

HPLC METHOD

For Analysis of Isomalto-oligosaccharide Mixture

Analyte: Isomaltooligosacchrides

Matrix: IMO Powder, IMO Syrup

Scope:

This method is for the analysis of the following compounds:

Glucose
Maltose
Isomaltose
Panose
Isomaltotriose
Maltotetraose (DP4)
Isomaltotetraose (DP4)
Maltopentaose (DP5)
Isomaltopentaose (DP5)
Maltohexaose (DP6)
Isomaltohexaose (DP6)
Maltoheptaose (DP7)
Isomaltoheptaose (DP7)

Unknown compounds may be present in the chromatogram. The amount of these compounds may be estimated by quantitating with a closely eluting standard.

Principle:

Samples are extracted with purified water and analyzed with High Performance Liquid Chromatography- Evaporative Light scattering Detection (HPLC-ELSD).

Apparatus:

- Laboratory Balance, 5 place
- Adjustable Volume Pipettes
- Disposable 15 ml Centrifuge Tubes, with Caps
- Volumetric Flasks, various volumes
- Volumetric Pipettes, various volumes
- Vortex Mixer
- HPLC Injection Vials
- HPLC, including:
 - Degasser
 - Pump, capable of Binary solvent delivery
 - Autoinjector
 - Column Oven
 - ELSD detector, Alltech 2000
- Column, Prevail Carbohydrate, 4.6 x 250 mm, Grace Scientific p/n 35101

BioNeutra Inc., Edmonton,

- Precolumn, Prevail Carbohydrate. 4.6 x 7.5 mm, Grace Scientific p/n 96435

Note- equivalent equipment may be substituted.

Reagents:

- Acetonitrile, HPLC grade
- Purified Water, HPLC grade

Note- equivalent reagents may be substituted.

Standards:

- Glucose
- Maltose
- Isomaltose
- Panose
- Isomaltotriose
- Maltotetraose
- Isomaltotetraose
- Maltopentaose
- Isomaltopentaose
- Maltohexaose
- Isomaltohexaose
- Maltoheptaose
- Isomaltoheptaose

Standard Preparation:

- Prepare a stock standard of each individual sugar at a concentration of approximately 15 mg/ml. Prepare the standard by accurately weighing standard material, adding an appropriate volume of Purified Water, then Vortexing for approximately 30 seconds. Example- 0.07500 g of Glucose is weighed in a 15 ml Disposable Centrifuge Flask, 5 ml of Purified Water is added, then the Flask is capped and Vortexed for 30 seconds.

- Prepare a Mixed Standard. Combine the following volumes of each Stock Standard in a 1 ml volumetric flask. Adjust the Mixed Standard Volume to 1 ml with Purified water and Vortex to mix.

Compound	Approximate Concentration of Stock (mg/L)	Volume of Stock (µL)	Final Volume (mL)	Approximate Concentration Of Mixed Standard (mg/mL)
Glucose	15	33	1	0.495
Maltose	15	66.5	1	0.9975
Isomaltose	15	233	1	3.495
Panose	15	66.5	1	0.9975
Isomaltotriose	15	133	1	1.995
Maltotetraose	15	133	1	1.995
Isomaltotetraose	15	167	1	2.505
Maltopentaose	15	66.5	1	0.9975
Maltohexaose	15	33	1	0.495
Maltoheptaose	15	33	1	0.495

Note- The concentration and volume of the Mixed Standard may be varied at the chemist's discretion. If needed, the mixed standard may be concentrated with a lyophilizer.

Note- Isomaltopentaose, Isomaltohexaose and Isomaltoheptaose may be added to the standard mix, or prepared as a separate standard at the chemist's discretion. A curve concentration range of 0.25 mg/ml to 1 .00mg/ml is recommended for these oligosaccharides.

- Preparation of Working Standards. The Mixed Standard will be the highest Working Standard. Prepare serial dilutions from the Mixed Standard to create four additional Working Standards. Mix each standard by Vortexing. Use the following Volumes-

Working Standard Level	Source Standard	Source Standard Volume (mL)	Volume of Purified Water added (mL)	Final Volume (mL)
4	Mixed Standard	0.5	0.39	0.89

3	Working Standard Level 4	0.5	0.39	0.89
2	Working Standard Level 3	0.5	0.39	0.89
1	Working Standard Level 2	0.5	0.39	0.89

Approximate Concentrations of Working Standards.

	Working Level 4 (mg/mL)	Working Level 3 (mg/mL)	Working Level 2 (mg/mL)	Working Level 1 (mg/mL)
Glucose	0.27809	0.15623	0.08777	0.049309
Maltose	0.56039	0.31483	0.17687	0.099365
Isomaltose	1.9635	1.10309	0.61971	0.34815
Panose	0.56039	0.31483	0.17687	0.099365
Isomaltotriose	1.1208	0.62966	0.35374	0.19873
Maltotetraose	1.1208	0.62966	0.35374	0.19873
Isomaltotetraose	1.4073	0.79062	0.44417	0.24953
Maltopentaose	0.56039	0.31483	0.17687	0.099365
Maltohexaose	0.27809	0.15623	0.08777	0.049309
Maltoheptaose	0.27809	0.15623	0.08777	0.049309

Note- Differing Working Standard Concentrations may be created at the chemist's discretion.

Sample Preparation:

1. IMO powder.
 - a. Weigh approximately 0.1000 g of powder in a 15 ml disposable centrifuge flask. Record the collected weight.
 - b. Using a Volumetric Pipette, add 10 ml of Purified Water to the flask and cap.
 - c. Vortex the flask for approximately 30 seconds. Transfer approximately 0.5 ml of extract to a suitable injection vial. The sample is ready for HPLC analysis.
2. IMO syrup.
 - a. Weigh approximately 0.2500 g of sample in a 15 ml disposable centrifuge flask. Record the collected weight.
 - b. Transfer approximately 7 ml of purified water to the flask and cap. Vortex the tube for approximately 30 seconds.
 - c. Transfer the extract to a 25 ml volumetric flask.

- d. Repeat steps b. and c. twice.
- e. Bring the volume of the extract to 25 ml volume with purified water. Mix the solution by inverting several times.
- f. Transfer approximately 0.5 ml of sample extract to a suitable injection vial and cap. The sample is ready for HPLC analysis.

Analysis:

HPLC conditions:

Mobile Phase A: Purified Water

Mobile Phase B: Acetonitrile

Flow Rate: 1 mL/min

Gradient:

Time:	Mobile Phase A (%)	Mobile Phase B (%)
0.0	24.0	76.0
20.49	24.0	76.0
20.50	33	67
27.09	33	67
45.0	47.5	52.5
45.10	24.0	76.0

Injection Volume: 8 μ L

Column Temperature: 35°C

ELSD:

Tube Temperature	100°C
Gas Type:	Nitrogen
Gas Flow:	2.0 L/min
Gain	1

Note- Chromatographic conditions may be modified to obtain desired chromatography.

Calculations:

Integration software is used to integrate the areas of the components of the standards. The areas of the standards are plotted against their corresponding concentrations to produce a quadratic curve with the following equation:

$$y=ax^2+bx+c$$

The area of each component in the sample chromatogram is compared to the standard curve of the same component to calculate a concentration.

The concentration derived from the standard curve is multiplied by the sample volume, and divided by the sample weight to determine the amount of each component in the sample.

In the sample chromatograms, peaks were present that did not correlate with the retention time, or peak shape of standard chromatograms (refer to following chromatograms). The peaks were quantitated using closely eluting standards to give a mass balance estimate. Similarity of analyte response may be confirmed by evaluating determined concentration versus theoretical concentration of known addition studies.

In the following sample chromatogram, the unknown peaks were quantitated using the specified standard curve:

Component	Curve Reference
Unknown 1	Maltose
Unknown 2	Maltose
Unknown 3	Panose
Unknown 4	Maltotetraose
Unknown5	Maltopentaose
Unknown6	Maltohexaose
Unknown 7	Maltoheptaose
