Method 6.6 – C-molasses: fructose, glucose and sucrose by HPLC

1. Rationale

This method is applicable to final molasses and is used to determine the glucose, fructose and sucrose contents of the sample by High Performance Liquid Chromatography (HPLC).

2. Principle

The method uses high performance ion (anion) exchange chromatography (HPAEC) with pulsed amperometric detection (PAD) and an internal standard (lactose) to determine the glucose, fructose and sucrose contents of a sample quantitatively. Results are based on the peak height to mass ratios of an integrated chromatograph.

An anion exchange column is used for the separation of the sugars. The separation mechanism of these columns relies on the fact that the sugars are complex alcohols that can be ionised at a high pH.

3. Definitions

3.1 Internal standardisation

The area of a specific chromatographic peak is determined by the amount of the analyte present in the sample. However, other factors such as column and detector temperatures may also influence the peak and will in turn affect the sensitivity response of the detector. Internal standardisation can be used to eliminate the effect of such variations by adding a known amount of a reference substance (the internal standard) to a fixed volume of the sample before injection into the column.

Standard solutions with known quantities of the analytes (glucose, fructose and sucrose) and the internal standard (lactose) are prepared and chromatographed. The standards are prepared to bracket the expected concentration range.

3.2 Relative response factor (RRF)

A relative response factor is determined for each sugar by dividing the mass to peak height ratio of each sugar by the mass to peak height ratio of the internal standard. The RRF values should be similar for standard solutions of different concentrations.

$$RRF_s = \frac{M_s}{P_s} \times \frac{P_I}{M_I}$$

where P ≡ peak area
M ≡ mass
S ≡ sugar
I ≡ internal standard

4. Apparatus

*All glassware must be boiled in a detergent (*e.g. *Extran, Merck), rinsed thoroughly and dried in an oven at approximately 110°C.*

4.1 Volumetric flasks: 10 × 100 and 2 × 2 000 cm^3

4.2 Beakers: 10 × 50 cm^3

4.3 Pipettes: 10 × 1 cm^3

4.4 Chromatograph

A high performance liquid chromatograph (HPLC) comprising a pump, autosampler or manual injection valve and a pulsed amperometric detector (PAD) with a cell containing a gold electrode (*e.g.* Dionex PAD-2) is used. The column must be an anion exchange column specifically designed for carbohydrate analysis (*e.g.* Dionex CarboPac PA1).

4.5 Membrane filters

Cellulose acetate filters of pore size 0.45 µm and 25 mm diameter for samples and 47 mm diameter for the solvent.

4.6 Autosampler vials: 1 cm^3

5. Reagents

5.1 Phosphorus pentoxide

Phosphorus pentoxide (P_2O_5) is toxic and corrosive to the eyes, skin and respiratory tract. Work in a fume cupboard while wearing gloves and safety glasses.

5.2 Glucose

Dry at approximately 40°C under vacuum over phosphorus pentoxide for 4 hours and store in a desiccator.

5.3 Fructose

Dry at approximately 40°C under vacuum over phosphorus pentoxide for 4 hours and store in a desiccator.

5.4 Sucrose

Dry at approximately 40°C under vacuum over phosphorus pentoxide for 4 hours and store in a desiccator.

5.5 Lactose solution

Weigh 64.00 g of lactose into a 100 cm^3 beaker. Dissolve, transfer to a 2 000 cm^3 volumetric flask and make to the mark. Sub-divide into 8 sachets (250 cm^3) and freeze. Allow the stock solution to reach room temperature before use.

5.6 Sodium hydroxide (50% solution)

Sodium hydroxide (NaOH) is a corrosive base and contact with the skin and eyes should be avoided.

Filter deionized water (18 mΩ) through a 0.45 µm membrane. Transfer 150 g of the water to a 250 cm^3 Schott bottle and sparge for 30 minutes with an inert gas [*e.g.* nitrogen (N_2)]. Mass 150 g of sodium hydroxide pellets just before adding it to the sparged and stirred water. The dissolution is exothermic and the solution will therefore heat up. Sparge and stir until all the pellets are dissolved. Allow to cool slightly, remove the stirrer and sparger and replace the lid.

Leave the stock to stand undisturbed for at least 7 days before use. Carbonates (CO_3^{2-}) will precipitate. Do not disturb the solution at any time. Pipette carefully from the clear portion of the solution. Close the bottle immediately after use. Do not use more than three-quarters of the solution.

5.7 Sodium azide solution (0.02%)

Sodium azide (N_3Na) is poisonous and irritating to the eyes. Contact with the skin and eyes should be avoided. Work in a fume cupboard while wearing gloves and safety glasses.

Weigh 0.4 g sodium azide into a 50 cm^3 beaker. Dissolve in some distilled water, transfer to a 2 000 cm^3 volumetric flask and make to the mark.

5.8 HPAEC solvent

Filter 4 litres of distilled water through the 0.45 µm membrane filter to remove dissolved carbon dioxide (CO_2). Transfer to a Pyrex bottle fitted with a screw cap containing three small holes (*i.e.* for the gas sparging tube, the eluant outflow line and a vent to ensure constant blanketing without build-up of pressure). Sparge with an inert gas [*e.g.* nitrogen (N_2)] for 30 minutes.

Pipette 31.2 cm^3 of the 50% NaOH solution into the sparged solvent (4 litres) to prepare 150 mM NaOH eluant. Ensure that the solvent is sparged continuously to eliminate CO_2. Decreasing retention times are a good indication that CO_2 contamination has occurred.

6. Procedure

6.1 Calibration standards

Prepare three standard solutions by weighing the quantities indicated in Table 1 accurately into 100 cm^3 volumetric flasks. Make to the mark with distilled water.

Table 1: Calibration standards

Component (g)	Calibration Standard		
	S1	**S2**	**S3**
Glucose (g)	0.020	0.060	0.100
Fructose (g)	0.030	0.070	0.110
Sucrose (g)	0.250	0.310	0.370
Lactose (g)	5.000	5.000	5.000

Sub-divide the standards into 10 sachets (10 cm^3 each) and freeze. Before each run, thaw a set of sachets (Sl, S2 and S3). Dilute the standards with 0.02% azide solution (2 cm^3 or 1 cm^3 to 100 cm^3 for PAD-2 or PAD-SC cells respectively). Filter through a 0.45 µm membrane into autosampler vials. Prepare two vials for each of the three standards, plus (x + 1) vials of S2, the prime calibration standard, where x is the number of samples. Theses S2 vials will be used to sandwich replicate vials of each molasses sample.

6.2 Samples

Weigh 1.0 g of the molasses samples and 5.0 g of the lactose solution in duplicate into 100 cm^3 beakers. Add about 50 cm^3 of distilled water and stir to dissolve. Transfer to a 100 cm^3 volumetric flask and make to the mark with distilled water. Dilute 2 cm^3 (PAD-2 cell) or 1 cm^3 (PAD-SC cell) of this solution to 100 cm^3 in a second volumetric flask with the 0.02% azide solution. Filter through a 0.45 µm membrane into autosampler vials.

6.3 Experimental conditions

An injection volume of 20 µl is used for all standards and samples. The column is operated at approximately 28°C. If possible the detector cell should be maintained in a column oven at approximately the same temperature. It is important to protect the detector and column from temperature fluctuations caused by draughts, central heating or air conditioners. This is to minimise response factor changes. The operating flow rate is 1 cm^3 per minute. The flow is reduced to 0.1 cm^3 per minute when the column is not in use. Column eluant is not recycled.

The settings shown below are used with a Dionex PAD-2. A measuring range of 3K is used for these settings.

Table 2: PAD-2 detector settings

Voltage constant	$E_{Applied}$ (V)	time constant	Time (milliseconds)
E1	0.05	t1	420
E2	0.75	t2	180
E3	-0.20	t3	360

6.4 Running the samples

Before analysing the samples, run the 3 standards to establish the system's linearity. If the sucrose response is not linear (*i.e.* if RF > 1.5% RSD), determine the source of error and correct before continuing. Run replicate vials from each sample sandwiched between vials of S2. Average response factors from the S2 vials bracketing the samples are used to calculate the sugar concentrations for each sample (*e.g.* S2, sample la, sample lb, S2, sample 2a, sample 2b, S2). This process is continued until all the samples have been chromatographed.

6.5 Integration

It is essential that an electronic integrator be used in order to achieve the required precision. The peak width, threshold settings and other integration options should be chosen to ensure that the same type of integration is used for the sugar peaks in both the samples and the standards.

Integrators that store all the raw data for a run are ideal, as peak width and threshold settings can later be changed to optimise integration without re-injection of standards and samples. Peak height quantification has greater freedom from interference by adjacent peaks, but the baseline selection must be carefully controlled to ensure uniformity between the standards and the samples.

7. Calculations

7.1 Calibration

Use the internal standard technique with peak areas as described in 3 for calibration.

7.2 Sugar % sample

Determine the amount of each sugar in the sample as follows:

$$\% \text{ sugar} = \frac{P_s}{P_I} \times M_I \times RRF_s \times \frac{100}{M_{sple}}$$

where P ≡ peak area
M ≡ mass
S ≡ sugar
I ≡ internal standard
RRF_s ≡ relative response factor
M_{sple} ≡ mass of sample

8. Precision

The relative standard deviation (RSD) for sucrose should be less than 1% for duplicate analyses. The RSD for the monosaccharides should he less than 2% for duplicate analyses.

9. References

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