Customs Laboratories
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Determination of Sugars in Food Products using High Performance Liquid Chromatography (HPLC)

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This table shows the most important changes that have been made compared with the latest former version	
Date of the latest former version: 25 July 2023	
Section	**Changes**
5.4.3	Minor change: filtering the sample extract with a syringe filter is facultative (and not compulsory)

Determination of Sugars in Food Products using High Performance Liquid Chromatography (HPLC)

1. Scope

This method describes the determination of the contents of free sugars: fructose, glucose, sucrose, maltose and lactose in food products for human consumption. It shall be used for implementation of Regulation (EC) N° 900/2008 (article 2 - calculation of sucrose/invert sugar/isoglucose content) and Regulation (EC) N° 904/2008 (article 2 - calculation of sugars). It shall also be used for the analysis of food for the determination of Tariff classification, except when specific methods apply.

2. Principle

Sugars are extracted using water and determined by HPLC using a refractive index detector and an external standard.

3. Reagents and materials

Use reagents of recognised analytical grade and demineralised or distilled water.

3.1. Glucose, min 99 %.

3.2. Fructose, min 99 %.

3.3. Sucrose, min 99 %.

3.4. Maltose monohydrate, min 99 %.

3.5. Lactose monohydrate, min 99 %.

3.6. Acetonitrile, HPLC quality.

3.7. Zinc acetate dihydrate, p.a.

3.8. Potassium hexacyanoferrate (II) trihydrate (K4[Fe(CN)]6.3H2O), extra pure.

3.9. Glacial acetic acid.

3.10. Reagent for clarification (Carrez I).

For example, dissolve 219.5g zinc acetate dihydrate (section 3.7) in water in a beaker glass. Rinse into a volumetric flask of 1000 ml and add 30 ml of glacial acetic acid (section 3.9). Mix thoroughly and dilute to the mark with water. This solution may be used for max 6 months while stored at ambient temperature.

Other clarification reagents, equivalent to Carrez I system, may be used.

3.11. Reagent for clarification (Carrez II).

For example, dissolve 106 g potassium hexacyanoferrate (II) trihydrate (section 3.8) in water in a beaker glass. Rinse into a volumetric flask of 1000 ml. Mix thoroughly and dilute to the mark with water. This solution may be used for max. 6 months while stored at ambient temperature.

Other clarification reagents, equivalent to Carrez II system, may be used.

3.12. HPLC Mobile phase.

Prepare a mobile phase which is conventionally used in the HPLC analysis of sugars.

When using an aminopropyl silica gel column, a common mobile phase is a mixture of HPLC grade acetonitrile (section 3.6) and water (e.g. 75/25 or 80/20 v/v).

The proportion of acetonitrile and water are adjusted depending on the state of the column in order to achieve a satisfactory separation of the sugars.

When using ion-exchange chromatography a common mobile phase is water or mixture of water and acetonitrile (up to 70/30 v/v).

4. Apparatus

4.1. Standard laboratory glassware

4.2. Fluted filters

4.3. Syringe filters, 0.45 µm, suitable for aqueous solutions

4.4. Sample vials suitable for the HPLC autosampler

4.5. 100 ml volumetric flasks

4.6. Plastic syringes, 5 ml or 10 ml

4.7. Analytical balance with precision of 0.1 mg

4.8. Apparatus suitable for high performance liquid chromatography, with:

- Column, (generally, alkylamine, 5 µm and 250 mm x 4,6 mm, or equivalent) placed in an thermostatic compartment to maintain a constant temperature.

The column and the mobile phase are chosen in order to obtain a separation between the peaks of fructose and glucose with a minimum resolution factor (RS) of 0.8, calculated with the following formula

$$RS = \frac{t_{R2} - t_{R1}}{0{,}5 \cdot (W_1 + W_2)}$$

where t_{R1} and t_{R2}: retention times of the first and second peaks,
and W_1 and W_2: baseline widths of the first and second peaks (in time units)

The separation can be improved with an additional column to achieve higher resolution.

- Refractive index detector

For stability, the temperature of the detector needs to be constant.

5. Procedure

5.1. Preparation of standard solutions

For a standard solution of 5 g/L, weigh 0.5 g of each sugar (section 3.1 to 3.5) to the nearest 0.1 mg in a 100 ml volumetric flask (section 4.5). Add 50 ml of water. Shake until the sugars are completely dissolved (eventually using a thermostatically-controlled water bath or ultrasonic bath) and dilute to the mark with water.

Suitability of stored standard solutions (refrigerated or deep freeze) must be verified.

Other or additional standard solutions can be prepared with different concentrations e.g. 2 g/L, for control or to establish a calibration curve. Multiple-point standardisation could be better than a single-point calibration; at least, the linearity of the system should be checked.

5.2. Sample preparation

The product is thoroughly homogenised generally by grinding, mixing, depending on its consistency.

5.3. Sample portion

The amount of sample is estimated from the ingredient declaration and the conditions of the HPLC analysis (concentration of the standard solutions), and shall not exceed:

amount of sample (g) = $\frac{0,5}{X}$ x V

where X is the highest estimated content among the sugars contained in the sample(% m/m) and V is the volume of the flask (ml).

Weigh the sample to 0.1mg accuracy.

5.4. Analysis

5.4.1. Preparation of the sample solution

The chosen test portion (section 5.3) is weighed into a volumetric flask (section 4.5). Add water up to 2/3 of the volume. Mix thoroughly.

For solid samples of a relatively complex consistency (e.g. halva, chocolate, praline etc.) a mild heating in a water bath or ultrasonic bath of around 40-70^{o}C for approximately 30 min is required to facilitate sugar extraction. However, products with thickening agents (such as starch) should not be heated to prevent degradation of the chromatography column.

For chewing gum samples, the addition of 10 ml of toluene before the addition of water to 2/3 of the volume of the flask facilitates sugar extraction. After clarification and dilution to the mark of the flask, as described below, the lower layer will be used to be filtered. The volume of toluene, which will not contain any sugar, will be subtracted from the volume of the flask for the dilution factor.

5.4.2. Clarification

For samples with a high content of proteins or fat, clarification is necessary by adding 1 (or up to 3) ml Carrez I (section 3.10) to the sample solution. After shaking, 1 (or up to 3) ml Carrez II (section 3.11) is added. Shake the sample again.

5.4.3. Processing for HPLC analysis

The sample in the volumetric flask is diluted to the mark with water at 20°C, homogenised and filtered through a fluted filter (section 4.2). Collect the sample extract. If necessary, filter the extract (for example, through a syringe filter (section 4.3) with a syringe (section 4.6) that has been preflushed with the extract). For fat clarification a C18 Cartridge Solid Phase Extraction (SPE) can also be used.

Collect the filtrates in vials (section 4.4).

5.5. Chromatography

Check the resolution with the standard solution and adjust if necessary the composition of the mobile phase (section 3.12).

The HPLC analysis of the standard solutions and the sample solutions are performed. The injected volume is generally 20 µL.

Check for a single point calibration that the variation coefficient of 5 injections of the standard solution is below 5% for each of the 5 sugars or if using a multiple-point calibration that the correlation factor is at least 0.995, or use the controls recommended by your method validation.

Examples of operating conditions:

HPLC-system with 2 columns:

Pre column: AJO-4302
Columns: SHODEX ASAHIPACK NH2P-50 4D 150 x 4,6 mm
LUNA OMEGA 3µm SUGAR 250 x 4,6 mm
Mobile phase: acetonitrile:water (77:23)
Runtime: 35 min.
Column temperature: 35°C
RI-temperature: 35°C
Backwards pressure in the system ca. 170 bar

HPLC-system with 1 column:

Column + precolumn: Waters carbohydrate high performance 4 µm (4.6 x 250 mm) + Waters high performance carbohydrate 4 µm (3.9 x 20 mm guard column)
Mobile phase: acetonitrile:water (80:20)
Runtime: 30 minutes
Column temperature: 35°C
RI temperature: 35°C
Backwards pressure in the system ca. 120 bar

If the sample contains sugar alcohols, the identification of each sugar must be checked and if necessary an additional analysis using other conditions (column, mobile phase) has to be performed. For example, glucose and sorbitol have close retention times.

Some amino acids can also interfere and the use of a different technique is then recommended, e.g. PAD-HPLC.

6. Calculation

For each sugar, the peak areas are determined after integration and the concentrations are calculated after calibration with standard solutions.

7. Expression of results

The expression of the results (sucrose/invert sugar/isoglucose content) is detailed in Regulation (EC) N° 900/2008 and in Regulation (EC) N° 904/2008.

Fructose, glucose, sucrose, maltose and lactose contents are expressed in g/100g.
Results are given with one decimal place (i.e. a maximum of 3 significant digits, with one decimal).

8. Precision

Eight CLEN inter laboratory tests were carried out between 2002 and 2021 (including the CLEN 9th Meursing test edition being the latest test performed in 2021), with the determination of sugars by HPLC by EU Customs laboratories.

Repeatability (r) and reproducibility (R) for sucrose and lactose

Sucrose			
concentration range	0-10%(m/m):	r = 0.8% (m/m)	R = 1.6% (m/m)
	10-33%(m/m):	r = 8% relative	R = 16.4% relative
	33-50%(m/m):	r = 2.6% (m/m)	R = 5.4% (m/m)
Lactose			
Concentration range	0-10%(m/m):	r = 0.9% (m/m)	R = 2.3% (m/m)
	10-30%(m/m):	r = 9% relative	R = 22.5% relative

Limits of detection (LOD) and limits of quantification (LOQ)

LOD and LOQ minimum requirements for the analysis of fructose, glucose and sucrose (for each sugar individually), established for the Meursing table code attribution:

LOD = 0.8% m/m	for respectively fructose, glucose and sucrose
LOQ =2.5% m/m	for respectively fructose, glucose and sucrose