Charcoal Column/Thin Layer Chromatographic Method for High Fructose Corn Sirup and Spectrophotometric Method for Hydroxymethylfurfural in Honey: Collaborative Studies

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A new spectrophotometric method is described for determining hydroxymethylfurfural in honey in which interfering background absorption of honey is corrected for by use of a bisulfite-treated sample as blank. Two procedures for detecting high-fructose corn sirup (HFCS) in honey were also tested. In one, charcoal column pretreatment is used to concentrate trace oligosaccharides, followed by thin layer chromatography to differentiate those of HFCS from those of honey. The other method depends on measurement of the isomaltose/maltose ratio by gas-liquid chromatography. The charcoal/thin layer chromatographic method for HFCS has been adopted official first action. The bisulfite method for hydroxymethylfurfural has been adopted interim first action.

Although the amount of hydroxymethylfurfural (HMF) in honey has been a quality factor for many years in Europe and elsewhere (1, 2), no completely satisfactory method for its estimation has been available. The most used quantitative procedures, those of Winkler (3), were tested collaboratively in 1978 (4), but both the chemical method and the ultraviolet absorption method failed to qualify as official AOAC methods. A new method (2) has been devised to overcome the shortcomings of both procedures. In the new method, the accuracy of the chemical method is retained and the precision of the ultraviolet procedure is incorporated. The procedure is based on ultraviolet absorption. The background absorption by honey is corrected for by destroying any HMF present with bisulfite and measuring the absorbance of an untreated solution with the bisulfite-containing solution as the blank. This method has now been subjected to collaborative study.

Until the publication of the carbon isotope ratio method for demonstrating the addition of high fructose corn sirup (HFCS) in honey (5), there was no way to detect the adulteration of honey with HFCS. Although unequivocal results are obtained by this method, the required instrumentation is quite expensive and is not available to the control laboratory. None of the 6 laboratories participating in the 1977 collaborative test is a control or enforcement laboratory, although several of them will carry out the complete analysis (combustion and $^{13}C/^{12}C$ ratio determination) for a fee.

A need thus exists for a test for the presence of HFCS in honey which can be performed with equipment and procedures common to control laboratories. Two such methods have been developed in this laboratory (6, 7). These have also been subjected to collaborative testing.

The method of Kushnir (6) depends on the demonstration by thin layer chromatography (TLC) of trace oligosaccharides from HFCS that are not found in pure honey. To attain the required sensitivity, the oligosaccharides are pre-concentrated by charcoal column chromatography. The procedure of Doner (7) is based on the quantitation of the isomaltose/maltose ratio in the sample by gas-liquid chromatography.

Collaborative Study

HMF.—Six samples were prepared as for the 1977 collaborative study (4); pairs of samples represented low, average, and high levels of HMF. Aliquots were shipped in 1 oz polypropylene wide-mouth screw-cap bottles with instructions to refrigerate Samples H1 and H2, the lowest in HMF.

HFCS.—Five adulterated samples were prepared by thoroughly mixing weighed amounts of HFCS and honey; portions of the same honey samples used in the 1977 study were taken to prepare the present collaborative samples. Commercial HFCS from 4 different sources was used, at levels from 5 to 50%. After admixture, the samples were concentrated to the density of

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Table 1. Composition of test samples for corn sirup adulteration collaborative test

Sample	Type of corn sirup: High-fructose, %	Type of corn sirup: Conventional, %	Source of corn sirup[a]
A-1	25	0	A
A-2	0	0	
A-3	5	0	B
A-4	50	0	C
A-5	0	5[b]	E
A-6	15	0	A
A-7	0	0	
A-8	35	0	D

[a] Each letter represents a manufacturer.
[b] High degree of conversion by acid-enzyme process.

the original honey in a rotating vacuum evaporator. One sample was prepared by admixing conventional corn sirup. In addition, 2 pure honeys were included in the test. Aliquots of the 8 samples were shipped to the collaborators in 2 oz polypropylene wide-mouth screw-capped bottles. The composition of these 8 samples (unknown to collaborators) is shown in Table 1.

For the convenience of the collaborators and to save time, certain materials and reagents were supplied. A practice sample, stated to be pure honey with known HMF content and isomaltose/maltose ratio, was included. A practice sample labeled as a mixture of honey and HFCS was also sent.

Collaborators were asked to report single values for HMF, an opinion on which of the 8 samples in the series (Table 1) were adulterated based on the TLC test, values for isomaltose/maltose by the GLC test, and an opinion on whether the sample was adulterated. Materials and reagents distributed included diphenylamine HCl for TLC, maltose.H_2O for GLC standard, weighed amount of isomaltose for GLC standard, cholestane for GLC internal standard, enough Darco G-60® charcoal and Dicalite 4200 (1+1) for 2 columns, each good for 10 samples.

Determination of HMF in Honey

Apparatus and Reagents

(a) *Spectrophotometer.*—To measure absorbance at 284 and 336 nm.

(b) *Carrez solution I.*—Dissolve 15 g potassium ferrocyanide ($K_4Fe(CN)_6.3H_2O$) in water and dilute to 100 mL.

(c) *Carrez solution II.*—Dissolve 30 g zinc acetate ($Zn(CH_3CO_2)_2.2H_2O$) in water and dilute to 100 mL.

(d) *Sodium bisulfite.*—0.20% ($NaHSO_3$) in water. Technical grade is adequate.

Procedure

Transfer ca 5 g honey (weighed to 1 mg in small beaker) to 50 mL volumetric flask with total of 25 mL water. Add 0.50 mL Carrez solution I, mix, add 0.50 mL Carrez solution II, mix, and dilute to volume with water. Drop of alcohol may be added to suppress surface foam. Filter through paper, rejecting first 10 mL filtrate.

Pipet 5 mL filtrate into each of two 18 × 150 mm test tubes; add 5 mL water to one (sample), and 5 mL 0.20% bisulfite to other (reference). Mix well (vortex mixer) and determine absorbance of sample against reference in 1 cm cells at 284 and 336 nm. If absorbance is too high for accuracy (>0.6), dilute sample solution as needed with water and reference solution to the same extent with 0.10% $NaHSO_3$. Multiply absorbance values by appropriate dilution factor before calculation.

Calculation

$$\text{mg HMF/100 g honey} = (A_{284} - A_{336}) \times 14.97 \times 5/\text{sample wt in g}$$

$$\text{Factor} = 14.97 = (126/16830)(1000/10)(100/5)$$

where 126 = mol. wt of HMF; 16830 = molar absorptivity of HMF at 284 nm; 1000 = mg/g; 10 = centiliters/L; 100 = g honey reported; 5 = nominal sample weight.

High Fructose Corn Sirup Thin Layer Chromatographic Method ( )—Official First Action

Apparatus and Reagents

(a) *Charcoal column.*—Use mixt. of Darco G-60 charcoal and rapid diat. earth filter aid (1+1). Use column ca 20–22 mm id with glass wool plug at lower end. Add ca 1–2 cm dry filter aid and wet from below. Pour in slurry of 12 g charcoal mixt. in 150 mL H_2O. Drain 5 min, apply 4 psi pressure until surface stabilizes, then 10 psi. Clean excess charcoal from surfaces by suction and reduce depth of packing to 10 cm, if necessary. Add slurry of filter aid sufficient for 1–2 cm depth, and wash column with 500 mL H_2O and 200 mL 50% alcohol, under which it may be stored. Before use, wash column with 250 mL H_2O. (Vac. operation may be used, but pressure is preferred.) Flow rate of 8.5 mL/min at 10 psi is commonly achieved.

(b) *Plates.*—Coated with 250 μm thickness of silica gel G.

(c) *Solvent*—*n*-Butanol-HOAc-H_2O (2+1+1).

(d) *Color reagent.*—Dissolve 1 mL redistd aniline

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and 1 g diphenylamine.HCl in 50 mL acetone and add 5 mL H_3PO_4. As alternative to redistg aniline, proceed as follows: Dissolve 1 g aniline in 50 mL acetone and decolorize with decolorizing carbon (not Darco G-60). Filter, dil. to 50 mL and add diphenylamine and H_3PO_4. Make fresh daily or store at 0°

Detection

(a) *Preparation of sample.*—Weigh to nearest mg 1 g sample in 30 or 50 mL beaker. Add 10 mL H_2O to dissolve and place on top of column. Force into column with suction but do not let run dry. Rinse beaker with two 5 mL portions H_2O and force into column. Wash with 300 mL 7% alcohol, which is discarded, and then with 100 mL 50% alcohol. Evap. eluate in tared 50 mL beaker on steam bath in current of air or N, and weigh air-dry residue.

Transfer residue to 13 × 100 mm test tube with total of 1 mL H_2O. Evap to dryness in bath at ca 60° in current of air or N. Dissolve residue in 0.1 mL H_2O for each 10 mg material.

(b) *Chromatograpy.*— Place solv. in tank 15 min before inserting plate. Apply 2 and 6 (2 × 3 or 3 × 2) μL of test soln to plate. Apply control spots of pure and adulterated honey, prepd as above. Control solns may be preserved by freezing or drying. Place spotted plate in developing tank until solv. front approaches top of plate. Remove plate, dry, and spray thoroly with color reagent. (*Caution:* Avoid contact with spray.) Let acetone evap., and place in oven at 90–95° until spots are well developed (ca 7–10 min).

(c) *Interpretation.*—Pure honey will show 1 or 2 large blue-gray or blue-brown spots at R_f ca >0.35. Any blue streaks or series of spots extending from origin indicate presence of corn sirups, including high fructose corn sirups (HFCS). Because of variability of HFCS from different manufacturers, intensity of streaks or spots from origin is not directly related to HFCS content of sample.

HFCS in Honey by Gas-Liquid Chromatography

This method has been described by Doner *et al.* (7).

Results and Discussion

HMF

Results received from 13 collaborators were statistically analyzed (Table 2). Use of Youden's ranking test (8) eliminated the data from Collaborator 6 from the calculation. No results were eliminated as outliers by Dixon's test (9). Variances for random error (s_r^2) and systematic error (s_b^2) are reasonably low, although significant systematic error is present at the low and high levels of HMF concentrations.

Table 2. Collaborative results for hydroxymethylfurfural in honey by bisulfite method[a]

	Sample					
Coll.	1	2	3	4	5	6
1	0.24	0.36	2.25	3.80	12.70	19.70
2	0.21	0.66	2.11	3.53	12.28	18.39
3	0.14	0.30	2.11	3.46	12.54	18.46
4	0.14	0.21	2.26	3.98	13.50	19.89
5	0.25	0.57	2.14	3.41	12.28	18.26
6[b]	0.15	0.17	1.91	3.33	12.26	17.83
7	0.12	0.40	2.42	3.49	12.66	18.68
8	0.12	0.22	2.36	3.58	12.49	17.94
9	0.15	0.26	2.14	3.61	13.02	19.45
10	0.28	0.43	2.40	3.34	12.16	16.89
11	0.11	0.18	2.27	3.37	13.17	19.38
12	0.21	0.33	2.29	3.45	13.25	19.28
13	0.00	0.21	2.04	3.49	12.75	20.00
Mean[c]	0.164	0.344	2.232	3.345	12.733	18.86
s_d		0.148		0.155		0.904
s_r		0.082		0.159		0.462
CV_r		32.28		5.70		2.58
s_b		0.087		0[d]		0.550
s_x[f]		0.120		0.159		0.718
CV_x		47.07		5.70		4.55
F[e]		3.27*		0.095		3.84*
DF		11		11		11

[a] As mg/100 g honey.
[b] Excluded from calculations by Youden ranking test (8).
[c] Without excluded values.
[d] Negative value for s_b^2.
[e] For presence of systematic error.
[f] $s_x = \sqrt{s_b^2 + s_r^2}$ = standard deviation of reproducibility.

Although the coefficients of variation for the lowest pair appear excessively high, HMF values this low are found only in unprocessed fresh honey, and are of no significance in regulatory work. Samples 3 and 4 are representative of normally processed market honey, while Samples 5 and 6 have HMF values usually present in old, over-processed or storage-abused honey. In these 2 latter ranges, the method shows acceptable coefficients of variation.

In the previous collaborative test of HMF methods (4), it was concluded that neither the Winkler ultraviolet procedure nor the chemical procedure could be recommended; they did not produce concordant results. The chemical method was considered more accurate. Results with the bisulfite method of the present study and with the Winkler chemical method were shown (2) to be equivalent in accuracy. However, the increased

This report of the Associate Referee, J. W. White, Jr, was presented at the 92nd Annual Meeting of the AOAC, Oct. 16–19, 1978, at Washington, DC.

Table 3. Comparison of collaborative studies of 2 methods for HMF in honey[a]

Statistic	Relative HMF concentration		
	Low	Medium	High
Total error	14.77**	25.7**	2.85*
Random error	3.83*	3.92*	7.44**
Systematic error	19.0**	—[b]	1.19

[a] F values calculated from variance of Winkler toluidine method (10 DF) ÷ variance of White bisulfite method (11 DF).
* $P < 0.05$.
** $P < 0.01$.
[b] Negative value for s_b^2 in bisulfite method.

precision which the present method permits was demonstrated by means of the F ratios (Table 3) in a comparison of variances obtained in the previous and the present collaborative studies. No collaborator reported difficulty with the bisulfite procedure.

HFCS Detection, TLC Method

Reports were received from 8 collaborators (Table 4). When the results were examined by Fisher's exact test (10) on 2 × 2 contingency tables, they significantly ($P < 0.01$) contradict the hypothesis that the procedure is indiscriminatory between adulterated and pure samples. For this calculation, the samples reported as inconclusive were classified as erroneous.

A thin layer chromatogram (Fig. 1) prepared by one collaborator is typical of ones prepared in this laboratory.

The official qualitative test for commercial glucose in honey (**31.134–31.136**) depends on paper chromatographic detection of starch dextrins precipitated from the sample by ethanol. The chromatographic part of this test (**31.136**) can be replaced advantageously by the TLC system used in the Kushnir procedure (6) and tested here, with significant saving of time and improvement in sensitivity.

The TLC procedure as tested here detects addition of less than 5% commercial glucose to honey, as well as HFCS. The official procedure for commercial glucose (**31.134–31.136**) has been reported (11) to detect 10% addition.

In a recent seizure action by a State authority based on the use of the quantitative estimation procedure for commercial glucose (**31.137**), examination by the Associate Referee of subsamples by Kushnir's TLC procedure indicated the absence of added corn sirup. The subsamples contained considerable honeydew and were dextrorotatory. In his description of this test, Browne (12) pointed out that the test should not be used for a dextrorotatory sample and that in all suspicious or doubtful cases confirmatory tests should be used. These cautions are not included in **31.137**. The application of 2 qualitative tests described by Browne to the sample by the Associate Referee confirmed the absence of added commercial glucose. Since **31.137** is a procedure rather than an official method, since it leads as written to erroneous results, and since better methodology now exists, section **31.137** should be deleted.

HFCS Detection, GLC Method (7)

Results of the isomaltose/maltose ratio and judgment on adulteration (Table 5) were re-

Table 4. Collaborative results for adulteration of honey with high-fructose or conventional corn sirup by TLC procedure

Coll.	Sample								Total
	A1	A2	A3	A4	A5	A6	A7	A8	
1	+[a]	–	+	+	+	+	–	+	
2	+	–	+	+	+	+	–	+	
3	+	–	+	+	+	+	–	+	
4	+	–	–	+	+	+	–	+	
5	+	–	+	+	+	+	+	+	
8	+	–	+	+	+	+	+	+	
10	+	–	?	+	+	+	?	+	
13	+	–	+	+	+	+	?	+	
Correct judgments	8	8	6	8	8	8	4	8	58
Incorrect judgments	0	0	1	0	0	0	2	0	3
Inconclusive[b]	0	0	1	0	0	0	2	0	3

[a] + indicates adulterated, – indicates pure honey, ? indicates inconclusive.
[b] Considered incorrect in the statistical treatment (see text).

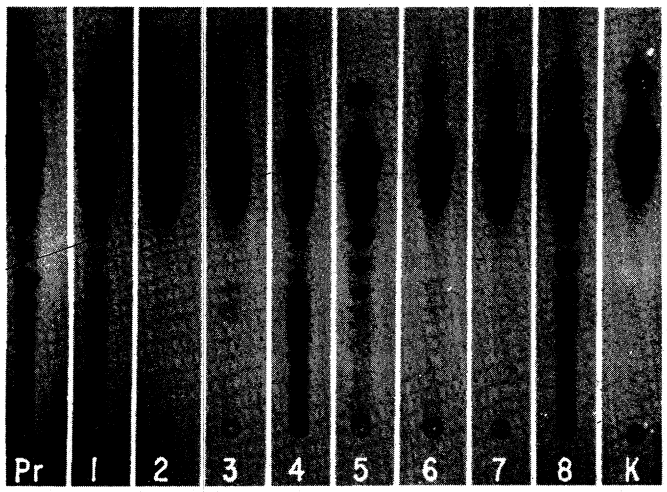

FIG. 1—TLC plate showing resolution obtained with 2 μL samples applied to plate. Pr, practice sample; 1–8, collaborative samples; K, known pure honey.

ceived from only 2 outside collaborators. Three collaborators attempted the method without success. Collaborator 8 obtained much lower values for the ratios than the other two. He reported a ratio of 0.29 for the practice sample which had a declared value of 0.63; then appeared to apply a conversion factor to his other values to arrive at his conclusions. Using all values, the 3 collaborators made correct judgments in 71% of the cases. The procedure as described (7) was calculated to provide about 80% correct judgments. The method has been useful in the laboratory of the Associate Referee and has led to identification of a number of commercial samples containing HFCS, confirmed by isotope ratio (5). It cannot be recommended for official AOAC action, however, in view of the small number of successful collaborative tests.

Comments of Collaborators

Collaborator 3: Directions for column preparation could be clarified and a flow rate should be specified.

Collaborator 8: HMF procedure is easy to follow; no difficulties were encountered. The TLC test is very time consuming. In the GLC test, the criterion for adulteration (IM/M >0.45) was not met by the practice sample; the practice HMF sample, a pure honey, contained more than the 3.9% maltose, indicating the presence of conventional corn sirup. In comparing our data for the (known) samples, we propose that Samples A1, A4, A6, A8 are adulterated. Although the column provided adequate separation of maltose and isomaltose, the maltose peak could not be resolved adequately in the analysis of an actual honey sample, leading to a lowering of the isomaltose/maltose ratios.

Table 5. Collaborative results[a] for adulteration of honey with high-fructose or conventional corn sirup by GLC procedure

	Sample								
Coll.	A1	A2	A3	A4	A5	A6	A7	A8	Total
1	0.48[b]	0.14	0.29	0.47[b]	0.22	0.42	0.22	0.50[b]	
8	0.26[b]	0.09	0.12	0.21[b]	0.10	0.18[b]	0.10	0.21[b]	
14	0.53[b]	0.15	0.30	0.48[b]	0.22	0.51[b]	0.19	0.53[b]	
Correct judgments	3	3	0	3	0	2	3	3	17
Incorrect judgments	0	0	3	0	3	1	0	0	7

[a] Values are isomaltose/maltose ratio.
[b] Judged adulterated by collaborator.

Collaborator 5: Cannot detect isomaltose in any sample by the GLC test.

Collaborator 8: Considerable time was spent on the TLC method but we were unable to visualize clearly any spots or streaks. It is possible that our column was not functioning as required, because spots were visible when the column was not used.

Collaborator 7: The HMF procedure is fast, relatively accurate, reproducible, and a superior method compared to the previous variety of Carrez methods. The TLC procedure yielded good, easy to interpret results. The procedure was easily understood and executed. However, it was also quite time consuming. The procedure could be streamlined with a disproportionately small sacrifice in results by effecting one or both of the following changes: excluding the constant weight portion of the procedure because attempts at quantification are unreliable; accepting flash evaporation as an alternative to evaporation by steam bath and nitrogen stream. In our laboratory, samples could be flash-evaporated in as little as 20 min per sample, whereas steam evaporation consumed 2½ hr and no more than 3–4 samples could be handled simultaneously (38–50 min per sample).

Comment by Associate Referee

The weighing of the 50% alcohol fraction from the charcoal column is included to facilitate putting comparable amounts of material on the TLC plate, independent of the size of the sample. This is thought necessary because HFCS from different sources vary widely in the amount of such material.

Recommendations

It is recommended that the bisulfite method for hydroxymethylfurfural in honey be adopted as official first action; that the charcoal column/TLC procedure for detecting high-fructose corn sirup and conventional corn sirup in honey be adopted as official first action; that the quantitative estimation procedure for commercial glucose in honey, **31.137**, be deleted.

The charcoal/thin layer chromatographic method for HFCS has been adopted as official first action; see *J. Assoc. Off. Anal. Chem.* (1979) **62**, 412, for the report of Subcommittee D. The bisulfite method for hydroxymethylfurfural has been adopted interim first action; see *J. Assoc. Off. Anal. Chem.* (1979) **62**, 703.

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