File No. 11023/53/2018-QA

Food Safety and Standards Authority of India

(A statutory Authority established under the Food Safety and Standards Act, 2006)

(Quality Assurance Division)

FDA Bhawan, Kotla Road, New Delhi – 110002

Dated, the 29th June, 2020

ORDER

Subject: **Method for the estimation of $\Delta\delta^{13}C_{fru\text{-}glu}$, $\Delta\delta^{13}C_{max}$, and Foreign Oligosaccharides in Honey by Elemental Analysis (EA)/Liquid Chromatography (LC) - Isotopic Ratio Mass Spectrometry (EA/LC-IRMS) - reg.**

The Scientific Panel on Methods of Sampling and Analysis and the Competent Authority, FSSAI has approved the Method for the estimation of $\Delta\delta^{13}C_{fru\text{-}glu}$, $\Delta\delta^{13}C_{max}$, and Foreign Oligosaccharides in Honey by Elemental Analysis (EA)/Liquid Chromatography (LC) - Isotopic Ratio Mass Spectrometry (EA/LC-IRMS) **[Annexure – I]**.

2. The food testing laboratories are hereby requested to use the aforesaid method, with immediate effect.

Encl: Method

29.6.2020

(Kumar Anil)
Advisor (QA)

To:

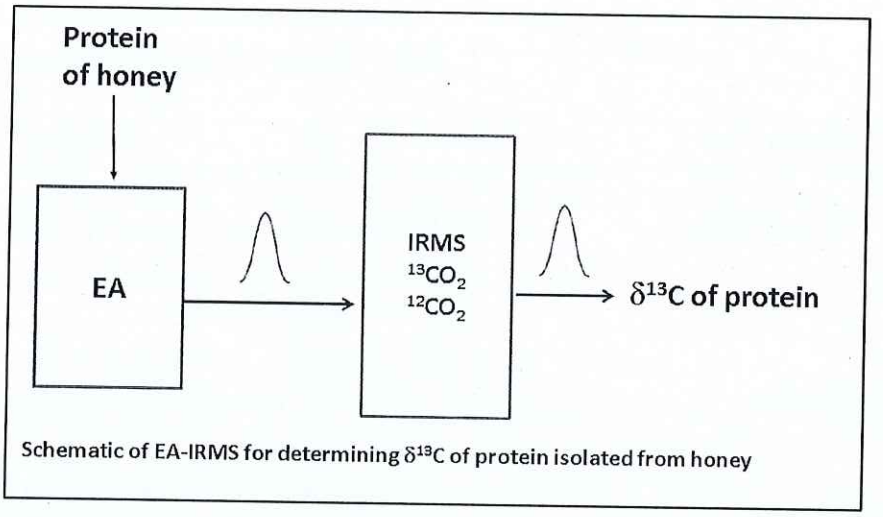
1. All FSSAI Notified Laboratories
2. All State Food Testing Laboratories

Copy to:

1. Executive Director (Regulatory Compliance), FSSAI
2. Advisor (Standards), FSSAI
3. Head (Regulations), FSSAI

Annexure -I

fssai FOOD SAFETY AND STANDARDS AUTHORITY OF INDIA Inspiring Trust, Assuring Safe & Nutritious Food	**Method for the estimation of $\Delta\delta^{13}C_{fru\text{-}glu}$, $\Delta\delta^{13}C_{max}$, and Foreign Oligosaccharides in Honey by Elemental Analysis(EA)/Liquid Chromatography(LC)-Isotopic Ratio Mass Spectrometry (EA/LC-IRMS)**		
Method No.	**02**	**Revision No. & Date**	**29.06.2020**
Abbreviations	IRMS: Isotope Ratio Mass Spectrometer LC: Liquid Chromatography EA: Elemental analyzer CEI: Compact Electron Ionization		
Caution	1. Always wear gloves and mask while doing sample analysis and reference material handling 2. Keep the eluent and reagent bottles under constant He purge to preventCO_2 contamination from ambient air. 3. Phosphoric acid and Sulphuric acid are highly corrosive. 4. Prepare the oxidation reagents fresh daily, store in dark brown bottle 5. Many of the routine gases for IRMS are hazardous. The laboratory should have an atmospheric monitoring system to warn of dangerous levels of gases.		
Principle	The method involves the determination of the relative isotopic ratios ($\delta^{13}C$) of 1) protein isolated from honey by EA-IRMS and 2) the $\delta^{13}C$ values of every individual sugar present in honey within a single HPLC run by LC-IRMS. Isotopic ratios are measured relative to a working reference gas calibrated using internationally accepted standards and are reported using the delta notation (δ) and expressed as 'per mill (‰)'. The delta notation is defined as $\delta^{13}C$ (o/oo) sample = [R(sample) / R(standard) - 1] × 1000 where R represents the ratio $^{13}CO_2/^{12}CO_2$. The $^{13}C/^{12}C$ carbon isotope ratios reported as $\delta^{13}C$ values are related to Vienna Pee Dee Belemnite (VPDB) according to the AOAC Official Method 998.12 δ is the$^{13}C/^{12}C$ ratio of the sample related to the $^{13}C/^{12}C$ ratio of a reference material to ensure international compatibility of data sets. The unit of expression is, per mill (‰). The CO_2 produced from combustion of the protein fraction isolated from the sample is analyzed to give $\delta^{13}C_{Protein}$‰by IRMS. The schematic for EA-IRMS is shown below		



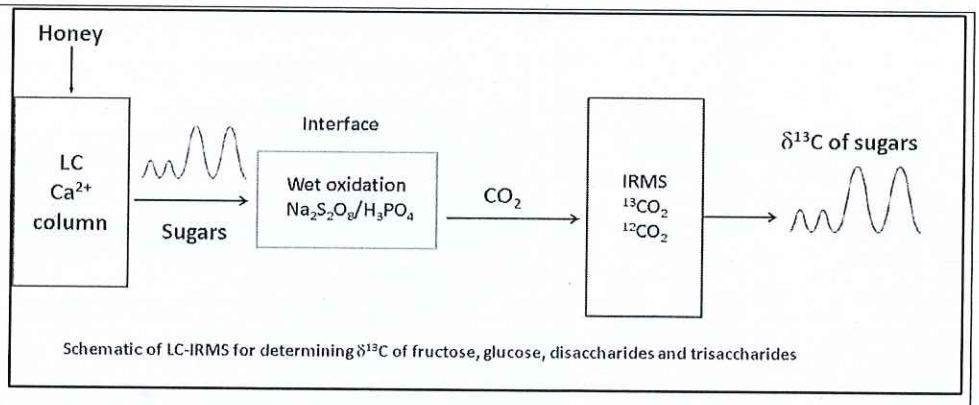
Schematic of EA-IRMS for determining $\delta^{13}C$ of protein isolated from honey

The $\delta^{13}C$ ‰ values of fructose, glucose, disaccharides, and trisaccharide and any other oligosaccharides present in honey are determined by LC-IRMS. The sugars are separated by LC using a cation exchange column. All individual sugars eluting from the LC column pass into the LC/IRMS interface. Here the carbon from organic samples in the mobile phase is converted into CO_2 by a wet chemical oxidation process using sodium peroxodisulfate either in the presence or absence of phosphoric acid. CO_2 and O_2 both diffuse through, which are subsequently dried in an online gas drying unit. The individual CO_2 peaks are subsequently admitted to the IRMS, which directly gives the $\delta^{13}C$ values for each individual sugar; $\delta^{13}C_{fru}$ ‰, $\delta^{13}C_{glu}$ ‰, $\delta^{13}C_{disaccharide}$ ‰, $\delta^{13}C_{trisaccharide}$ ‰ and $\delta^{13}C$‰ of any other oligosaccharides (see chromatograms below). The schematic of a typical LC-IRMS is shown below.

The difference in the carbon isotope ratio between $\delta^{13}C_{fru}$ ‰ and $\delta^{13}C_{glu}$ ‰gives $\Delta\delta^{13}C_{fru\text{-}glu}$‰.

$\Delta\delta^{13}C_{max}$ is the maximum difference observed between all possible isotopic ratios measured ($\Delta\delta^{13}C_{fru\text{-}disaccharides}$ / $\Delta\delta^{13}C_{fru\text{-}trisaccharides}$/ $\Delta\delta^{13}C_{fru\text{-}protein}$ / $\Delta\delta^{13}C_{flu\text{-}disaccharides}$ / $\Delta\delta^{13}C_{glu\text{-}trisaccharides}$ / $\Delta\delta^{13}C_{glu\text{-}protein}$/ $\Delta\delta^{13}C_{disaccharides\text{-}trisaccharides}$ / $\Delta\delta^{13}C_{disaccharides\text{-}protein}$ / $\Delta\delta^{13}C_{trisaccharides\text{-}protein}$)

The peak area (%) for foreign oligosaccharides is calculated from the areas appended in the LC chromatogram.

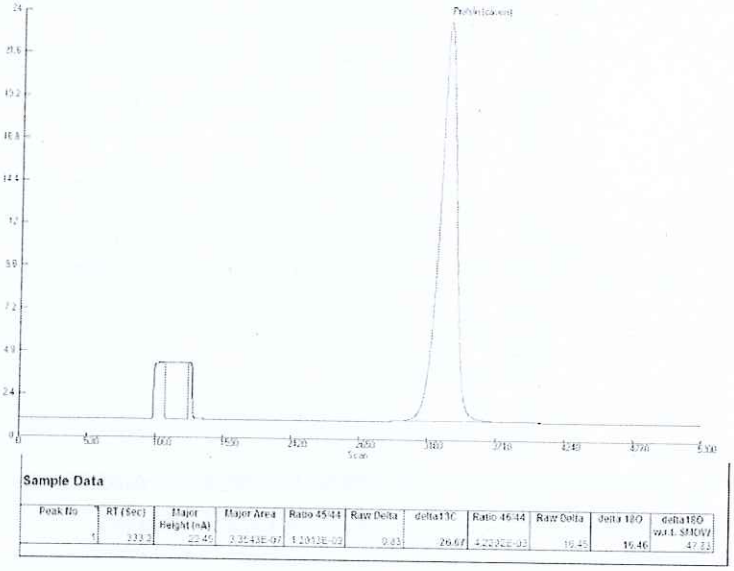


Schematic of LC-IRMS for determining $\delta^{13}C$ of fructose, glucose, disaccharides and trisaccharides

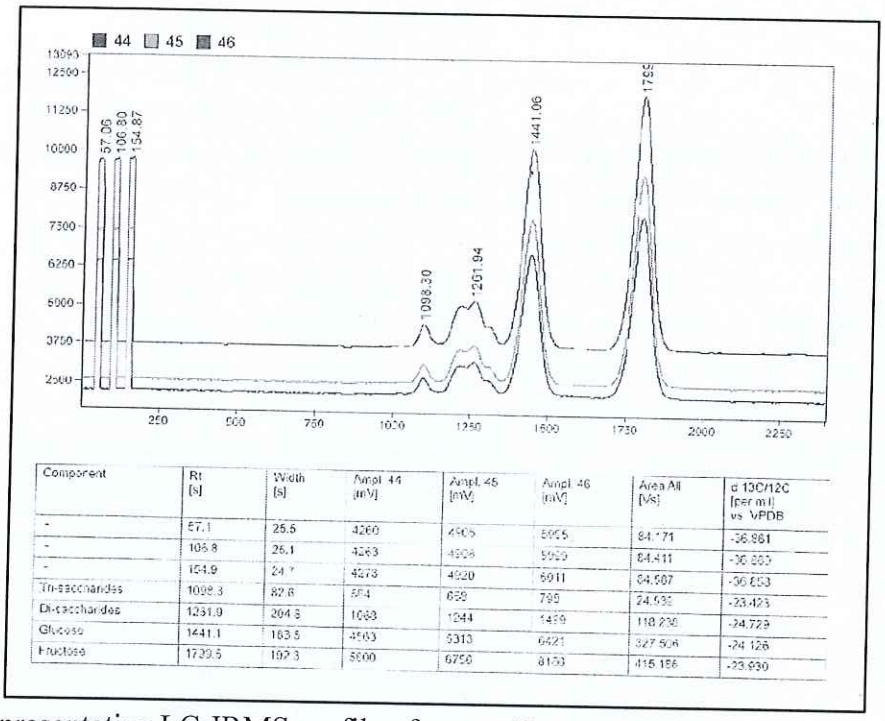
Apparatus	1. An integrated EA-IRMS instrument equipped with an automated combustion system and mass spectrometer designed or modified for isotope ratio measurement at natural abundance 2. An integrated LC-IRMS comprising of a HPLC/UPLC and in line oxidation reactor for aqueous oxidation of LC elute and a mass spectrometer designed or modified for isotope ratio measurement at natural abundance 3. LC comprises of a binary pump, autosampler, column oven (set at 80°C), and cation exchange column (Ca^{2+}, 300 × 7.7 mm, 8µm or equivalent) 4. Analytical microbalance: 0.0001g 5. Micropipette: 10 – 100 µL, 20 – 200 µL and 100 – 1000 µL 6. Volumetric flasks: 10 mL Class A 7. Vortex mixer 8. Sonicator 9. Centrifuge (capable of 10,000 × g) 10. Water bath (80 °C) 11. Convection Oven 12. Centrifuge tubes (50mL, 15mL) 13. Spatula 14. Forceps (blunt end and pointed curved end) 15. Tin capsules 16. Capsule holding tray 17. Nylon stocking material (100-150 mesh) 18. Syringe filters (0.45µm and 0.22 µm) 19. Vacuum concentrator
Chemicals	1. Ultra-pure water (Electrical Resistivity, Min.,18.18 MΩ cm, at 25°C) 2. Phosphoric acid (H_3PO_4) (puriss. P.a. ≥ 99%), crystalline 3. Sodium peroxodisulfate ($Na_2S_2O_8$, Sodium persulfate) (purum p.a. ≥ 99%) 4. Sodium tungstate dihydrate ($Na_2WO_4 \cdot 2H_2O$) (puriss. p.a. ≥ 99%) 5. Sulfuric acid (p.a. 98%)

<table>
<tr><td></td><td>6. CO_2 (working standard reference gas): 99.999% Pure
7. O_2 (flash combustion gas): >99.999% Pure
8. Helium: 99.999% pure</td></tr>
<tr><td>Reference standards</td><td>Stable isotope reference standards: Any of these certified reference standards defined below can be used as secondary working standards. These are not recommended for daily use as the commercial availability are restricted.
<table>
<tr><td>Standard</td><td>$\delta\ ^{13}C$ (‰)</td></tr>
<tr><td>Sucrose</td><td>-10.449</td></tr>
<tr><td>Casein</td><td>-26.98</td></tr>
<tr><td>NBS 22 Oil</td><td>-30.031</td></tr>
<tr><td>Beet sugar</td><td>-26.027</td></tr>
<tr><td>Galactose</td><td>-21.415</td></tr>
<tr><td>Fructose</td><td>-10.985</td></tr>
<tr><td>Glucose</td><td>-10.97</td></tr>
<tr><td>Cane sugar</td><td>-11.64</td></tr>
</table>
The following sugars are used as in-house standards for normalization and verified against any of the listed secondary standards.
1. D- (-)-fructose≥99% pure
2. D-(+)-glucose monohydrate ≥99.5% pure
3. D-(+)-sucrose ≥99% pure
4. D-(+)-maltose monohydrate ≥99% pure
5. D-(+)-Raffinose pentahydrate ≥99% pure</td></tr>
<tr><td>Preparation of reagents</td><td>Reagents for protein isolation
1. 10% aqueous solution of Sodium tungstate: Dissolve 10 g of $Na_2WO_4 \cdot 2H_2O$ in 100 mL of pure water. Prepare fresh daily
2. 0.335 M H_2SO_4: Dilute 1.88 mL concentrated H_2SO_4 to 100 mL with ultra-pure water
Chemical oxidation reagents
1. 20% Sodium peroxodisulfate: Dissolve 200 g Sodium peroxodisulfate in 1000 mL ultra-pure water in a brown glass bottle using an ultrasonic bath. Use a water-jet pump for vacuum degassing to remove all dissolved CO_2.
2. 1.5 M H_3PO_4 in water: Weigh 147.0 g of crystalline H_3P0_4. Dissolve in ~250 mL of ultra-pure water and make up to 1 L with water.
LC reagents
Ultra-pure water: (Electrical Resistivity, Min.18.18 MΩ cm, at 25°C)</td></tr>
<tr><td>Preparation of standards for EA IRMS</td><td>1. Weigh protein standard (Casein), approximately between 0.1-0.2 mg, with the help of spatula in the tin capsule.
2. Fold the tin capsule with the help of the forceps in such a way so as to remove air.</td></tr>
</table>

	3. Gently fold it from all the sides and place the folded tin capsule in the carousel and start the sequence of operation following the manufacturer's instruction
Sample preparation for EA-IRMS	a) Prepare in triplicate b) Strain honey through 100–150 mesh nylon stocking material to remove insoluble material. c) Add 4 mL H_2O to 10–12 g honey (in triplicate) in a 50 mL centrifuge tube and mix well to get a homogenous solution. d) Prepare fresh by mixing 2.0 mL 10% Na_2WO_4 solution and 2.0 mL 0.335 M H_2SO_4 in a small test tube. e) Add this mixture immediately to the diluted honey solution and mix well. f) Swirl the tube in ca 80°C water bath until a visible flocculant (precipitate) forms with a clear supernatant. *Note: If no visible flocculant forms, or if supernatant remains cloudy, add 2 mL aliquots of 0.335 M H_2SO_4 with repeating heating between additions.* g) Fill tube with water, mix, centrifuge for 5 min at 6000 × g h) Decant supernatant. i) Repeat washing, mixing, and centrifuging steps nine times with ca 40 mL portions of water, thoroughly dispersing the pellet each time. j) Dry protein at least for 3 h in ca 75°C oven. k) Weigh approximately 0.1-0.2 mg isolated protein in tin capsules. l) Gently fold the tin capsule with the help of forceps and place it on the carousel of EA-IRMS for determining $\delta^{13}C_{protein}$ ‰. ***Precautions*** i. Decant the supernatant immediately after centrifugation to avoid the mixing of pellet with the supernatant. ii. Protein washing must be done very carefully to avoid any loss of pellet with the water. iii. Fold the tin capsules gently to avoid the leakage or loss of sample iv. Be careful during tin capsule folding to avoid air trapping
EA-IRMS chromatography (*Instrument tuning varies with make and model. Set parameter as per manufacturer's instructions and optimize for best resolution*)	Operate the system according to the manufacturer's instructions. **Sample analysis** 1. Placed the isolated protein samples and standard in the tin capsules 2. Completely seal each capsule with tweezers 3. Place sealed capsules in the appropriate place on the tray of the automatic sampler 4. Operate the EA-IRMS following the manufacturer's instructions after calibration with CO_2 reference gas

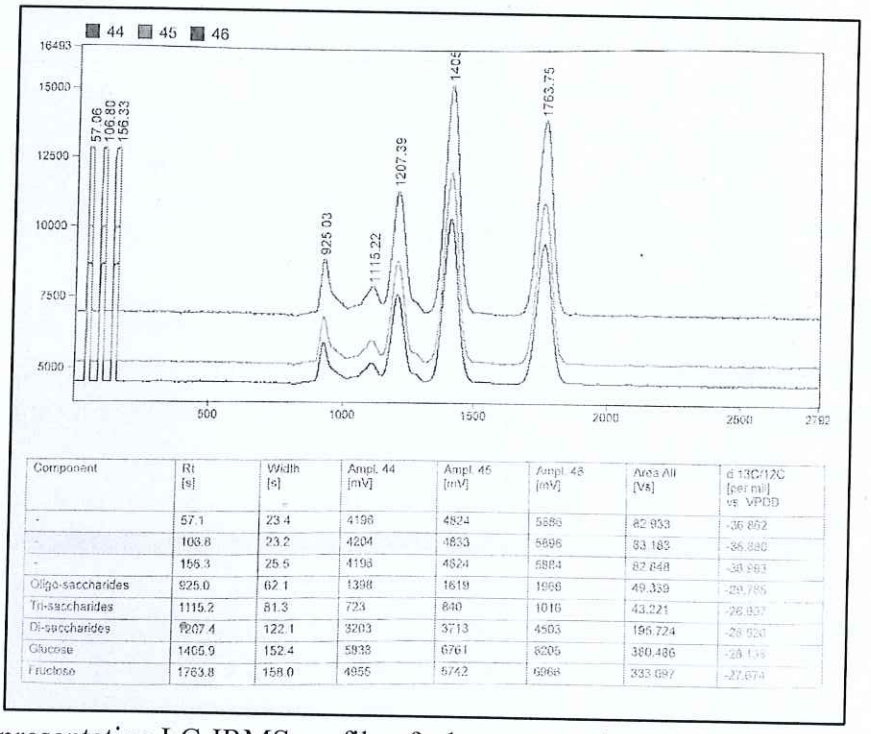
	Instrumental settings for EA (vario ISOTOPE cube, Elementar, UK) Temperature: Oxidation tube: 950°C Reduction tube: 650°C Pressure: 1300-1400 mbar He flow: 230 mL/min CO_2 flow: 230 mL/min O_2 flow: 18 mL/min ***Instrumental setting for IRMS (Isoprime IRMS)*** Ion Source: CEI — High Vacuum: 5e-6 Turbo Speed: 100% — TCD temperature: 59°C Focus point: >0.5 — Accelerating Voltage: 4000v Extraction Voltage: 76.00v — Half Plate differential (v): -121.00 Z-Plate Voltage (v): -53.00 — Trap Current (μA) : 200.00 Electron Volts (9ev): 75.00 — Ion Repellor Voltage (v): -9.00 Magnet Current: 4000 ***The gas cylinders (associated valves etc) which supply working gases to the IRMS must be stored in a temperature-controlled environment.***
Isotopic calculation **$\delta\ ^{13}C_{protein}$ ‰**	Isotope ratios are expressed relative to international standard. The $\delta^{13}C_{protein}$ ‰ is directly read of the chromatogram. See representative chromatogram below  Sample Data Peak No | RT (Sec) | Major Height (nA) | Major Area | Ratio 45/44 | Raw Delta | delta13C | Ratio 46/44 | Raw Delta | delta 18O | deltaD18O w.r.t. SMOW 1 | 333.2 | 22.45 | 3.3543E-07 | 1.1013E-02 | 0.83 | -26.67 | 4.2230E-03 | 19.45 | 19.46 | 47.83 Representative chromatogram: EA-IRMS profile of casein analysis showing $^{13}C_{protein}$ ‰= -26.67
Preparation of standards for LC-IRMS	**Preparation of reference sugar working standards** Prepare a solution of Fructose, Glucose, Sucrose and Raffinose containing 250 mg/L of each in ultra-pure water. Filter through 0.22 μm syringe filter
Sample Preparation for LC-IRMS analysis	1. Strain honey through a 100-150 mesh size nylon stocking material 2. In triplicate accurately weigh about 200 mg sample in a 15 ml centrifuge tube. Mix well with 5 ml of Ultra-pure water.

	3. Sonicate the mixture and make the volume up to 10 mL with water in a 10 mL volumetric flask. 4. Filter through 0.22 μm syringe filter into the HPLC injection vials. *Note: Prepare sample solutions fresh everyday*
LC-IRMS conditions (*Instrument setting and tuning vary depending on make and model. Set parameter and tune as per manufacturer's instructions and optimize for best results*)	The conditions shown below have been optimized with the following: **LC conditions** i. Column: Ca^{2+} (300 × 7.7 mm, 8μm) ii. Solvent: Ultra-pure Water iii. Flow rate: 0.3 mL/min iv. Column Oven temperature: 80 °C v. Injection volume: 10 μL **Interface for wet oxidation** (Isoprime Liquiface, Elementar, UK) i. Reactor temperature: 95°C ii. Oxidation reagent: 20% Sodium peroxodisulfate (Purge the solution with Helium gas before use) *iii.* Flow rate: 60 μL/min *Note: Some instruments use 20% Sodium peroxodisulfate and 1.5 M H_3PO_4 for wet oxidation. Follow the manufacturer's instructions.* **IRMS Parameters (*Isoprime IRMS*):** Ion Source: CEI — High Vacuum: 5e-6 Turbo Speed: 100% — TCD temperature: 59°C Focus point: >0.5 — Accelerating Voltage: 4000v Extraction Voltage: 76.00v — Half Plate differential (v): -121.00 Z-Plate Voltage (v): -53.00 — Trap Current (μA): 200.00 Electron Volts (9ev): 75.00 — Ion Repellor Voltage (v): -9.00 Magnet Current: 4000
LC-IRMS chromatography of samples	1. Introduce CO_2 reference gas pulse three times (20 s each) at the beginning of each run. 2. The constant flow rate during this period gives the peaks a flattop appearance (see figure below). 3. A level of CO_2 corresponding to 2–5 V (depending on the instrument) at m/z 44 is used to calibrate the system. 4. Inject standard mixture (10 μL) of fructose, glucose, disaccharide and trisaccharide. Repeat 10 times to obtain the mean and standard deviation for the $\delta^{13}C$ ‰ of individual sugars. 5. Inject Honey sample (10 μL) in triplicate. 6. The IRMS chromatogram provides details of the $\delta^{13}C$ ‰ of each of the sugars in the sample and the area under the curve of each of the resolved sugars (see representative chromatograms below). 7. The $\Delta\delta^{13}C_{fru\text{-}glu}$ ‰, $\Delta\delta^{13}C_{max}$ ‰, and foreign oligosaccharide content are calculated from the chromatogram data.
Representative LC-IRMS profiles	



Component	Rt [s]	Width [s]	Ampl. 44 [mV]	Ampl. 45 [mV]	Ampl. 46 [mV]	Area All [Vs]	d 13C/12C [per mil] vs. VPDB
-	57.1	25.5	4260	4905	5965	84.171	-36.861
-	106.8	25.1	4263	4908	5929	84.411	-36.880
-	154.9	24.7	4273	4920	5911	84.567	-36.853
Tri-saccharides	1098.3	82.6	564	659	799	24.536	-23.423
Di-saccharides	1261.9	204.8	1068	1244	1459	118.236	-24.729
Glucose	1441.1	183.5	4563	5313	6421	327.506	-24.126
Fructose	1739.5	192.3	5800	6756	8153	415.186	-23.930

Representative LC-IRMS profile of a pure Honey sample



Component	Rt [s]	Width [s]	Ampl. 44 [mV]	Ampl. 45 [mV]	Ampl. 46 [mV]	Area All [Vs]	d 13C/12C [per mil] vs. VPDB
-	57.1	23.4	4198	4824	5886	82.953	-36.852
-	106.8	23.2	4204	4833	5896	83.183	-36.880
-	156.3	25.5	4198	4824	5884	82.848	-36.883
Oligo-saccharides	925.0	62.1	1398	1619	1966	49.339	-29.785
Tri-saccharides	1115.2	81.3	723	840	1016	43.221	-26.937
Di-saccharides	1207.4	122.1	3203	3713	4503	196.724	-28.920
Glucose	1405.9	152.4	5833	6761	8205	380.486	-28.135
Fructose	1763.8	158.0	4955	5742	6968	333.697	-27.674

Representative LC-IRMS profile of a honey sample containing foreign oligosaccharides

Calculations

1. **$\Delta\delta^{13}C_{fru-flu}$ ‰**

Subtract the $\delta^{13}C_{Glu}$ (‰) value given in the chromatogram from the $\delta_{13}{}^{13}C_{Fru}$ (‰) value. Report as **$\Delta\delta^{13}C_{fru-flu}$‰**

2. **$\Delta\delta^{13}C_{max}$ ‰**

Extract the $\delta^{13}C$ ‰ values of fructose, glucose, disaccharides and trisaccharides from the LC-IRMS profile.

Extract the $\delta^{13}C$ ‰ of protein from EA-IRMS profile and tabulate as shown

A $\delta^{13}C$ ‰	B $\delta^{13}C$ ‰	A-B $\Delta\delta^{13}C$ ‰
Fructose	Disaccharide	
Fructose	Trisaccharide	
Fructose	Protein	
Glucose	Disaccharide	
Glucose	Trisaccharide	
Glucose	Protein	
Disaccharide	Trisaccharide	
Disaccharide	Protein	
Trisaccharide	Protein	

The highest value observed in column three gives $\Delta\delta^{13}C_{max}$ ‰

3. Foreign oligosaccharides (% peak area)

Extract the area of individual peaks and calculate using the formula

$$\text{Foreign oligosaccharide (area \%)} = \frac{\text{Sum of the peak area of all peak(s) other than Fructose, Glucose, disaccharides and trisaccharides}}{\text{Total peak area}} \times 100$$

Quality control

a. The laboratory working standard must be analysed in each sequence of sample measurements at least once.
b. A quality control sample of a chosen pure honey must be analysed in each LC-IRMS measurement sequence.

Reference

1. AOAC Official Method 998.12 C-4 Plants Sugars in Honey. Internal Standard Stable Carbon Isotope Ration Method First Action 1998
2. Improved detection of honey adulteration by measuring differences between $^{13}C/^{12}C$ stable carbon isotope ratios of protein and sugar compounds with a combination of elemental analyzer - isotope ratio mass spectrometry and liquid chromatography - isotope ratio mass spectrometry (δ13C-EA/LCIRMS). Lutz Elflein, Kurt-Peter Raezke; Apidologie 2008, 39 (5), 574–587.
3. Liquid chromatography coupled to isotope ratio mass spectrometry: A new perspective on honey adulteration detection. Ana I. Cabanero, Jose L. Recio, Mercedes RupeäRez; J. Agric. Food Chem. 2006, 54, 9719-9727.
4. LC-IRMS: Authenticity control of honey using the Thermo Scientific LC IsoLink LC-IRMS. Andreas W. Hilkert, Michael Krummen, Dieter Juchelka; Thermo application note 30024.
5. "Scientific support to the implementation of a Coordinated Control Plan with a view to establishing the prevalence offraudulent practices in the marketing of honey" N° SANTE/2015/E3/JRC/SI2.706828. E.

	Aries, J. Burton, L. Carrasco, O. De Rudder, and A. Maquet. JRC Technical Report 2016, JRC104749, 38 p.
Approved by	Scientific Panel on Methods of Sampling and Analysis