Table 999.05. Interlaboratory study results for naringin and neohesperidin in orange juice, liquid chromatography

	1% Grapefruit	3% Grapefruit	5% Grapefruit	5% Sour orange	5% K-Early	5 ppm Spiked	20 ppm Spiked	100% Orange	100% Orange
					Naringin				
Total labs	12	12	12	12	12	11	12	11	11
Amount added, μg/g[a]	3.9	11.7	19.5	46.5	5.2	5.0	20.0	0.0	0.0
Overall mean	3.43	10.24	16.87	44.76	7.21	5.20	17.57	—	—
s_r	0.52	0.98	1.43	1.32	0.80	0.67	1.33	—	—
s_R	1.10	1.92	3.04	5.07	1.40	1.19	3.54	—	—
RSD_r	15.23	9.55	8.40	2.95	11.04	12.94	7.57	—	—
RSD_R	1.94	18.73	18.00	11.34	19.39	22.85	20.12	—	—
r	1.46	2.74	4.00	3.69	2.23	1.88	3.73	—	—
R	3.07	5.37	8.50	14.21	3.92	3.33	9.90	—	—
No. positive detection[b]	12	12	12	12	12	11	12	4	4
					Neohesperidin				
	1% Grapefruit	3% Grapefruit	5% Grapefruit	5% Sour orange	5% K-Early	1.25 μg/g Spiked	5 μg/g Spiked	100% Orange	100% Orange
Total labs	11	11	11	11	11	11	10	11	11
Amount added, μg/g[a]	0.14	0.40	0.68	33.8	35.6	1.25	5.00	0.0	0.0
Overall mean	—	—	—	31.71	35.45	—	4.85	0.0	0.0
s_r	—	—	—	1.90	1.06	—	0.57	—	—
s_R	—	—	—	3.50	3.71	—	1.27	—	—
RSD_r	—	—	—	6.00	3.00	—	11.74	—	—
RSD_R	—	—	—	11.03	10.45	—	26.17	—	—
r	—	—	—	5.33	2.98	—	1.59	—	—
R	—	—	—	9.79	10.37	—	3.55	—	—
No. positive detection[b]	0	1	3	11	11	10	11	0	0

[a] Value based on amount determined in whole juice or standard.

[b] Number of laboratories with distinct component peak at correct retention time.

C. Apparatus

(a) *Liquid chromatograph.*—With pump, manual or automatic injector, UV detector, integrator or data collection system, and column heater. Operating conditions: flow rate 1.0 mL/min; injection volume 20 μL (40 μL if test portions are diluted); UV at 280 nm, 0.05 absorbance units full scale (AUFS), column temperature 25°–30°C regulated.

(b) *Column.*—High purity silica packing material with very low metal impurities bonded to C18 phase. New columns should exceed the following specifications to allow for deterioration:

Theoretical plate count (N) for naringin is 2000. $N = 5.545 (t_r/W_h)^2$, where t_r = retention time of peak at apex in seconds, and W_h = peak width at half height in seconds.

Resolution (R) between naringin and hesperidin is ≥1.5.

$$R = \frac{2\,(t_{r(\text{naringin})} - t_{r(\text{hesperidin})})}{1.699\,(W_{h(\text{naringin})} - W_{h(\text{hesperidin})})}$$

Peak symmetry (S) is between 0.9–1.4 for hesperidin. $S = A_{0.1h}/B_{0.1h}$. Using a line drawn from the apex of the peak to the baseline and perpendicular to the baseline, $A_{0.1h}$ = peak width in seconds to left of perpendicular at 10% of peak height, and $B_{0.1h}$ = peak width in seconds to right of perpendicular at 10% of peak height.

Column capacity factor (k') < 10 for neohesperidin. $k' = (t_r - t_d)/t_d$, where t_r is defined above and t_d = retention time of injection spike (time it takes for unretained solvent to flow through the column in seconds).

The following columns have been found to be satisfactory without addition of acetic acid to the mobile phase: Kromasil C18 5 μm, 15 ×4.6 mm (Cat. No. KS-1546-C185) with a flow rate of 1 mL/min (Higgins Analytical, 1235 Pear Ave, Suite 105, Mountain View, CA 94043, USA); Prodigy C18 5μm, 150 ×3 mm (Cat. No. 00F-4097-Y0) with a flow rate of 0.5 mL/min (Phenomenex, 2320 W. 205th St, Torrance, CA 90501-1456, USA); Inertsil ODS 2 5 μm, 150 × 4 mm (Cat. No. 2070210) with a flow rate of 0.8–1.0 mL/min (SGE, Inc., 2007 Kramer Ln, Austin, TX 78758, USA). Many other C18 columns can be used, but may require addition of acetic acid to the mobile phase to obtain acceptable symmetry and plate count values.

(c) *Precolumn.*—A high quality C18 bonded silica packed precolumn containing few metal impurities.

(d) *Filters.*—If test portions are diluted 1:1 with 40% aqueous acetonitrile, use 25 mm × 0.45 μm nylon filter with glass fiber prefilter. If test portions are filtered without prior dilution, use a 25 mm × 0.2 μm Anotop Plus (Whatman No. 6809-4022 or 6809-4025) or cellulose acetate membrane filter to remove particulates.

D. Reagents

(a) *Acetonitrile.*—LC grade.

(b) *Deionized water.*

(c) *Acetic acid.*—LC grade.

(d) *Dimethylformamide.*—LC grade.

(e) *Dimethylsulfoxide.*—LC grade.

(f) *Mobile phase.*—Aqueous acetic acid–acetonitrile (81 + 19.) Add 1–10 mL LC grade glacial acetic acid to LC grade deionized water to give a total volume of 2 L. Acetic acid may minimize peak tailing and improve column efficiency. Amount of acetic acid necessary depends on the column used and addition of acetic acid to the mobile phase increases the probability of interference from Na benzoate and K sorbate with peaks of interest. Mix 1620 mL water or acetic acid solution with 380 mL LC grade acetonitrile.

(g) *Naringin, hesperidin, and neohesperidin stock A solution.*—500 μg/g. Standard grade. Atomergic Chemetals Corp. (71 Carolyn Blvd Ave, Farmingdale, NY 11735, USA); Indofine Chemical Co. (PO Box 473, Somerville, NJ 08876, USA); or Extrasynthese S.A. (BP62, Z.I. Lyon Nord, 69730 Genay, France). Accurately weigh 0.05 g each of naringin, hesperidin, and neohesperidin into a 100 mL volumetric flask. Add 10 mL dimethylformamide or dimethylsulfoxide, mix until dissolved, and dilute to volume with mobile phase.

(h) *Sodium benzoate and potassium sorbate stock B solution.*—500 μg/g. Accurately weigh 0.05 g K sorbate and 0.05 g Na benzoate, transfer to a 100 mL volumetric flask, and dilute to volume with mobile phase.

(i) *Working standards.*—Make 5 dilutions (1:5, 1:10 , 1:20, 1:50, 1:100) of stock standard A in mobile phase to provide 100, 50, 25, 10, and 5 μg/g working standards. Add 10 mL stock A and 10 mL stock B into a 100 mL volumetric flask to provide a standard for checking the mobile phase and column suitability. Dilute to volume with mobile phase.

E. Preparation of Test Solutions

Centrifuge 10–20 mL test portion at 10 000 × g for 10 min at 25°C. Filter through a 25 mm × 0.2 μm Anotop Plus, or membrane filter.

Alternatively, if a centrifuge is not available, mix test samples well and dilute 1 + 1 with aqueous acetonitrile (40 + 60). Filter test solutions through 25 mm × 0.45 μm nylon filter with glass fiber prefilter. Adsorption of flavonoids by nylon in aqueous solutions is overcome by the acetonitrile. Do not use a filter with cellulose acetate, Versapore, or polysulfone membranes as they are not resistant to acetonitrile. Do not use filters with a PVDF or PTFE membrane as these materials adsorb flavonoids from the matrix.

F. Chromatography

Inject working standard containing stock B solution and adjust solvent strength so resolution (R) is at least 1.5 between naringin and hesperidin, both naringin and neohesperidin are resolved from Na benzoate and K sorbate (R ≥ 1.5), and k′ < 10 for neohesperidin. Use an injection volume of 20, 10, or 5 μL dependent on the internal diameter (4.6, 3.0, or 2.0 mm, respectively) of the analytical column used. Inject one orange juice test solution and set run time for 60 min. Interfering peak will elute between 25–60 min on columns 150 mm or shorter. For longer columns (250–300 mm) interfering peak may not elute for 80–100 min. Time injection interval so late peak does not interfere with subsequent analyses. Temperature control of the analytical column and use of an automatic injection system are recommended. Calibrate instrument with standards and check instrument for linearity. If response is nonlinear, a multipoint calibration of the instrument is necessary each time a set is analyzed. If linear, duplicate injections of a single standard for calibration is sufficient for subsequent sets.

G. Calculations

Identification of peaks is improved if relative retention times (RRT) are used instead of retention times. Base relative retention times on retention time of hesperidin.

$$\text{RRT of component } x = \frac{\text{retention time of } x}{\text{retention time of hesperidin}}$$

Quantitate naringin and neohesperidin by peak area. Calculations may be programmed into the integrator following manufacturer's instructions or by standard curve if detector response is nonlinear. If response is linear, calculate response factor (RF) for each standard component and determine concentration of naringin and neohesperidin in each test solution as follows:

$$\text{RF of component } x = \frac{\text{average area of component } x}{\text{concentration of } x \text{ in standard } (\mu g / g)}$$

$$\text{Concentration of } x \text{ in test portion} = \frac{(\text{area of component } x)(\text{dilution factor})}{\text{RF of component } x}$$

Reference: *J. AOAC Int.* **83**, 1155(2000).

Revised: March 2002

37.1.67

AOAC Official Method 2004.01 Carbon Stable Isotope Ratio of Ethanol Derived from Fruit Juices and Maple Syrups

Isotope Ratio Mass Spectrometry (IRMS)
First Action 2004

[Applicable to determination of the $^{13}C/^{12}C$ ratio of ethanol derived by alcoholic fermentation of fruit juices (orange, apple, and pineapple) and maple syrup.]

See Table **2004.01** for results of the interlaboratory study supporting acceptance of the method.

Caution: Ethanol is flammable: use caution when distilling; Karl Fischer reagent is harmful, flammable, corrosive, and readily absorbed through skin: handle with care. Collect ethanol and Karl Fischer titrant as hazardous waste. If a spill of these products occurs, turn off or unplug all electrical equipment. *See Appendix B, Laboratory Safety*.

Table 2004.01. Interlaboratory study results for determination of $\delta^{13}C$ of ethanol derived from fruit juices and maple syrup by IRMS

Sample ID	Sugar added, %	Mean $\delta^{13}C$ (‰)[a]	No. of labs[b]	s_r, ‰	s_R, ‰	r[c]	R[d]
Orange juice	—[e]	–26.67	12 (0)	0.10	0.22	0.28	0.62
Orange juice + cane sugar	16[f]	–24.36	12 (0)	0.08	0.22	0.21	0.62
Apple juice + cane sugar	41[f]	–20.44	10 (1)	0.06	0.23	0.17	0.65
Maple syrup + cane sugar	26[f]	–20.73	12 (0)	0.12	0.17	0.32	0.49
Pineapple juice + beet sugar	41[f]	–20.0	11 (0)	0.08	0.23	0.22	0.65
Cane sugar	—[e]	–12.45	12 (0)	0.16	0.26	0.46	0.73

[a] C^{13}/C^{12} ratio of ethanol derived from fruit juices and maple syrup by IRMS, expressed as $\delta^{13}C$ per mil relative to V.PDB carbonate standard.

[b] Each value is number of laboratories retained after elimination of outliers; each value in parentheses is number of laboratories removed as outliers.

[c] $r = 2.8 \times s_r$.

[d] $R = 2.8 \times s_R$.

[e] — = Blind duplicates.

[f] Youden pairs.

A. Principle

Sugars are converted to ethanol by fermentation, and ethanol is purified by distillation. The determination of the isotope ratio $^{13}C/^{12}C$ of ethanol is performed on the carbon dioxide resulting from combustion of ethanol distilled from the product. The different ratios of the masses 44, 45, and 46 of carbon dioxide are determined with an isotope ratio mass spectrometer. The carbon-13 isotopic deviation $\delta^{13}C$ is then calculated on a delta per mil scale by comparison of results obtained on a working standard (e.g., glutamic acid), previously calibrated with respect to the international reference Vienna-Pee Dee Belemnite (V.PDB) carbonate standard [Craig, H. (1957)].

Ethanol produced by fermentation constitutes a convenient probe for characterizing precursor sugars and can be used to detect various kinds of sugar additions to fruit juices and maple syrup. The addition of C_4 plant sugar in C_3 plants (all common fruits except pineapple) and in maple syrup increases the $\delta^{13}C$ value of ethanol. Conversely, the addition of C_3 plant sugar in pineapple juice decreases the $\delta^{13}C$ value of ethanol.

B. Apparatus

(**a**) *Abbe refractometer.*—For determination of Brix value.

(**b**) *Fermentation vessel.*—1.5 L capacity, fitted with trap device that prevents air entry while allowing CO_2 emission.

(**c**) *Computerized system for monitoring fermentation.*—For recording of fermentation parameters.

(**d**) *Centrifuge.*—With minimum 3000 × *g* force.

(**e**) *Steam distillation system.*—For quantitative isolation of ethanol from alcoholic product or beverage; used in determination of alcohol content.

(**f**) *Electronic densitometer.*—For determination of alcohol content.

(**g**) *Preparatory distillation system.*—For isolation of ethanol, equipped with manual Cadiot column with spinning band (Teflon moving part), or for better efficiency and quality control, computerized distillation system (*see* Figure **2004.01A**); electric heating mantle with voltage regulator; 1 L round-bottom flask with ground glass neck joint; 125 mL conical flasks with ground glass neck joints; 125 and 60 mL glass bottles with plastic stoppers.

Performance characteristics: preparatory distillation system must be capable of extracting ≥96% ethanol present in fermented products of 3–20% (v/v) alcoholic content. Alcoholic content of distillate must be ≥90% (w/w) to guarantee that isotopic fractionation of distillate is <0.2‰ (parts per 1000) for $\delta^{13}C$.

Equipment available from EUROFINS (44323 Nantes, France; www.eurofins.com).

(**h**) *Karl Fischer titrator.*

(**i**) *Isotope ratio mass spectrometer.*—With ability to determine the ^{13}C content of CO_2 gas at natural abundance with internal precision of 0.05‰ or better (expressed in relative δ value). The internal precision is here defined as the difference between 2 measurements of the same CO_2 specimens. The mass spectrometer will generally be fitted with collectors that measure the current intensities for *m/z* 44, 45, and 46. The mass spectrometer should either be fitted with a dual inlet system, for alternatively measuring the unknown specimen and a standard, or use a continuous-flow (CF) technique [CF-isotope ratio mass spectrometry (IRMS)].

(**j**) *Combustion system.*—Able to convert quantitatively the ethanol into carbon dioxide and eliminate all other combustion products including water, without any isotopic fractionation. In case of CF technique (CF-IRMS), test portions are quantitatively combusted and the resulting CO_2 is eluted to the ionization chamber of the isotope ratio mass spectrometer. Commercially available CF-IRMS techniques are elementary analyzer (EA)-IRMS (equipped for liquid or solid injection) or gas chromatography–combustion (GC-C)-IRMS. In case of manual off-line preparation system, the CO_2 resulting from combustion of the test and of the reference portions are collected in containers which are then fitted to the dual inlet system of the isotope ratio mass spectrometer.

(**k**) *Analytical balance.*—With repeatability of 0.1 mg.

(**l**) *Volumetric pipet.*—20 µL.

(**m**) *Tin capsules.*—Suitable for liquid (2 × 5 mm, 12 µL capacity) and for solid (4 × 6 mm, 70 µL capacity).

(**n**) *Pliers.*—Suitable for encapsulation (microanalyzer).

(**o**) *Syringe.*—10 µL. For liquid injection.

(**p**) *Vials.*—2 mL. With tight closing and inert septa to contain the liquid for injection.

Notes: (*1*) Items (**a**), (**c**), (**f**), and (**h**) may be replaced by any appropriate system (e.g., pycnometer for alcoholic content measurement and by liquid chromatography (LC) for sugar content

CADIOT COLUMN

(Automated Distillation Control System for SNIF-NMR analysis)

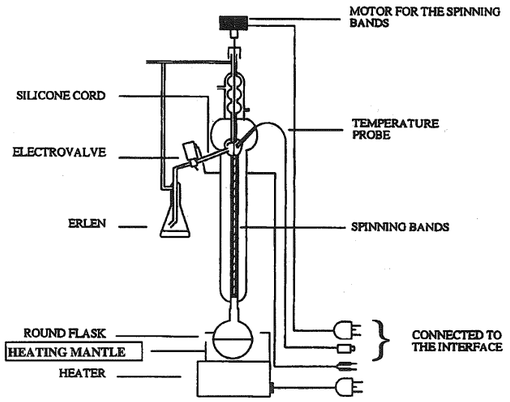

Figure 2004.01A. Distillation apparatus for isolation of ethanol.

determination). Alcoholic content of fermented juice (3–12% alcohol, v/v) must be measured with absolute precision of 0.05% alcohol (v/v), and alcoholic content of distillate (90–96%, w/w) must be measured with absolute precision of 0.1% alcohol (w/w).

(*2*) For manual off-line combustion, the materials and consumables depend on the combustion system used in the laboratory. The above list was established for the most commonly used systems (EA-IRMS and GC-C-IRMS). Any material can be replaced by other material having an equivalent efficiency.

C. Reagents

(**a**) *Active dry yeast.—Saccharomyces bayanus cerevisiae.*

(**b**) *Karl Fischer reagent.*

(**c**) *Working standard of known $^{13}C/^{12}C$ ratio.*—Calibrated against international standards (e.g., glutamic acid, of high purity, >99%).

(**d**) *International reference of known $^{13}C/^{12}C$ ratio.*—NBS 22 (NIST reference material 8539) or equivalent [International Atomic Energy Agency (IAEA), PO Box 100, Wagramer Strasse 5, A-1400, Vienna, Austria].

D. Fermentation

Notes: (*1*) Do not add additional nutrients to the fermentation medium, because this would be a source of isotopic fractionation.

(*2*) Accurately perform all weighing operations in **D** and **E**. Record all weights of preparation flasks and masses of products placed into preparation flasks. Use average of duplicate weights in calculations.

The amount of product submitted to fermentation should always be sufficient to result in at least 10 g ethanol. The potential alcoholic content of the product can be estimated by the following equation:

$$T^{Q}pot\%\mathrm{Vol} = \frac{C_s}{17}$$

where $T^{Q}pot\%$Vol is the potential alcoholic content expressed in % (v/v); C_s is the concentration of sugar in the product in g/L and 17 (g/L) is the practical concentration of sugar which produces 1% (v/v) of alcohol.

(**a**) *Fermentation of fruit juice.*—Into fermentation vessel, **B(b)**, place 0.6 L single strength juice for which soluble solids content (Brix) has been previously determined. Add 3 g dry yeast directly to juice. Install fermentation device to prevent air entry. Allow juice to ferment at ca 20°C until all sugars are converted to alcohol. Use optional system, **B(c)**, for monitoring fermentation, if desired. Check for complete fermentation of fermentable sugars by measuring residual sugars using LC or color reaction (e.g., Clinitest). The fermentation of orange or pineapple juice lasts approximately 2 days (it may last 4–5 days for apple). Centrifuge fermented liquid ca 2 min at 4000 rpm.

Determine alcoholic content of fermented juice (t^{Q}, %, v/v) as follows: Pipet 20 mL supernate into 200 mL volumetric flask and dilute with distilled water (10-fold dilution). Transfer the 200 mL diluted supernate into round bottom flask and steam-distill using steam distillation system, **B(e)**. Collect all distillate in 200 mL volumetric flask, dilute with distilled water, and measure alcohol content by densitometry at 20° ± 0.01°C (absolute uncertainty for 5% alcoholic solution is 0.05%). Alternatively, determine alcoholic content of fermented product by pycnometric method.

(**b**) *Fermentation of maple syrup.*—Determine soluble solids content (Brix) of syrup. Dilute concentrate in fermentation vessel, **B(b)**, with H_2O to 0.6 L or slightly more to obtain ca 12° Brix in diluted product (for optimal fermentation speed, 12° ± 1° Brix is recommended). Add 3 g dry yeast and homogenize by shaking fermentation vessel. Install fermentation trap device to prevent air entry. Allow juice to ferment at 20°C until all sugars are converted to alcohol. Check for complete fermentation of fermentable sugars by measuring residual sugars using LC or color reaction (e.g., Clinitest). Centrifuge fermented liquid. Determine alcoholic content of fermented juice as in (**a**).

(**c**) *Fermentation of cane sugar.*—Weigh 72 g crystallized cane sugar. Dilute in fermentation vessel, **B(b)**, with H_2O to 0.6 L or slightly more to obtain ca 12° Brix in diluted product (for optimal fermentation speed, 12° ± 1° Brix is recommended). Add 3 g dry yeast and homogenize by shaking fermentation vessel. Install fermentation trap device to prevent air entry. Allow juice to ferment at 20°C until all sugars are converted to alcohol. Check for complete fermentation of fermentable sugars by measuring residue sugars using LC or color reaction (e.g., Clinitest); centrifuge fermented liquid. Determine alcoholic content of fermented juice as in (**a**).

Keep fermented juices or sugars in a refrigerator before distillation if storage is required. Start distillation within 24 h of the end of the fermentation.

E. Distillation of Ethanol

Place 3 pumice stones (to prevent bumping) into round-bottom distillation flask. Weigh empty flask (W_0^B). Place at least 400 mL homogenized fermented juice or syrup (V) into flask and weigh again (W_1^B). Calculate weight of fermented juice or syrup added to flask:

$$W^J = W_1^B - W_0^B$$

...t water circulating in condenser of extraction apparatus, **B(g)**. ...n 125 mL conical flask with ground glass joint, previously ...ed (W_0^E), to collect distillate. Attach round-bottom flask ...ning fermented juice to Cadiot column and heat contents to ...g. When boiling liquid is refluxing, switch on motor of ...ng band, and wait 5 min to attain equilibrium.

...er setting constant reflux ratio observed at top of column, ...t boiling liquid between 78° and 78.5°C (*see* Figure **2004.01A** ...propriate location of temperature probe). When temperature ...ds 78.5°C, discontinue collection for 5 min to prevent water ...tion. After temperature returns to 78°C (pure alcohol is ...ed), start again collecting distillate until 78.5°C. Repeat this ...dure until the temperature remains constant, indicating that all ...ol has distilled.

...*e*: Complete distillation requires ca 4 h. Generally 98–98.5% ...alcohol in fermented juice is recovered from distillate, with ...gth 91–93%, w/w (93–95%, v/v).

...igh conical flask containing distillate (W_1^E) and calculate exact ...t of distillate:

$$W^D = W_1^E - W_0^E$$

...ol round-bottom flask containing residue and then weigh (W_2^B). ...ulate weight of residue:

$$W_R = W_2^B - W_0^B$$

...lue represents H_2O in fermented product.

...re distillates in glass bottles (with as little headspace as ...ble) with Teflon-sealed screw-caps. Close the flasks tightly to ...nt isotopic fractionation (associated with evaporation). After ...distillation, clean the column by blowing nitrogen or dry air for ...in to eliminate residual ethanol.

...etermination of Alcoholic Strength of Distillate

...termine water content (m_w, in g) in distillate from **E** by Karl ...er method using ca 0.25 mL distillate of exactly known mass ...n g).

...lculate alcoholic strength (t_m^D, %, w/w) of distillate as follows:

$$t_m^D = [(m - m_w) / m] \times 100$$

...e m = mass of distillate used in Karl Fisher method and m_w = ... of water measured.

...lculate weight losses of distillation (W^L) and yield of ethanol ...llation (%) as follows:

$$\text{Weight losses } W^L = W^J - (W^D + W^R)$$

...ield of distillation, % = $100 \times t_m^D \times W^D / (0.78924 \times V \times t^Q)$

...re V = exact volume of fermented juice used for distillation, mL; ... = weight of distillate, in g; t^Q = alcoholic content of fermented ..., % v/v.

...ield of extraction should be ≥96%; otherwise isotope ratios of ...nol in distillate are modified because of significant isotopic ...ionation during distillation.

Calculate relative weight losses (Rw^L, %) as follows:

$$Rw^L = 100 \times W^L/W^J$$

Rw^L >0.5% indicates abnormal losses during distillation step (e.g., leak in distillation system or error in weighing).

Note: Conditions of high yield of ethanol distillation and alcoholic content are strictly required to keep isotopic fractionation at a minimum. These conditions can be achieved by using optional computerized distillation system, **B(g)**.

G. Determination of Carbon-13 Deviation of Ethanol

The following descriptions refer to the most commonly used procedures for combustion of ethanol specimens prior to stable carbon isotope analysis. Any other method, ensuring that the ethanol specimen is quantitatively converted into carbon dioxide without any evaporative loss of ethanol, is suitable for the preparation of the carbon dioxide for isotope analysis.

(**a**) *Continuous flow method based on the utilization of an elemental analyzer coupled with the mass spectrometer.*—Insert at intervals a solid working standard, which can be encapsulated easily, into the series of examinations; e.g., glutamic acid. Always insert a capsule of working standard at the beginning and end of a series of examinations. Check the cleanness of capsules, pliers, and tray. Take a capsule of adequate size with the curved end of the pliers. Introduce the necessary volume of liquid into the capsule using the 20 µL pipet. Crimp the capsule with special nippers. Prepare 2 capsules for each specimen and standard. If doubt exists concerning tightness of capsule, it must be reprepared. Take the greatest care during the sealing of capsules containing ethanol, a volatile compound. Position the capsule in the automatic sample changer of the microanalyzer at the appropriate place carefully referenced by an ordered number.

Notes: (*1*) About 3.8 mg absolute ethanol or 4.2 mg distillate having an alcoholic content of 92% (w/w) is needed to obtain 2 mg carbon. Calculate the appropriate amount of distillate in the same way according to the amount of carbon needed (which depends on the spectrometer characteristics).

(*2*) When using an injector for liquids, considerations regarding the procedure of encapsulation of solids are irrelevant.

Check the pressure of the manometers which regulate the output of compressed gas cylinders; also check the furnace temperatures according to manufacturer's specifications.

Check the absence of leaks in the EA-IRMS system (e.g., by monitoring the ion current *m/z* 28 corresponding to N_2).

Adjust IRMS for measuring the intensities of the ionic currents *m/z* 44, 45, and 46.

Allow system to equilibrate.

Place encapsulated specimens in the carousel of the analyzer and start the measurements (combustion to CO_2 and analysis).

Operate spectrometer according to manufacturer's instructions.

(**b**) *Off-line method based on the off-line combustion of ethanol and dual inlet measurement.*—Application of an automated elemental analyzer and an automated trapping box system should use the same working procedure as described under (**a**).

Convert the carbon content of the specimen quantitatively into CO_2 by the following devices: (*1*) closed combustion system filled with circulating oxygen gas; (*2*) elemental analyzer with helium/oxygen gas stream; (*3*) sealed glass ampule filled with

Analytical sequence	Attributed numbers	Measured values (mean of 2 capsules)	Correction factor:	Corrected values:
W.S.	1	-20.0	-0.2	-20.2
sample 1	2	-23.4	-0.2	-23.6
sample 2	3	-27.7	-0.1	-27.8
sample 3	4	-14.0	-0.1	-14.1
sample 4	5	-12.2	0.0	-12.2
sample 5	6	-18.9	0.0	-18.9
sample 6	7	-18.8	0.1	-18.7
sample 7	8	-15.0	0.1	-14.9
sample 8	9	-16.6	0.2	-16.4
sample 9	10	-26.8	0.2	-26.6
sample 10	11	-25.9	0.3	-25.6
W.S.	12	-20.5	0.3	-20.2

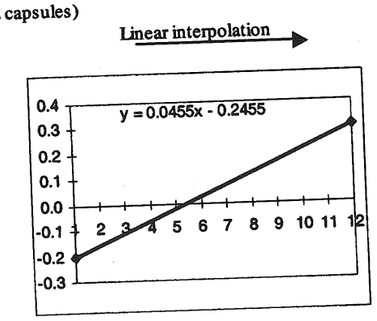

Figure 2004.01B. Example of drift correction.

copper oxide as oxidizing agent, but take care with this method to avoid formation of copper carbonate.

After conversion of ethanol to CO_2, fix specimen container to inlet system and perform carbon isotope analysis. Introduce CO_2 into the mass spectrometer by a dual inlet system so that the ion currents of the specimen can be compared to those of a reference CO_2 calibrated against an international standard. Operate spectrometer according to manufacturer's instructions.

H. Quality Control of Measurements

First check the system by combusting an international reference (e.g., NBS 22) and adjusting the subsequent results to the accepted $\delta^{13}C$ value, or to give the results obtained for 3 independent determinations of an international reference for normalization of the results. The mass/area ratio of CO_2 can be determined by using a device (such as an integrator) to measure the area of the corresponding CO_2 peak (usually measured by a catharometer).

Notes: (*1*) Check that the $\delta^{13}C$ value for the working standard does not differ by >0.5‰ of the reference value; otherwise retune the spectrometer.

(*2*) For each specimen, check the 2 replicate values run in succession. If the deviation between the 2 capsules is <0.3‰, accept the measurement; the final result for a given specimen is the mean of 2 capsules. If the deviation is >0.3‰, repeat the measurement.

(*3*) It is essential to check that for both working standard and ethanol specimens, the amplitude of the recorded signal remains in the appropriate range defined by the spectrometer manufacturer (a lower amplitude may indicate an evaporation of ethanol from insufficient sealing of the capsule).

(*4*) In case of on-line measurements, correct the $\delta^{13}C$ values of the specimens according to the difference between the measured $\delta^{13}C$ value of the working standard and its true value, previously determined with respect to the international reference. This drift correction is usually done automatically by the software of the instrument. The drift can be considered as linear between 2 measurements of the $\delta^{13}C$ of the working standard. Measure the working standard at the beginning and end of all series of specimens. Make a correction for each specimen by a linear interpolation between the 2 values of the differences between the assigned value of the working standard and the measurements. An example of such correction is given in Figure **2004.01B**. Values refer to $\delta^{13}C$ (‰) values. In this example, the accepted true value of the working standard (WS) is –20.2‰.

I. Calculations

All $\delta^{13}C$ values are expressed with respect to the international reference V.PDB [Craig, H. (1957)], according to the following equation:

$$\delta^{13}C = \frac{R_{specimen} - R_{reference}}{R_{reference}} \times 1000$$

where $R_{specimen}$ and $R_{reference}$ are, respectively, the isotopic ratios $^{13}C/^{12}C$ of the specimen and of the international reference ($R_{reference}$ = 0.0112372).

References: Craig, H. (1957) *Geochim. Cosmochim. Acta* **12,** 133–149.

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37.1.68

AOAC Official Method 2005.02
Total Monomeric Anthocyanin Pigment Content of Fruit Juices, Beverages, Natural Colorants, and Wines

pH Differential Method
First Action 2005

(Applicable to the determination of monomeric anthocyanins in fruit juices, beverages, natural colorants, and wines within the range of 20–3000 mg/L as cyanidin-3-glucoside equivalents.)

See Table **2005.02** for the results of the interlaboratory study supporting acceptance of the method.

A. Principle

Monomeric anthocyanin pigments reversibly change color with a change in pH; the colored oxonium form exists at pH 1.0, and the colorless hemiketal form predominates at pH 4.5. The difference in the absorbance of the pigments at 520 nm is proportional to the pigment concentration. Results are expressed on a cyanidin-3-glucoside basis. Degraded anthocyanins in the polymeric form are resistant to color change regardless of pH and