

# SUGAR PROCESSING RESEARCH INSTITUTE, INC.

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August 6, 1997

Dr. Jonathan W. White  
President  
Honeydata Corporation  
217 Hillside Drive  
Navasota, TX 77868

Dear Jack:

The attached has been retyped and will be recommended in the  
General Referee Report.

Sincerely yours,

Margaret A. Clarke

**HONEYDATA CORPORATION**  
217 Hillside Drive Navasota, Texas 77868

FAX message: 5 pages including this.

TO: Margaret A. Clarke, General Referee, Sugars and Sugar Products  
FROM: J. W. White, Associate Referee, Honey  
SUBJECT: Corrections to rewrite of Method 991.41, recently received.  
DATE: August 13, 1997

There are several errors in the above document draft:

Omissions: The method number is not included.

The captions for the diagrams are missing.

Commission: A number of misprints, wrong spellings, etc. as noted in the draft.

*Jack White*

991.41 C-4 Plant Sugars in Honey  
Internal Standard  
Stable Carbon Isotope Ratio Method

Final Action 1996

Method is used to demonstrate C-4 (corn or cane) sugars in honey at any concentration over 7%.

Method performance:

Range -0.30‰ (2.1%) to -1.9‰ (13.6%)  
 $s_r = 1.25 - 2.69$ ;  $s_R = 11.25 - 2.69$ ;  $RSD_r = 9.22 - 90.0\%$ ;  $RSD_R = 14.5 - 92.0\%$

A. Principle

Stable carbon isotope ratio value for protein isolated from a honey provides a standard to which stable carbon isotope ratio value of the whole honey is compared. The difference between these values (the ISCIRA index) is a measure of the C-4 sugar content of the honey.

Both honey and protein must be analyzed on the same instrument.

HONEY

I

Alternative ~~X~~-Batchwise Method  
Final Action 1979

(a) Combustion system.—Use one of the following options. (1) Craig procedure.—Vacuum-tight glass manifold including quartz combustion tube half-filled with CuO in tubular furnace, liquid N trap, automatic Toepler pump, and high-vacuum source. To prepare CuO for Craig procedure, purify CuO (wire form) by firing in electric furnace ca 1 h at 900°. Store in closed bottle after cooling.

(2) Sofer procedure.—Combustion tube: standard wall borosilicate glass (20 cm x 9 mm), sealed at 1 end. Before use, purge by heating ca 1 h at 550°. To prepare CuO for Sofer procedure, crush CuO (wire form) to pass ca 1.5 mm sieve and heat 2 h at 750° before use.

(b) Purification system (Craig).—Glass manifold interconnected with combustion system including trap, sample collection tube, and manometer [see FIG. 991.41A and *Geochimica et Cosmochimica Acta* 3, 54-55(1953)].

(c) Mass spectrometer.—Micromass 602 (new Model 602D) (Kearns Group, 58 Buckingham Dr, Stamford, CT 06902), Model 6-60-RMS (Measurement and Analysis Systems, Inc., 1155 Zion Rd, Bellefonte, PA 16823), Varian MAT G D150 (superseded by MAT 250) (Varian MAT Mass Spectrometry, 25 Hanover Rd, Florham Park, NJ 07832), or equivalent instrument designed or modified for isotope ratio measurement and capable of accuracy of 0.01% of abundance at mass 45.

(d) Standards.—For calibration purposes (available every 3 years in amount of 400 mg, except oil, 1 mL; graphite, 0.8 g; sucrose, 1 g, from Office of Standard Reference Materials, National Institute of Standards and Technology, Gaithersburg, MD 20899): (1) NIST 19 Limestone.— $\delta^{13}\text{C} = -1.95\%$  against Pee Dee Belemnite. (2) NIST 22 crude oil.— $\delta^{13}\text{C} = -29.73 \pm 0.09\%$ . (3) ANU sucrose.— $\delta^{13}\text{C} = -10.47 \pm 0.13\%$  (4) USGS 24 Graphite.— $\delta^{13}\text{C} = -15.9 \pm 0.13\%$  (5) PEFI Polyethylene Foil.— $\delta^{13}\text{C} = -31.77 \pm 0.08\%$ .

### C. Preparation of Sample

(a) Craig procedure.—Place 20-50 mg sample, weighed to nearest 0.1 mg, in ceramic boat, position boat in tube, and evacuate system. Admit to 600 mm Hg, tank O purified over CuO at 700°, followed by liquid N trap. Heat sample to ≥850° in manifold in tubular furnace, condensing CO<sub>2</sub> in liquid N trap. Recirculate gases over CuO 10-30 min at 850°. Isolate collection trap and purification system from combustion system and Toepler pump by valves, and pump off O. Cool purification trap with solid CO<sub>2</sub>—acetone; cool sample tube with liquid N. Let collection trap warm, condensing impurities in solid CO<sub>2</sub> trap and CO<sub>2</sub> in sample tube.

*procedure*  
 (b) Sofer ~~procedure~~.—Use 9-in. Pasteur pipet to place 3-5 mg sample on side wall of prepared combustion tube, spreading as thin film in strip along axis of tube. Avoid placing sample within 3-4 cm of open end. Cover sample with 3-5 g CuO and allow tube to remain horizontal for ≥5 min. Place tube in drying oven at 60-65° for ≥8 h. Remove tubes from oven, hold vertically, and tap firmly to dislodge CuO particles from wall area where seal will be made.)

While sample tubes are still warm place 1-6 sample tubes on vacuum manifold and evacuate by mechanical pump for 3-4 minutes, then seal tubes with torch. Place tubes horizontally in oven with loose CuO covering sample and bottom of tube end to end. Combust samples at 585-590° for 1 h. Let samples cool in oven at least 1 h to below 400°. Attach tubes to vacuum purification line and break seal or open with a tube cracker [see Anal. Chem. 48, 2652 (1976)]. Purify and analyze CO<sub>2</sub> as described in C(a).

### D. Determination

Operate mass spectrometer according to manufacturer's instructions. Calibrate with ≥2 standards, B(d). Correct values obtained for zero enrichment in inlet system, mixing between sampling and standard valves, tailing of major onto minor peak signal, and combustion of <sup>17</sup>O to mass 45 signal. Calculate:

$$\delta^{13}\text{C}(\text{‰}) = \{[(^{13}\text{C}/^{12}\text{C} \text{ sample}) / (^{13}\text{C}/^{12}\text{C} \text{ standard})] - 1\} \times 1000$$

Convert laboratory analyses, relative to whatever standard was used, to PDB base by the following relationship:

$$\delta(\text{X} - \text{PDB}) = \delta(\text{X} - \text{B}) + \delta(\text{B} - \text{PDB}) + 10^{-3} \delta(\text{X} - \text{B}) \times \delta(\text{B} - \text{PDB})$$

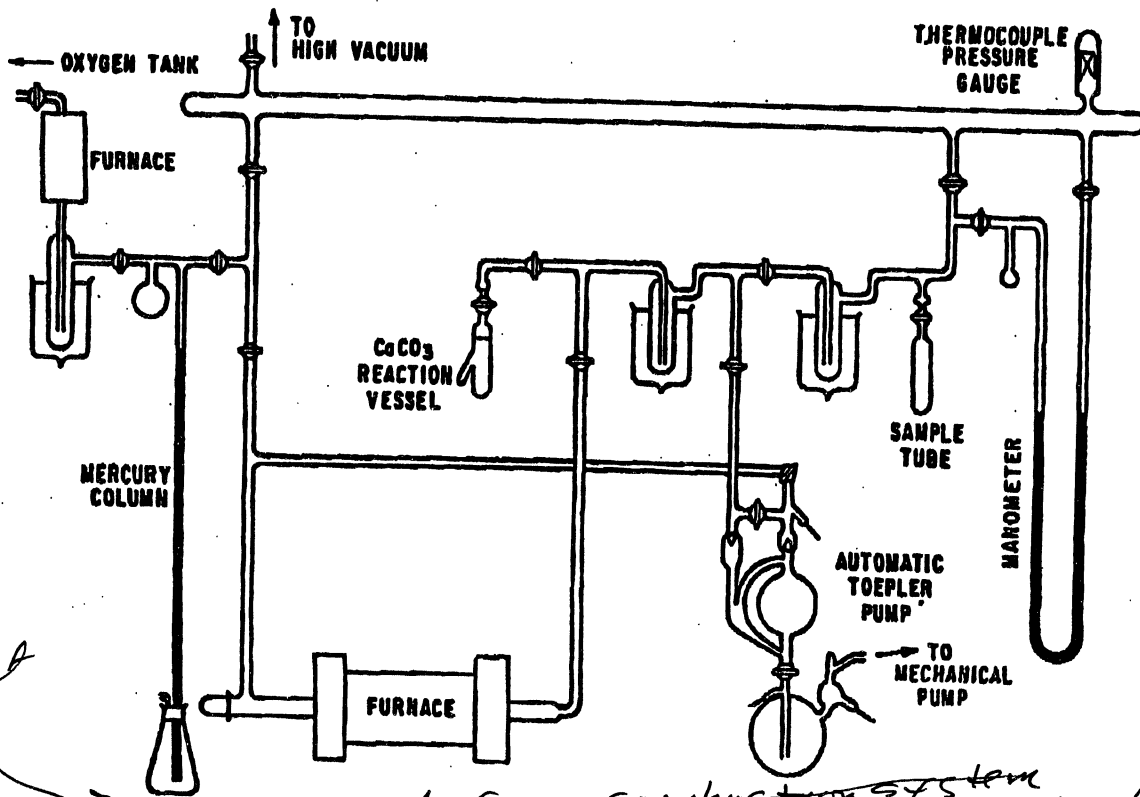
where δ(X - B) and δ(X - PDB) refer to analyses of sample (X) relative to standard (B) and relative to PDB, and δ(B - PDB) is analysis of standard (B) relative to PDB, and all δ's in parts per thousand.

Alternative II—Continuous-Flow Method  
~~First Action 1993~~  
 Final <sup>1996</sup>

#### A. Principle

Sample is burned by in-line automated Dumas combustion, with Cr<sub>3</sub>O<sub>3</sub> catalyst and software-selected pulse of O<sub>3</sub>, purified and carried by He to ion source of mass spectrometer. Continuous-flow combustion and purification process with on-line measurement by single-inlet bench-top mass spectrometer is completely automated, under software control, with sample δ<sup>13</sup>C value obtained directly from computer printout.

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see enlargement next

Fig 991.41A ~~Gas combustion system~~ Carbon combustion and purification system for Coal Powder

B. Apparatus

Integrated system.—Equipped with Rotoprep-CN automated Dumas combustion system with GC gas purification, Tracermass bench-top mass spectrometer, and IBM-compatible computer software control for combustion parameters and calculation of results (ANCA-MS, Europa Scientific, Inc., 1776 Mentor Ave., Cincinnati, OH 45212-3597) (see Fig. 991.41B).

[calibrated against ≥2 reference standards as Alternative I, B(d)] following every 8 or fewer honey samples.

D. Determination

Operate system according to manufacturer's instructions. Carrier flow rate is 60 mL/min, with computer-controlled 15 mL pulse of high-purity O<sub>2</sub> (99.999%) injected into oxidation tube at 1000°. Set reduction stage at 600° and GC column at 150°. Ion currents at m/z 45, 46, 47 are simultaneously integrated, corrected for background, <sup>17</sup>O contribution at mass 45, and any drift between references. Since only 1 measurement can be made for each sample, precision is determined by measuring 5 samples of NIST 22 Crude Oil against itself

C. Preparation of Sample

In triplicate, accurately weigh ca 3 mg undiluted honey, to nearest 0.1 mg, in 6 x 4 mm tin capsule, seal, and place on autosampler of combustion unit. Place working standard reference sample

as reference. Computer printout may be in  $\delta^{13}\text{C}$  units.

## PROTEIN

### A. Apparatus

(a) Centrifuge.—With horizontal 4-head rotor for 50 mL tubes, to provide 1500 x g.

### B. Reagents

(a) Tungstic acid, sodium salt.—10% aqueous solution.

(b) Sulfuric acid.—0.67N. Dilute 1.88 mL  $\text{H}_2\text{SO}_4$  to 100 mL.

### C. Determination

If appreciable amounts of solid matter are present, strain honey through 100-150 mesh (nylon stocking material is excellent); any insoluble material heavier than water will contaminate protein precipitate.

Use one of the following options for protein isolation and purification:

Repetitive washing procedure.—Add 4 mL  $\text{H}_2\text{O}$  to 10-12 g honey in clear 50 mL centrifuge tube; mix well. Add 2.0 mL 10% sodium tungstate solution and 2.0 mL 0.67N  $\text{H}_2\text{SO}_4$  to small test tube, mix, and immediately add to honey solution; mix well. Swirl tube in ca 80° water bath until visible floc forms, with clear supernate. If no visible floc forms, or if supernate remains cloudy, add 0.67 N  $\text{H}_2\text{SO}_4$  in 2 mL increments, repeating heating between additions.

Fill tube with water, mix contents and centrifuge 5 min at 1500 x g, and decant supernate. Repeat washing, mixing, and centrifuging steps 5 times with ca 50 mL

portions of water, thoroughly dispersing pellet each time.

Dialysis procedure.—Use cellulose dialysis tubing retaining proteins with mol wt greater than 12,000, 25 mm (flat) x 30 cm (Sigma 250-9U is suitable). Hydrate tubing, closely tie 2 knots at one end. Heat 5-7 g honey to incipient boil (microwave oven is useful), add ca 3-5 mL  $\text{H}_2\text{O}$ , mix, place in sac, tie 2 knots in end, dialyze against running tap water for at least 16 h. Transfer contents of sac to 50 mL centrifuge tube and centrifuge 5 min at 1500 x g. Decant supernate into 100 mL beaker, discard pellet. Mix 6.0 mL 10% Na tungstate and 6.0 mL 0.67 N  $\text{H}_2\text{SO}_4$ , add to dialysate. Heat on hot plate with stirring until visible floc forms, with clear supernate. Additional increments of acid may be needed. Transfer to 50 mL centrifuge tube and centrifuge 5 min at 1500 x g. Discard supernate, disperse pellet thoroughly, fill tube with water, mix well, and centrifuge.

Place appropriate amount of protein in ceramic combustion boat similar to that used for honey samples. Combust protein by same method used for honey. If necessary to hold for later isotope ratio analysis, either transfer (Pasteur pipet) washed pellet with minimum amount of water to small vial, cap, and place in boiling water for 2 min, or dry protein for at least 3 h in ca 75° oven.

Calculate apparent C-4 sugar content as follows:

$$\% \text{ C-4 sugars} = \{[\delta^{13}\text{C}_P - \delta^{13}\text{C}_H] / [\delta^{13}\text{C}_P - (-9.7)]\} \times 100$$
where  $\delta^{13}\text{C}_P$  and  $\delta^{13}\text{C}_H$  are  $\delta^{13}\text{C}$  values, ‰, for protein and honey, respectively; and -9.7 is the average  $\delta^{13}\text{C}$  value for corn syrup, ‰.

CF

5

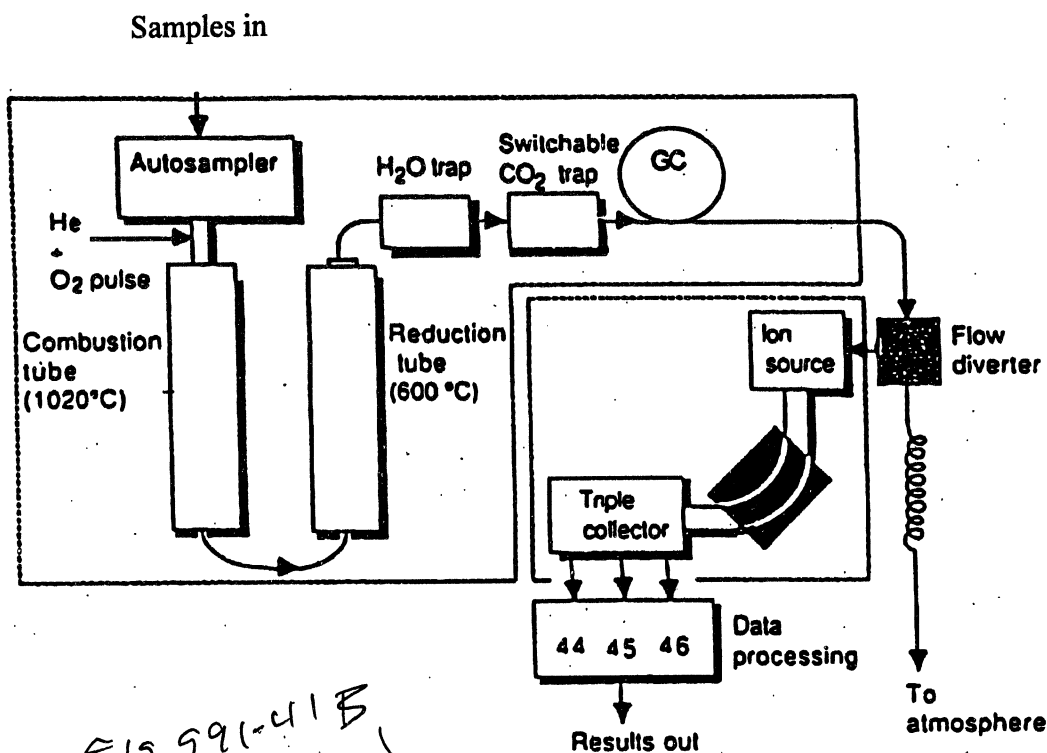


Fig 991-41B Integrated system for continuous-flow method

Report negative values from this calculation as 0%.

Sample is considered to contain significant C-4 sugars (primarily corn or cane) only at or above a value of 7%.

Ref.: JAOAC 72, 907(1989). J. AOAC Int. 75, 543(1992).

JAOAC 61, 746(1978); 71, 88(1988); 74, 627(1991). J. AOAC Int. 75, 543(1992); 76, 140(1993). Geochim et Cosmochim Acta 12, 133 (1957). Spectroscopy 4(7) 42 (1989). Anal. Chem. 48, 1651(1976); 52, 1389(1980).

see enlargement sent earlier