AOAC Official Method 998.12
C-4 Plant Sugars in Honey

Internal Standard
Stable Carbon Isotope Ratio Method
First Action 1998

[Method is used to demonstrate C-4 (corn or cane) sugars in honey at a concentration >7%.]

Results of the interlaboratory study supporting acceptance of the method:
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 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 honey.

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

Honey

Alternative I—Batchwise Method
Final Action 1979

B. Apparatus

(**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°C. Store in closed bottle after cooling.

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

(**b**) *Purification system (Craig).*—Glass manifold interconnected with combustion system including trap, collection tube, and manometer [*see* Figure **998.12A** and *Geochimica et Cosmochimica Acta* **3**, 54–55(1953)].

(**c**) *Mass spectrometer.*—Instrument especially designed or modified for isotope ratio measurement at natural abundance 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, USA): (*1*) *NIST 19 Limestone.*—$\delta^{13}C$ = –1.95‰ against Pee Dee Belemnite. (*2*) *NIST 22 Crude Oil.*—$\delta^{13}C$ = –29.73 ± 0.09‰. (*3*) *ANU sucrose.*—$\delta^{13}C$ = –10.47 ± 0.13‰. (*4*) *USGS 24 Graphite.*—$\delta^{13}C$ = –15.9 ± 0.13‰. (*5*) *PEFI polyethylene foil.*—$\delta^{13}C$ = –31.77 ± 0.08‰.

C. Preparation of Honey

(**a**) *Craig procedure.*—Place 20–50 mg test portion, 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°C followed by liquid N trap. Heat test portion to ≥850°C in manifold in tubular furnace, condensing CO_2 in liquid N trap. Recirculate gases over CuO 10–30 min at 850°C. Isolate collection trap and purification system from combustion system and Toepler pump by valves, and pump off O. Cool purification trap with solid CO_2–acetone; cool analyte tube with liquid N. Let collection trap warm, condensing impurities in solid CO_2 trap and CO_2 in analyte tube.

(**b**) *Sofer procedure.*—Use 9 in. (22.9 cm) Pasteur pipet to place 3–5 mg test portion on side wall of prepared combustion tube, spreading as thin film in strip along axis of tube. Avoid placing material

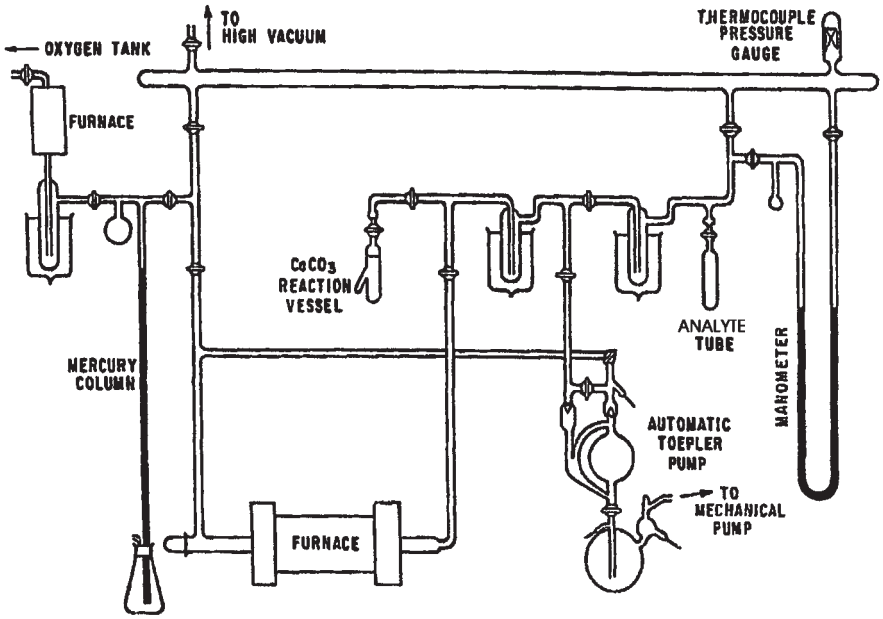

Figure 998.12A—Carbon combustion and purification system for Craig procedure.

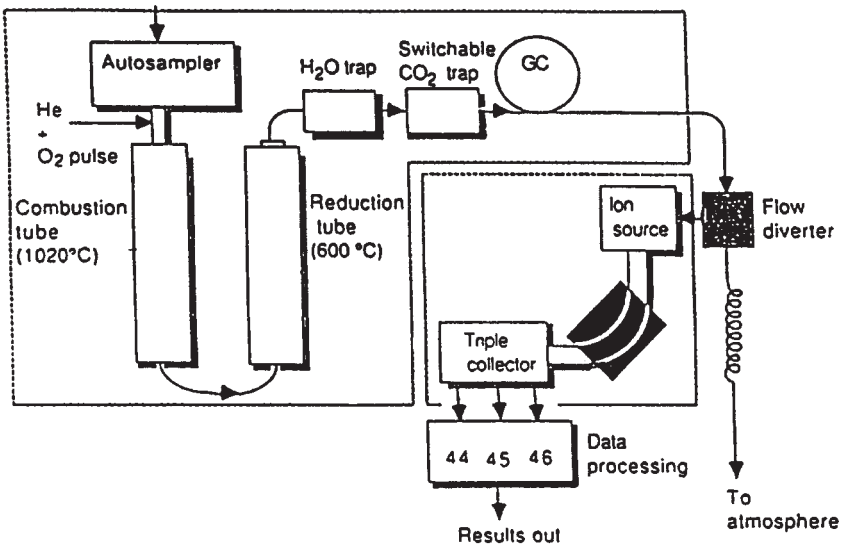

Figure 998.12B—Integrated system for continuous-flow method.

within 3–4 cm of open end. Cover test portion with 3–5 g CuO and let tube remain horizontal ≥5 min. Place tube in drying oven at 60–65°C 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 tubes are still warm, place 1–6 analyte tubes on vacuum manifold and evacuate by mechanical pump 3–4 min, then seal tubes with torch. Place tubes horizontally in oven with loose CuO covering test portion and bottom of each tube end-to-end. Combust test portions at 585–590°C for 1 h. Let tubes cool in oven at least 1 h at <400°C. Attach tubes to vacuum purification line, and break seal or open with a tube cracker [*see Anal. Chem*. **48**, 1652(1976)].

Purify and analyze CO_2 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 ^{17}O to mass 45 signal.

Calculate:

$$\delta^{13}C, ‰ = \left(\frac{^{13}C/^{12}C \text{ test}}{^{13}C/^{12}C \text{ standard}} - 1 \right) \times 1000$$

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

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

where $\delta_{(X-B)}$ and $\delta_{(X-PDB)}$ refer to analyses of test (X) relative to standard (B) and relative to PDB, and $_{(B-PDB)}$ is analysis of standard (B) relative to PDB, and all δs are in parts per thousand.

Alternative II—Continuous-Flow Method
Final Action 1996

A. Principle

Test portion is burned by in-line automated Dumas combustion, with Cr_3O_3 catalyst and software-selected pulse of O_3, 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 analyte $\delta^{13}C$ value obtained directly from computer printout.

B. Apparatus

Integrated system.—Equipped with automated Dumas combustion system with GC gas purification, mass spectrometer designed or modified for isotope ratio measurement at natural abundance, and IBM-compatible computer software control for combustion parameters and calculation of results (PDZ Europa Service Centre, Europa House, Electra Way, Crewe, Cheshire CW1 62A UK; *see* Figure **998.12B**).

C. Preparation of Test Portion

In triplicate, accurately weigh 3 mg undiluted honey, to nearest 0.1 mg, in 6 × 4 mm tin capsule, seal, and place on autosampler of combustion unit. Place working standard reference sample [calibrated against ≥2 reference standards as in *Alternative I,* **B**(**d**)] following every 8 or fewer test portions.

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 (99.999%) injected into oxidation tube at 1000°C. Set reduction stage at 600°C and GC column at 150°C. Ion currents at *m/z* 45, 46, 47 are simultaneously integrated, corrected for background, ^{17}O contribution at mass 45, and any drift between references. Because only one measurement can be made for each test portion, precision is determined by measuring 5 test portions of NIST 22 Crude Oil against itself as reference. Computer printout may be in $\delta^{13}C$ units.

Protein

A. Apparatus

Centrifuge.—With horizontal 4-head rotor for 50 mL tubes, to provide 1500 × *g*.

B. Reagents

(**a**) *Tungstic acid, sodium salt.*—10% aqueous solution of $Na_2WO_4 \cdot 2H_2O$.

(**b**) *Sulfuric acid.*—0.335M. Dilute 1.88 mL H_2SO_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:

(*1*) *Repetitive washing procedure.*—Add 4 mL H_2O to 10–12 g honey in clear 50 mL centrifuge tube; mix well. Add 2.0 mL 10% $NaWO_4$ solution and 2.0 mL 0.335M H_2SO_4 to small test tube, mix, and immediately add to honey solution; mix well. Swirl tube in ca 80°C water bath until visible floc forms with clear supernate. If no visible floc forms, or if supernate remains cloudy, add 0.335M H_2SO_4 in 2 mL increments, repeating heating between additions.

Fill tube with water, mix, centrifuge 5 min at 1500 × *g*, and decant supernate. Repeat washing, mixing, and centrifuging steps 5 times with ca 50 mL portions of water, thoroughly dispersing pellet each time.

(*2*) *Dialysis procedure.*—Use cellulose dialysis tubing retaining proteins with mol wt >12 000, 25 mm (flat) × 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 H_2O, mix, place in sac, tie 2 knots at end, and dialyze against running tap water for ≥16 h. Transfer contents of sac to 50 mL centrifuge tube, and centrifuge 5 min at 1500 × *g*. Decant supernate into 100 mL beaker, and discard pellet. Mix 6.0 mL 10% Na_2WO_4 and 6.0 mL 0.335M H_2SO_4, and add to dialysate. Heat on hot plate, 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 × *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 test portions. 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 2 min, or dry protein at least 3 h in ca 75°C oven.

Calculate apparent C-4 sugar content as follows:

$$\text{C-4 sugars, \%} = \frac{\delta^{13}C_P - \delta^{13}C_H}{\delta^{13}C_P - (-9.7)} \times 100$$

where $\delta^{13}C_P$ and $\delta^{13}C_H$ are $\delta^{13}C$ values, ‰, for protein and honey, respectively, and −9.7 is the average $\delta^{13}C$ value for corn syrup, ‰. Report negative values from this calculation as 0%. Product is considered to contain significant C-4 sugars (primarily corn or cane) only at or above a value of 7%.

References: *JAOAC* **61**, 746(1978); **71**, 88(1988); **72**, 907(1989); **74**, 627(1991).
J. AOAC Int. **75**, 543(1992); **76**, 140(1993).
Geochim et Cosmochim Acta **12**, 133(1957).
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