7) Ough, C. A. (1969) *J. Food Sci.* **34**, 228-230
- (8) White, J. W., Jr, & Rudyj, O. N. (1978) *J. Apicul. Res.* **17**, 89-93
- (9) White, J. W., Jr (1964) *J. Assoc. Off. Anal. Chem.* **47**, 488-491
- (10) Turner, J. H., Rebers, P. A., Barrick, P. L., & Cotton, R. H. (1954) *Anal. Chem.* **26**, 898-901
- (11) Youden, W. J., & Steiner, E. H. (1975) *Statistical Manual of the AOAC*, AOAC, Washington, DC
- (12) Dixon, W. J. (1953) *Biometrics* **9**, 74
- (13) White, J. W., Jr, & Subers, M. H. (1960) *J. Assoc. Off. Anal. Chem.* **43**, 774-777
- (14) White, J. W., Jr (1979) *J. Assoc. Off. Anal. Chem.* **62**, 509-514
- (15) Gautier, J. A., Renault, J., & Julia-Alvarez, M. (1961) *Ann. Fals. Fraudes* **XX**, 397-411
- (16) Romann, E., & Staub, M. (1961) *Mitt. Geb. Lebensm. Hyg.* **52**, 44-58