PERIODIC TABLE OF FOOD INITIATIVE

Total Sugar Analysis

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1.0 Overview

This standard operating procedure is used to extract total sugar, measured as glucose, fructose, sucrose, lactose, maltose and isomaltose, from animal and plant samples. The samples are diluted in 75% organic solution with an added internal standard mixture. The sugar components are separated on a Water BEH Amide column and detected by the Agilent Triple Quadrupole LC/MS.

2.0 Definitions

IS: Internal Standard
ACN: Acetonitrile
IPA: Isopropanol
MeOH: Methanol

3.0 Materials and Equipment

The equivalent materials and equipment may be used if not stated as **required**.

3.1 Reagents

1. Sodium Acetate, JM Baker, 3462-01
2. Calcium chloride, Fisher Bioreagents, 10035-04-8
3. Glacial Acetic Acid, 97.5%, VWR, 64-19-7
4. Water, HPLC Grade, Fisher Chemical, W5-4
5. Ammonium Hydroxide (28-30%, NH_3 basis), Sigma Aldrich, 2211228-500ML-A
6. 50% Sodium hydroxide, VWR, 1310-73-2
7. Acetonitrile, Optima LC/MS Grade, Fisher Chemical, A955-4
8. Ammonium Acetate (99.99%), Sigma Aldrich, 372331-100G
9. Isopropanol (HPLC Grade), Sigma Aldrich, 34863-4L
10. Methanol (HPLC Grade), Sigma Aldrich, 34860

3.2 Standards

1. Sugar Calibration Curve (see appendix) **(Required)**
2. IS C13 Sugar Mixture, 500 ug/mL (see appendix) **(Required)**
3. Sugar QC Stock, 2.5 ug/mL (see appendix) **(Required)**

3.3 Sample Matrix

1. Food samples (prepared following the PTFI sampling protocols)
2. Reference Food Sample: NIST standard

3.4 Consumables

1. 15 mL Falcon tubes, Corning, 352096
2. 96 well plate (PCR), Eppendorf, 0030129318
3. Autosampler 96 well plate mat (PCR), Axygen, AM96-PCR-RD
4. 0.2uM WWPTFE 96 well filter plate, Pall Corp, 8682 **(Required)**
5. 10µL, 20µL, 200µL pipette tips
6. 1mL Combitip, Eppendorf, 4008-9430
7. pH strips, Hydrion pH 0-13, #93
8. 0.25mL autosampler vial, Thermo Scientific, 200 050
9. Blue snap-it seal caps, Thermo Scientific, C4011-54B
10. Disposable Weigh Boats, VWR, 10770-454
11. Disposable Polypropylene Spatula, LevGo 17231
12. General Purpose Laboratory Tape, VWR, 89097-916
13. Polystyrene Reservoirs, non-sterile, 100ml, VWR, 89094-660
14. Aluminum Foil Seals for PCR and Storage, VWR 60941-126

3.5 Equipment

1. Waters Acquity BEH Amide Column, 2.1x150mm, 1.7µm, PN 186004802 **(Required)**
2. Waters Acquity BEH Amide VanGuard Pre-Column, 2.1x50mm, 1.7µm, PN 186004799 **(Required)**
3. pH Meter, Oakton, 54X409903
4. 1L clear reagent bottles with caps, DWK Life Sciences, 218815457
5. 50mL glass graduated cylinder, VWR, 10124-056
6. 1000mL glass graduated cylinder, VWR, 470228-972
7. 10µL, 20uL, 200µL multichannel pipette
8. 10µL, 20uL, 200µL pipette
9. Bottle Top dispenser,1-10 mL, VWR, 76319-586
10. Vortex, VWR, 710213
11. Centrifuge, MedLab, TDA5A with 96 well plate capacity
12. Fridge, 4°C
13. Freezer, -20°C

14. Analytical Scale

4.0 Safety

Please be sure to use the proper Personal Protection Equipment (PPE). At a minimum, use safety shields, gloves and a lab coat.

5.0 Preparation Reagents

A “batch” is defined as a set of samples extracted at the same time. One extraction blank, 1 calibration curve (14 calibrators), 1 reference food, and 1 QC needs to be extracted for every batch of samples. The QC will be re-injected every 24 samples. For example, for a 60 sample run, a batch consists of 1 blank, 1 reference food, 1 calibration curve (14 calibrators), 1 QC and 60 samples (77 total extractions).

The following reagent preparation is for a sample batch of 77 total extractions. Adjust ratios according to your specific batch size. Note: There are few limitations to the batch size. Limitations will be dependent on lab capabilities (eg. instrument time and reagent bottle sizes).

5.1 Total Sugar Reagents

The following reagent preparation is enough for 1 batch of 77 total extractions. Adjust ratios according to your batch extraction limit.

100mM Sodium Acetate Buffer (pH 5.0 + 5mM Calcium Chloride)

1. Use a 1000 mL graduated cylinder to measure 900 mL of Water.
2. Using a pipette to slowly add 5.8 mL of glacial acetic acid to the Water.
3. Use the pH meter to adjust the solution to pH 5.0 using sodium hydroxide solution.
4. Adjust to 1000 mL with water. Transfer the solution to a 1 L reagent bottle.
5. Add 0.74g of calcium chloride dihydrate to the solution. Cap the bottle and mix to fully dissolve.
6. This solution can be stored in the fridge, 4°C.

Solution “A1”: 10 mM Ammonium Acetate in H2O: ACN (90:10) (pH adjusted to 10.2 w/ Ammonium Hydroxide)

1. Fill a graduated cylinder to measure 900 mL of Water and transfer to a 1 L reagent bottle.
2. Weigh 0.77g of ammonium acetate into a weighing boat. Transfer the ammonium acetate into the 1 L reagent bottle of Water.
3. Cap the reagent bottle and invert a few times to fully dissolve the ammonium acetate.

4. Use a graduated cylinder to measure 100 mL of ACN and add to the solution in the 1 L reagent bottle.
5. Adjust the pH to 10.2 using ammonium hydroxide (~4.5 mL of ammonium hydroxide is needed to reach a pH of 10.2). Use pH test strips or a pH meter to confirm.
6. Cap the bottle and invert a few times to mix.
7. This solution can be prepared as needed.

Solution “B1”: 10 mM Ammonium Acetate in ACN:H2O (90:10) (pH adjusted to 10.2 w/ Ammonium Hydroxide)

1. Fill a graduated cylinder to measure 100 mL of Water and transfer to a 1L reagent bottle.
2. Weigh 0.77g of ammonium acetate into a weighing boat. Transfer the ammonium acetate into the 1L reagent bottle of Water.
3. Cap the reagent bottle and invert a few times to fully dissolve the ammonium acetate.
4. Use a graduated cylinder to measure 900 mL of ACN and add to the solution in the 1L reagent bottle.
5. Adjust the pH to 10.2 using ammonium hydroxide (~4.5 mL of ammonium hydroxide is needed to reach a pH of 10.2). Use pH test strips or a pH meter to confirm.
6. Cap the bottle and invert a few times to mix.
7. This solution can be prepared as needed.

Needle wash: ACN/IPA/MeOH/Water (1:1:1:1, v/v/v/v)

1. Transfer 200 mL of each solvent (ACN, IPA, MeOH and water) to a 1 L graduated cylinder.
2. Transfer into a clear 1 L reagent bottle.
3. Cap the bottle and invert a few times to mix.
4. This solution can be prepared as needed.

Seal Wash: 10% IPA

1. Transfer 100 mL of IPA to a 1 L graduated cylinder.
2. Add 900 mL of Water to the IPA in the 1 L graduated cylinder.
3. Transfer the solution into a clear 1 L reagent bottle.
4. This solution can be prepared as needed.

6.0 Sample Extraction

6.1 Sample Aliquoting

1. Allow food samples, reference sample, calibration curve (14 calibrators), QC stock and IS to reach room temperature (~30 minutes).
2. For each food and reference samples, weigh 50.0 + 2.0 mg in 15mL tubes. Record all weights as additional metadata that will be entered into Freezerworks or submission of

data into the PTFI Lab Portal. Note: To prepare the blank, calibration curve and QC skip to step 6.3.

6.2 Sugar Extraction

1. To each sample tube, use a bottle top dispenser to add 5 mL of the 100 mM Sodium Acetate buffer.
2. Cap and vortex the sample tubes for 5 seconds or longer to fully resuspend.
3. Stack a 96 well filter plate (0.2 um WWPTFE) on top of a 96 well analysis plate. Tape the two plates together to ensure they will stay attached during centrifugation.
4. Use a multichannel pipette to transfer 75 µL of Acetonitrile from a solvent reservoir to each sample well.
5. Use a multichannel pipette to transfer 10 µL of Water from a solvent reservoir to each sample well. Pipette the solvents up and down a few times to mix the contents of the well.
6. Use a pipette to transfer 5 µL of the Sugar IS to each sample well. Pipette the solvents up and down a few times to mix the contents of the well.
7. Use a pipette to transfer 10 µL of each sample into each sample well. Pipette the solvents up and down a few times to mix the contents of the well.
8. Centrifuge the plates at 2000 rpm for 2 minutes.
9. After centrifugation, all contents from the filter plate should be transferred to the analysis plate. Note: The filter plate can be discarded. Filter plates can only be used once.
10. Place a PCR lid on the analysis plate. If sample analysis will not run the same day, replace the plate lid with a foil PCR plate lid and store in a -20°C freezer. On the day of analysis, thaw the plate to room temperature and replace the foil lid with an autosampler PCR plate lid.

6.3 Blank, Calibration Curve and QC Preparation

Depending on the number of samples extracted in step 6.2, the calibration curve may be added to 14 empty wells in the same PCR 96 well plate rather than a new PCR plate.

Blank 75% ACN

1. Use a pipette to transfer 75 µl of Acetonitrile to an autosampler vial.
2. Use a pipette to transfer 25 µl of Water to the vial.
3. Cap and vortex to mix. If sample analysis will not run the same day, store the blank in a -20°C freezer. On the day of analysis, thaw the blank to room temperature.

Calibration Curve

1. Use a multichannel pipette to transfer 75 µl of Acetonitrile from a solvent reservoir to 14 sample wells in a 96 well PCR plate.

2. Use a pipette to add 5 µL of the IS to each sample well. Pipette the solvents up and down a few times to mix the contents of the well.
3. Use a pipette to transfer 20 µL of L1 calibrator into a sample well. Pipette the solvents up and down a few times to mix the contents of the well.
4. Repeat transferring 20 µL of L2-L14 calibrators into separate sample wells. Pipette the solvents up and down a few times to mix the contents of the well.
5. Place a PCR lid on the plate. If sample analysis will not run the same day, replace the plate lid with a foil PCR plate lid and store in a -20°C freezer. On the day of analysis, thaw the plate to room temperature and replace the foil lid with an autosampler PCR plate lid.

QC

1. Use a pipette to transfer 75 µl of Acetonitrile to an autosampler vial.
2. Use a pipette to transfer 5 µL of the IS to the vial. Pipette the solvents up and down a few times to mix the contents of the well.
3. Use a pipette to transfer 20 µL of QC stock into the vial. Pipette the solvents up and down a few times to mix the contents of the well.
4. Cap and vortex to mix. If sample analysis will not run the same day, store the blank in a -20°C freezer. On the day of analysis, thaw the QC to room temperature.

7.0 Total Sugar LC/MS Operating Parameters

7.1 Instrumentation

1. Infinity 1290 UHPLC, Agilent Technologies, equipped with:
    a. Column compartment (MCT), Model G7116B
    b. Multisampler, Model G7167B
    c. Binary Pump, Model G7120A
    d. Waters Acquity UPLC BEH Amide Column, 2.1x150mm, 1.7µm, PN 186004802 **(Required)**
    e. Waters Acquity UPLC BEH Amide VanGuard Pre-column, 2.1x5mm, 1.7µm, PN 186004799 **(Required)**

2. 6470 QqQ Mass Spectrometer, Agilent Technologies

3. Mobile Phases, Needle Wash and Seal Wash
    a. Solution “A”: 10 mM Ammonium Acetate in Water: ACN (90:10) pH 10.2
    b. Solution “B”: 10 mM Ammonium Acetate in ACN: Water (90:10) pH 10.2
    c. Needle wash: ACN/IPA/MeOH/Water (1:1:1:1, v/v/v/v)
    d. Seal Wash: 10% IPA

7.2 Method

1. LC Gradient **Table 1**.

Time (min)	%B
0.00	95.00
4.00	95.00
5.50	80.00
9.50	72.00
9.51	60.00
12.50	60.00
12.51	95.00
15.50	95.00

2. Injection Volume: 5 µL
3. Flow Rate: 0.450 mL/min
4. Column Temperature: 50°C
5. Autosampler Temperature: 4°C
6. Needle wash: Flush Port 3 seconds
7. MS Parameters **Table 2.**

Parameter	Setting
Gas Temperature	225°C
Gas Flow	13 L/min
Nebulizer	35 psi
Sheath Gas Temp	350°C
Sheath Gas Flow	12 L/min
Capillary (Negative)	3500 V
Nozzle Voltage (Negative)	500 V

8. Dynamic MRM Parameters

Polarity: Negative
MS1 and M2 Res: Unit
Cell Accelerator Voltage: 5
Table 3. Dynamic MRM parameters

Compound Name	Precursor Ion	Product Ion	Ret Time (min)	Delta Ret Time	Fragmentor	Collision Energy
C13 Glucose & C13 Fructose	185.1	92.0	6	8	80	4
C13 Lactose	353.2	167.1	8.5	8	135	4
C13 Maltose	353.2	167.1	8.5	8	70	4
C13 Sucrose	353.2	105.0	8.5	8	135	20
Glucose & Fructose	179.1	89.1	6	8	100	4

Isomaltose	341.1	221.1	8.5	8	70	20
Maltose & Lactose	341.1	161.1	8.5	8	70	4
Sucrose	341.1	101.1	6	8	135	20

8.0 Data Analysis

Total sugar results should be routed to the Verso team via email to Steve@versobio.com or Tracy@versobio.com for processing.

See Appendix for additional data analysis information.

9.0 References

9.1 Cheang et al. 2024 Combined Alcohol Soluble Carbohydrate Determination (CASCADE) of Food. *ACS Food Sci. Technol*. 2024, 4, 3, 554–560 https://doi.org/10.1021/acsfoodscitech.3c00641

10.0 Appendix

10.1 IS Mixture – 500 µg/mL

Reagents

1. D-[UL-13C6] Fructose, Omicron Biochemicals, FRU-011, 201595-65-5
2. [UL-13C12] Sucrose, Omicron Biochemicals, SUC-006, 57-50-1
3. [UL-13C12] Lactose, Omicron Biochemicals, LAC-008, 64044-51-5
4. [UL-13C12] Maltose, Omicron Biochemicals, MAL-002, 6363-53-7
5. D-[UL-13C6] Glucose, Omicron Biochemicals, GLC-082, 110187-42-3
6. Water, HPLC Grade, Fisher Chemical, W5-4

Consumables

1. 1.5 mL Microcentrifuge tubes, screwcap, Sarstedt Inc, 72.703.600

2. Disposable Polypropylene Spatula, LevGo 17231
3. 200, 1000 µL pipet tips

Equipment

1. Analytical scale
2. 200 and 1000 µL Pipette
3. Freezer, -20°C
4. Vortex

Mixture Preparation

1. Weigh 5.0 - 10.0 mg of each C13 standard (C13 Fructose, C13 Glucose, C13 Maltose, C13 Lactose and C13 Sucrose) into separate 1.5 mL microcentrifuge tube.
2. Use a pipette to add water to each weighed C13 standard to create a 10 mg/mL solution. For example, for a weight of 7.9 mg, add 790 µL of water to create a 10 mg/mL solution.
3. Cap each C13 standard solution and vortex to fully dissolve.
4. Use a pipette to transfer 750 µL of water into a new microcentrifuge tube.
5. Use a pipette to add 50 µL of each C13 10mg/ml solutions to the aliquoted water. Note: Rinse the pipette up and down 4 times when dispensing to ensure all C13 solutions are transferred to the water. Change pipet tips between C13 solutions.
6. Cap and vortex the IS mixture.
7. Freeze the IS mixture and any remaining C13 solutions in the -20°C freezer.

10.2 Total Sugar Calibration Curve

Reagents

1. Water, HPLC Grade, Fisher Chemical, W5-4
2. D-(+) Glucose, Sigma-Aldrich, 50-99-7
3. D-(-) Fructose, Sigma-Aldrich, 57-48-7
4. Maltose, VWR, 6363-53-7
5. Sucrose, VWR, 57-50-1
6. Isomaltose, Tokyo Chemical Industry, 499-40-1
7. Lactose, Biosynth Carbosynth, 63-42-3

Consumables

1. 1.5 mL Microcentrifuge tubes, screwcap, Sarstedt Inc, 72.703.600
2. Disposable Polypropylene Spatula, LevGo 17231
3. 200µL and 1000 µL pipet tips

Equipment

1. Analytical scale

2. 200 µL, 1000 µL Pipettes
3. Freezer, -20°C
4. Vortex

Mixtures Preparation

1. Weigh 10.0 - 20.0 mg of each sugar standard (Fructose, Glucose, Sucrose, Maltose, Lactose and Isomaltose) into separate 1.5mL microcentrifuge tubes.
2. Use a pipette to add water to the weighed sugar standards to create a 20 mg/mL solution. For example, for a weight of 16 mg, add 800 µL of water to create a 20 mg/mL solution.
3. Cap each standard solution and vortex to fully dissolve.
4. Use a pipette to transfer 100 µL of water into a new microcentrifuge tube.
5. Use a pipette to add 150 µL of each 20 mg/mL solutions to the aliquoted water. Note: Rinse the pipette up and down 4 times when dispensing to ensure all 20 mg/mL solutions are transferred to the water. Change pipet tips between 20 mg/mL solutions.
6. Cap and vortex the microcentrifuge tube. This mixture is a 3000 µg/mL stock mixture that will be used for calibration point L14. All stock mixtures will be 5x the concentration of the desired final calibration point. (ex. The final concentration of calibration point L14 is 600 µg/mL and the stock solution of calibration point L14 is 3000 µg/mL).
7. Create the rest of the calibration curve stock mixtures (L1-L13) according to the dilution scheme below. The stock will be 5x the concentration of the desired final calibration point. (ex. Cal L14 = 600 ug µg/mL, stock solution of Cal L14 = 3000 µg/mL). For example, to create stock mixture L13 use a pipette to transfer 147 µL of water into a new microcentrifuge tube. Use a pipette to transfer 735 µL of stock solution L14 to the water for a total volume of 882 µL. Cap and vortex the microcentrifuge tube. This mixture is a 2500 µg/mL stock mixture that will be used for calibrator L13. The remaining volume of stock solution L14 is 265 µL.

	Cal Level [ug/mL]	Cal Stock Level [ug/mL]	volume previous standard [ul]	volume H2O [ul]	Final volume of sample before subsequent dilution [ul]	remaining volume after subsequent dilution [ul]
L14	600	3000	N/A	N/A	1000	265
L13	500	2500	735	147	882	254
L12	400	2000	628	157	785	251
L11	300	1500	534	178	712	252
L10	200	1000	460	230	690	255
L9	150	750	435	145	580	252
L8	100	500	328	164	492	252
L7	50	250	240	240	480	255
L6	25	125	225	225	450	254
L5	10	50	196	294	490	255
L4	5	25	235	235	470	250
L3	2.5	12.5	220	220	440	250
L2	1.25	6.25	190	190	380	255
L1	0.625	3.125	125	125	250	250

10.4 QC Stock

Reagents

1. Water, HPLC Grade, Fisher Chemical, W5-4
2. Sugar Calibrator Stock L14 (3000 µg/mL)

Consumables

1. 1.5 mL Microcentrifuge tubes, screwcap, Sarstedt Inc, 72.703.600
2. Disposable Polypropylene Spatula, LevGo 17231
3. 10 µL and 1000 µL pipet tips

Equipment

1. Analytical scale
2. 10 µL, 1000 µL Pipettes
3. Freezer, -20°C
4. Vortex

QC Stock Mixture

The QC stock mixture is prepared using an aliquot from calibrator stock L14.

1. Thaw calibrator stock L14 to room temperature (~30 minutes).
2. Use a pipette to add 1195 µL of water to a microcentrifuge tube.
3. Use a pipette to add 5 µL of calibrator stock L14 to the water. Pipette the solvents up and down a few times to rinse the pipet tip.
4. Cap the tube and vortex to mix the microcentrifuge tube. This mixture is a 12.5 µg/mL QC stock mixture that will be used for the QC.
5. This QC stock can be stored in the -20°C freezer until use.

10.5 Total Sugar Data Analysis

The quantitation of all 6 sugar analytes is performed by employing an individual standard curve (quadratic, non-forced origin, with a weight of 1/x) and peak area is normalized by an employing internal standard. Levels for the calibration curve are as follows:

- 0.625 µg/ml = L1
- 1.25 µg/ml = L2
- 2.5 µg/ml = L3
- 5 µg/ml = L4
- 10 µg/ml = L5
- 25 µg/ml = L6

- 50 µg/ml = L7
- 100 µg/ml = L8
- 150 µg/ml = L9
- 200 µg/ml = L10
- 300 µg/mL = L11
- 400 µg/mL = L12
- 500 µg/mL = L13
- 600 µg/mL = L14

When optimizing the standard curve, any level with accuracy outside of a +25% range for all except isomaltose +30% range of the intended concentration should not be used to calculate the standard curve. To ensure proper quantification of samples, prioritize removing the outer (e.g. lowest or highest) points of the calibration curve first and ensure that per analyte, the minimum and maximum responses for the included levels are respectively lower and higher than the responses of each sample.

Final concentrations (µg/mg) for each sugar can be calculated by inserting the responses into the calibration curve equation. Due to the nature of the sample preparation, there is no multiplication factor needed to convert from µg/ml to µg/mg. Assuming the sample preparation input is exactly 50.0 mg, then the final concentrations values are accurate. If sample preparation input weights are not exactly 50.0mg, a conversion factor (50.0/(input mass)) can be applied to the final concentration to get the corrected values. Units are then converted from ug/mg to g/100g by multiplying the data by 0.1. Any missing values are set to 0.

The limit of quantitation (LOQ) for this assay is 0.06g/100g. Any values below the LOQ are designated as “<LOQ” in the final data file. Finally, the measured compounds are summed for each sample to get a “Total Sugar” measurement.