

## **CARBOHYDRATES BY HPLC**

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### **UOP Method 780-92**

#### **SCOPE**

This method is for determining saccharides in aqueous solutions by high performance liquid chromatography (HPLC). Specific saccharides typically quantitated are tetrasaccharides (DP4+) and unresolved higher polysaccharides; maltotriose including other unresolved trisaccharides (DP3); maltose, maltulose plus other unresolved disaccharides; glucose; mannose plus sorbose; fructose; and psicose. The lower limit of quantitation for an individual component in the aqueous solution is typically 0.01 mass-%.

#### **OUTLINE OF METHOD**

Samples having a solids concentration above 20 mass-% are diluted. The sample or sample dilution is filtered through a 0.22- $\mu$ m filter. A reproducible sample volume is injected into a series arrangement of two packed resin-based calcium-form columns connected to a differential refractive index detector. The peak area for each saccharide is determined and concentrations are calculated using factors derived from the analysis of calibration blends. Results are reported in mass-% and/or normalized mass-% composition (dry basis).

#### **APPARATUS**

References to catalog numbers and suppliers are included as a convenience to the method user. Other suppliers may be used.

*Balance*, readability 0.1-mg

*Bottle*, wide-mouth, with teflon-lined cap, 120-mL capacity, Fisher Scientific, Cat. No. 03-321-1A

*Bottle*, four-liter, polyethylene, Fisher Scientific, Cat. No. 02-923J, for collection of mobile phase eluant

*Bubble trap*, in-line, 1/16-inch, with bracket, Alltech Associates, Cat. No. 01-0221

*Chromatographic column*, resin-based, calcium-form, HPX-87C, 300  $\times$  7.8 mm, Bio-Rad, Cat. No. 125-0095 (two required)

|                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                       |
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| <p><b>IT IS THE USER'S RESPONSIBILITY TO ESTABLISH APPROPRIATE PRECAUTIONARY PRACTICES AND TO DETERMINE THE APPLICABILITY OF REGULATORY LIMITATIONS PRIOR TO USE. EFFECTIVE HEALTH AND SAFETY PRACTICES ARE TO BE FOLLOWED WHEN UTILIZING THIS PROCEDURE. FAILURE TO UTILIZE THIS PROCEDURE IN THE MANNER PRESCRIBED HEREIN CAN BE HAZARDOUS. MATERIAL SAFETY DATA SHEETS (MSDS) OR EXPERIMENTAL MATERIAL SAFETY DATA SHEETS (EMSDS) FOR ALL OF THE MATERIALS USED IN THIS PROCEDURE SHOULD BE REVIEWED FOR SELECTION OF THE APPROPRIATE PERSONAL PROTECTION EQUIPMENT (PPE).</b></p> |
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*Column heater*, capable of maintaining a column temperature of  $85 \pm 1^\circ\text{C}$ , Bio-Rad, Cat. No. 125-0425

*Cryobox*, polycarbonate,  $9 \times 9$  array storage to hold 81 cryovials, Fisher Scientific, Cat. No. 15-350-107B

*Cryovial*, polypropylene, 2.0-mL capacity, Fisher Scientific, Cat. No. 03-337-7D

*Desiccator*, glass, 250-mm ID, Fisher Scientific, Cat. No. 08-615B

*Detector*, differential refractive index, Waters Associates, Model 410, Cat. No. 70000

*Dish*, petri, glass,  $100 \times 10$ -mm, Fisher Scientific, Cat. No. 08-747B

*Filter*, 0.22- $\mu\text{m}$  Durapore, 25-mm, Millipore, Cat. No. GVWP 025 00

*Filter*, solvent inlet filter, Upchurch Scientific, Cat. No. A310

*Flask*, 4-liter, filtering, borosilicate glass, Fisher Scientific, Cat. No. 10- 180H, used as the mobile phase reservoir

*Freezer*, -50 to  $-85^\circ\text{C}$  range, Fisher Scientific, Cat. No. 13-989-96

*Guard columns*, de-ashing cation/anion system and Carbo-C micro guard, Bio-Rad, Cat. Nos. 125-0118 and 125-0128, respectively

*Guard column holder assembly*, two end cap assemblies, one union, and one body, 30-mm, Pierce Chemical, Cat. Nos. B-34402, B-34400 and B-34403, respectively

*Hot plate/stirrer*, square, ceramic top, 60- to 100-rpm, 115-V, 60-Hz, Fisher Scientific, Cat. No. 11-498-7H

*Injector*, autosampler, capable of a 10- $\mu\text{L}$  injection, Waters, WISP Model 712, Cat. No. 77207

*Integrator*, electronic, for obtaining peak areas

*Oven*, vacuum,  $30 \times 20 \times 20$ -cm interior dimensions, temperature to  $200^\circ\text{C}$ , vacuum to 102 kPa below ambient, Fisher Scientific, Cat. No. 13-264A

*Pump*, HPLC, capable of maintaining 0.6 mL/min flow rate, Waters Model 510, Cat. No. 21000

*Recorder*, multi-range, single pen, Fisher Scientific, Cat. No., 13-939-85A

*Silicone stopper*, solid, size No. 14, Cole-Parmer, Cat. No. L-06298-26

*Stir bar*, magnetic, octagonal, Teflon-coated, Fisher Scientific, Cat. No. 14-511-63

*Syringe*, 3-mL, disposable, with Luer-Lok fitting, Fisher Scientific, Cat. No. 14-823-40

*Vial*, glass, molded screw cap, 14.8-mL capacity, Fisher Scientific, Cat. No. 03-339-21F

*Vial*, glass, molded screw cap, 22.2-mL capacity, Fisher Scientific, Cat. No. 03-339-21K

*Vial assembly*, consisting of cap with self-sealing silicone septa, WISP, Waters Associates, Cat. No. 73010

*Vial*, glass, 4-mL capacity, WISP suitability, Waters, Cat. No. 72710

*Water purification system*, MilliQ-Plus UF, Millipore, Cat. No. ZD4011595

## REAGENTS

All reagents shall conform to the specifications established by the Committee on Analytical Reagents of the American Chemical Society, when such specifications are available, unless otherwise specified.

References to catalog numbers and suppliers are included as a convenience to the method user. Other suppliers may be used.

*Desiccant*, indicating Drierite adsorbent, 8-mesh, Fisher Scientific, Cat. No. 07-578-3A

*D-(-)Fructose*, 99% minimum purity Baker Analyzed, VWR Scientific, Cat. No. JTM 556-5

*D-Glucose*, anhydrous, 99% minimum purity, Mallinckrodt, Cat. No. 4912

*D-(+)Maltose monohydrate*, 94% minimum purity, Baker analyzed, VWR Scientific, Cat. No. JTP 533-5

*D-Mannose*, 99% minimum purity, Aldrich, Cat. No. 11,258-5

*Maltotetraose (DP4)*, 99% minimum purity, Boehringer Mannheim, Cat. No. 691-780

*Maltotriose (DP3)*, 98% minimum purity, Boehringer Mannheim, Cat. No. 691-771

*Psicose*, 95% minimum purity, Sigma, Cat. No. P 8043

*D-Sorbose*, 98% minimum purity, Aldrich, Cat. No. 29,433-0

*Water*, double-deionized only, MilliQ grade

## PROCEDURE

### Preparation of Apparatus

1. Prepare the guard columns according to the manufacturer's specifications.
2. Assemble the guard columns in the proper sequence from inlet to outlet in the following order: de-ashing cation/anion guard and Carbo-C micro guard.
3. Degas MilliQ grade water in the 4-L filtering flask mobile phase reservoir for a minimum of two hours at 90°C under vacuum while constantly stirring.
  - Heating the water while applying the vacuum and constantly stirring, will not only facilitate the release of gas from the water, but will also help to sterilize the eluant.
  - The mobile phase reservoir must be kept clean, free of particulate and microbial growth.
4. Install the guard columns and analytical columns. Assemble, test and prepare all system components (see Fig. 1) as per the manufacturer's specifications.

### Operating Conditions

1. Establish the recommended operating conditions listed in Table 1.
  - Other conditions may be used provided they produce the required sensitivity and chromatographic separations equivalent to those shown in the typical chromatogram.

2. Ensure that the linear range of the refractive index detector and the integrator system are not exceeded when operated at the recommended conditions.
  - This can be determined by inspection of the chromatogram for flat or rounded top peaks for the components of interest or by injecting a larger and/or smaller volume of blend and comparing the results.

### Sample Preparation

1. Determine the refractive index of the sample using UOP Method 816, or equivalent.

**Table 1**  
**Operating Conditions**

|                            |                                                                                    |
|----------------------------|------------------------------------------------------------------------------------|
| <b>Mobile Phase</b>        | <b>MilliQ degassed water,<br/>maintained at 85°C with constant stirring</b>        |
| <b>Pump flow</b>           | <b>0.6 mL/min</b>                                                                  |
| <b>Sample size</b>         | <b>10 <math>\mu</math>L</b>                                                        |
| <b>Column temperatures</b> |                                                                                    |
| <b>Guard</b>               | <b>ambient</b>                                                                     |
| <b>Chromatographic</b>     | <b>85°C</b>                                                                        |
| <b>Detector</b>            | <b>refractive index</b>                                                            |
| <b>Temperature</b>         | <b>40°C</b>                                                                        |
| <b>Recorder</b>            | <b>10-mV full scale</b>                                                            |
| <b>Chart speed</b>         | <b>2.5 mm/min</b>                                                                  |
| <b>Integration system</b>  | <b>acquisition and analysis parameters as<br/>per manufacturer's specification</b> |

2. Read the corresponding mass-% solids value from Table 2.
3. Perform a quantitative dilution (Steps 4 through 6) on those samples that have a total mass-% solids greater than 20%.
  - Samples having a solids content less than 20 mass-% are analyzed as received (neat) omitting Steps 4 through 6.
4. Dilute the sample on a mass/mass basis to  $15 \pm 2$  mass-% with water.
5. Record the mass of the sample and water to the nearest 0.1 mg.
6. Shake the diluted sample well.
7. Withdraw an aliquot using a 3-mL disposable syringe.
8. Attach the 0.22- $\mu$ m filter unit to the syringe Luer-Lok and filter the aliquot into a WISP vial.

### Chromatographic Technique

1. Ensure that the system is equilibrated as per the recommended operating conditions and that a stable recorder baseline is established.
2. Inject 10  $\mu$ L of the sample or sample dilution using the autosampler and immediately start the integrator.

**Table 2**  
**Percent Solids Based on Refractive Index<sup>a</sup>**

| <b>Solids,<br/>%</b> | <b>Refractive<br/>Index,<br/>20°C</b> | <b>Solids,<br/>%</b> | <b>Refractive<br/>Index,<br/>20°C</b> |
|----------------------|---------------------------------------|----------------------|---------------------------------------|
| 0                    | 1.33299                               | 38                   | 1.39506                               |
| 1                    | 1.33439                               | 39                   | 1.39694                               |
| 2                    | 1.33583                               | 40                   | 1.39882                               |
| 3                    | 1.33728                               | 41                   | 1.40072                               |
| 4                    | 1.33874                               | 42                   | 1.40263                               |
| 5                    | 1.34021                               | 43                   | 1.40456                               |
| 6                    | 1.34169                               | 44                   | 1.40650                               |
| 7                    | 1.34318                               | 45                   | 1.40846                               |
| 8                    | 1.34468                               | 46                   | 1.41042                               |
| 9                    | 1.34619                               | 47                   | 1.41241                               |
| 10                   | 1.34771                               | 48                   | 1.41440                               |
| 11                   | 1.34924                               | 49                   | 1.41642                               |
| 12                   | 1.35079                               | 50                   | 1.41844                               |
| 13                   | 1.35234                               | 51                   | 1.42048                               |
| 14                   | 1.35391                               | 52                   | 1.42254                               |
| 15                   | 1.35549                               | 53                   | 1.42461                               |
| 16                   | 1.35708                               | 54                   | 1.42669                               |
| 17                   | 1.35868                               | 55                   | 1.42879                               |
| 18                   | 1.36029                               | 56                   | 1.43091                               |
| 19                   | 1.36191                               | 57                   | 1.43304                               |
| 20                   | 1.36355                               | 58                   | 1.43519                               |
| 21                   | 1.36519                               | 59                   | 1.43735                               |
| 22                   | 1.36685                               | 60                   | 1.43952                               |
| 23                   | 1.36852                               | 61                   | 1.44172                               |
| 24                   | 1.37020                               | 62                   | 1.44393                               |
| 25                   | 1.37190                               | 63                   | 1.44615                               |
| 26                   | 1.37361                               | 64                   | 1.44839                               |
| 27                   | 1.37532                               | 65                   | 1.45056                               |
| 28                   | 1.37706                               | 66                   | 1.45293                               |
| 29                   | 1.37880                               | 67                   | 1.45522                               |
| 30                   | 1.38055                               | 68                   | 1.45752                               |
| 1                    | 1.38232                               | 69                   | 1.45985                               |
| 32                   | 1.38410                               | 70                   | 1.46219                               |
| 33                   | 1.38590                               | 71                   | 1.46455                               |
| 34                   | 1.38771                               | 72                   | 1.46692                               |
| 35                   | 1.38952                               | 73                   | 1.46932                               |
| 36                   | 1.39136                               | 74                   | 1.47173                               |
| 37                   | 1.39320                               | 75                   | 1.47416                               |

<sup>a</sup>Critical Data Table J, Corn Refiners Association

- Identify the components from the resultant chromatogram and obtain the peak areas. A typical chromatogram is shown in Fig. 2.

- The terms DP3 and DP4+ are abbreviations for the degree of polymerization and refer to trisaccharides and tetrasaccharides plus higher polysaccharides, respectively.

### Calibration

- Dry each saccharide in a petri dish for 4 hours at 70°C under vacuum.
- Transfer each saccharide into a desiccator to cool.
- Prepare analytical standards, Blend 1 through Blend 12 as shown in Table 3, by weighing each component in the blend to the nearest 0.1 mg as described in ASTM Method D 4307.
  - The mass of maltose in the blend is multiplied by 0.94 to account for water of hydration.

**Table 3**  
**Calibration Standards**

| Blend Number | DP4, g | DP3, g | Maltose, g | Glucose, g | Fructose, g | Psicose, g | Water, g |
|--------------|--------|--------|------------|------------|-------------|------------|----------|
| 1            | 3.0    |        |            |            |             |            | 16.0     |
| 2            |        | 3.0    |            |            |             |            | 16.0     |
| 3            |        |        | 15.0       |            |             |            | 85.0     |
| 4            |        |        |            | 21.0       |             |            | 79.0     |
| 5            |        |        |            |            | 21.0        |            | 79.0     |
| 6            |        |        |            |            |             | 1.0        | 9.0      |
| 7            | 0.01   |        | 0.01       | 0.01       | 0.01        |            | 99.96    |
| 8            | 0.5    |        | 2.0        | 2.5        | 5.0         |            | 40.0     |
| 9            | 2.0    |        | 0.5        | 5.0        | 2.5         |            | 40.0     |
| 10           |        | 0.8    |            |            |             | 0.5        | 8.7      |
| 11           |        | 0.2    |            |            |             | 0.1        | 9.7      |
| 12           |        | 0.01   |            |            |             | 0.01       | 99.98    |
| QC1          | 1.1    | 0.5    | 1.3        | 30.9       |             |            | 66.2     |
| QC2          |        |        | 0.4        | 19.9       | 14.9        |            | 64.8     |

- Mix each blend well and allow them to stand for 2 hours to permit mutarotation to occur.
- Withdraw an aliquot using a 3-mL disposable syringe.
- Attach the 0.2- $\mu$ m filter unit to the syringe Luer-Lok and filter the aliquot into a WISP vial.
- Analyze each blend in triplicate as described under *Chromatographic Technique*.
- Determine the purity of each saccharide by normalized area percent based on the analysis of Blends 1 through 6.
  - For purity determination an equal response is taken for all saccharides.
- Calculate the mass-% of each component in the blends, taking into account any impurities found in Step 8, as outlined in ASTM Method D 4307.

10. Average the peak areas obtained at each concentration for each component.
11. Using the average peak areas as the independent variable and the concentrations of the component as the dependent variable, perform a second order non-linear regression forced through zero to determine the coefficients of regression (Eq. 1 in *CALCULATIONS*).
  - The coefficients calculated for maltose are used for maltose.
  - For other components not found in the calibration blends, use the coefficients calculated for glucose.
  - Many data acquisition software packages will perform these calculations automatically.
12. Prepare QC Blend 1 and QC Blend 2, Table 3, as described in *Calibration*, Steps 1 through 3.
  - Daily analysis of the QC samples is used to verify that the system is operational as per Statistical Process Control (SPC) procedures.
13. Portion out unused quantities of each calibration blend and the QC blends into 2-mL cryovials. Store at -79°C in a freezer for future use.
  - Samples can be stored indefinitely at -79°C. After removal from the freezer, a blend is used only one day and cannot be refrozen.

## CALCULATIONS

### Blends

Calculate the coefficients of regression for each component based upon the analysis of the calibration blends, as follows:

$$R_i = K_0 + K_i M_i + J_i M_i^2 \quad (1)$$

where:

$J_i$  = second-order constant, based on component  $i$

$K_0$  = 0, y-intercept constant

$K_i$  = first-order constant, based on component  $i$

$M_i$  = concentration of component  $i$ , mass %

$R_i$  = average response of calibration component  $i$ , area count

### Samples

Calculate the concentration of each component in the injected sample to the nearest 0.01 mass-% using Eq. 2.

$$P_i = \frac{\sqrt{(K_i^2 + 4J_i A_i)} - K_i}{2J_i} \quad (2)$$

where:

$A_i$  = response of sample component  $i$ , area count

$J_i$  = previously defined, Eq. 1

$K_i$  = previously defined, Eq. 1

$P_i$  = concentration of component,  $i$ , in the injected sample, mass-%

Where no dilution of the sample was required, report the concentrations determined in Eq. 2. If the sample required dilution, calculate a dilution factor using Eq. 3.

$$D = \frac{S + W}{S} \quad (3)$$

where:

**D** = dilution factor

**S** = mass of sample, g

**W** = mass of water added, g

Calculate the concentration of each component in the as-received sample, before dilution, to two decimal places using Eq. 4.

$$C_i = DP_i \quad (4)$$

where:

**C<sub>i</sub>** = concentration of component, i, in the as-received sample, before dilution, mass-%

**D** = previously defined, Eq. 3

**P<sub>i</sub>** = previously defined, Eq. 2

Calculate normalized mass-% composition (dry basis) to two decimal places using Eq. 5.

$$N_i = \frac{100 W_i}{\sum W_i} \quad (5)$$

where:

**N<sub>i</sub>** = concentration of component, i, dry basis, mass-%

**W<sub>i</sub>** = concentration of component, i, in the as-received sample, mass-% (**P<sub>i</sub>** for undiluted samples, **C<sub>i</sub>** for diluted samples)

**100** = factor to convert to percent

## PRECISION

### Repeatability

Based on two tests performed by each of two analysts, on each of two days (8 tests) in one laboratory, the within-laboratory estimated standard deviations (esd) were calculated for components at specific concentrations in 2 commercial blends and are listed in Table 4. Two tests performed in one laboratory by different analysts on different days should not differ by more than the allowable difference values listed in Table 4 at the concentrations listed (95% probability).

### Reproducibility

There is insufficient data to calculate the reproducibility of the test at this time.

**Table 4**

| <b>Blend and Component</b>        | <b>Concentration, mass-%</b> | <b>Within-Lab esd, mass-%</b> | <b>Allowable Difference, mass-%</b> |
|-----------------------------------|------------------------------|-------------------------------|-------------------------------------|
| <b>High Glucose</b>               |                              |                               |                                     |
| DP4+                              | 1.06                         | 0.007                         | 0.03                                |
| DP3                               | 0.52                         | 0.003                         | 0.01                                |
| Maltose                           | 1.34                         | 0.009                         | 0.04                                |
| Glucose                           | 30.95                        | 0.119                         | 0.47                                |
| <b>High Glucose/<br/>Fructose</b> |                              |                               |                                     |
| Maltose                           | 0.43                         | 0.014                         | 0.05                                |
| Glucose                           | 20.18                        | 0.089                         | 0.35                                |
| Fructose                          | 15.08                        | 0.064                         | 0.25                                |

**TIME FOR ANALYSIS**

The elapsed time for one analysis is 1 hour. The labor requirement is 0.5 hour.

**REFERENCES**

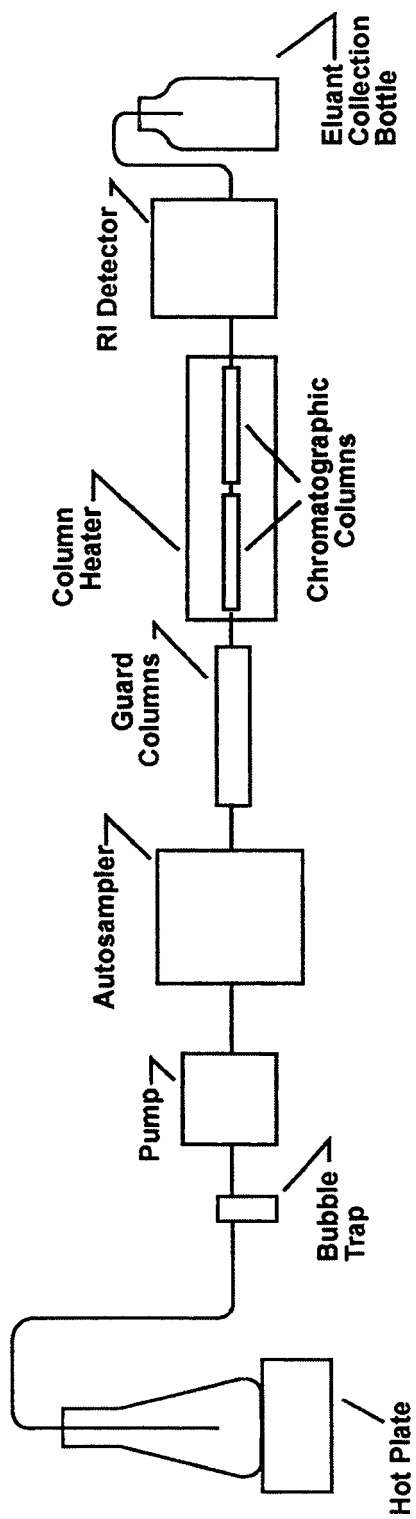
ASTM Method D 4307, [www.astm.org](http://www.astm.org)

*Critical Data Table J*, pp 42J-1, 2, published by Corn Refiners Association Inc., 1001 Connecticut Ave., N.W., Washington, DC 20036

UOP Method 816, "Refractive Index of Hydrocarbons"

**SUGGESTED SUPPLIERS**

Aldrich Chemical Co., 1001 W. Saint Paul Ave., Milwaukee, WI 53233 (414-273-3850)  
 Alltech Associates, 2051 Waukegan Rd., Deerfield, IL 60015 (708-948-8600)  
 Bio-Rad Laboratories, 2200 Wright Ave., Richmond, CA 94804 (415-234-4130)  
 Boehringer Mannheim Corp., P.O. Box 50414, Indianapolis, IN 46250-0414 (317-849-9350)  
 Cole-Parmer Instrument Co., 7425 N. Oak Park Ave., Chicago, IL 60648 (708-647-7600)  
 Fisher Scientific Co., 1600 Parkway View Drive, Pittsburgh, PA 15205 (412-562-8300)  
 Mallinckrodt Inc., 675 McDonnell Blvd. St. Louis, MO 63134 (314-895-2000)  
 Millipore Corp., 80 Ashby Rd., Bedford, MA 01730 (617-275-9200)  
 Pierce Chemical Co., P.O. Box 117, Rockford, IL 61105 (815-968-0747)  
 Sigma Chemical Co., 3050 Spruce St., St. Louis, MO 63103 (314-771-5765)  
 Upchurch Scientific Inc., P.O. Box 1529, Oak Harbor, WA 98277 (206-679-2528)  
 VWR Scientific Inc., P.O. Box 7900, San Francisco CA 94120 (415-468-7150)  
 Waters Associates, 34 Maple St., Milford, MA 01757 (508-478-2000)



**Figure 1**  
Assembly of Apparatus

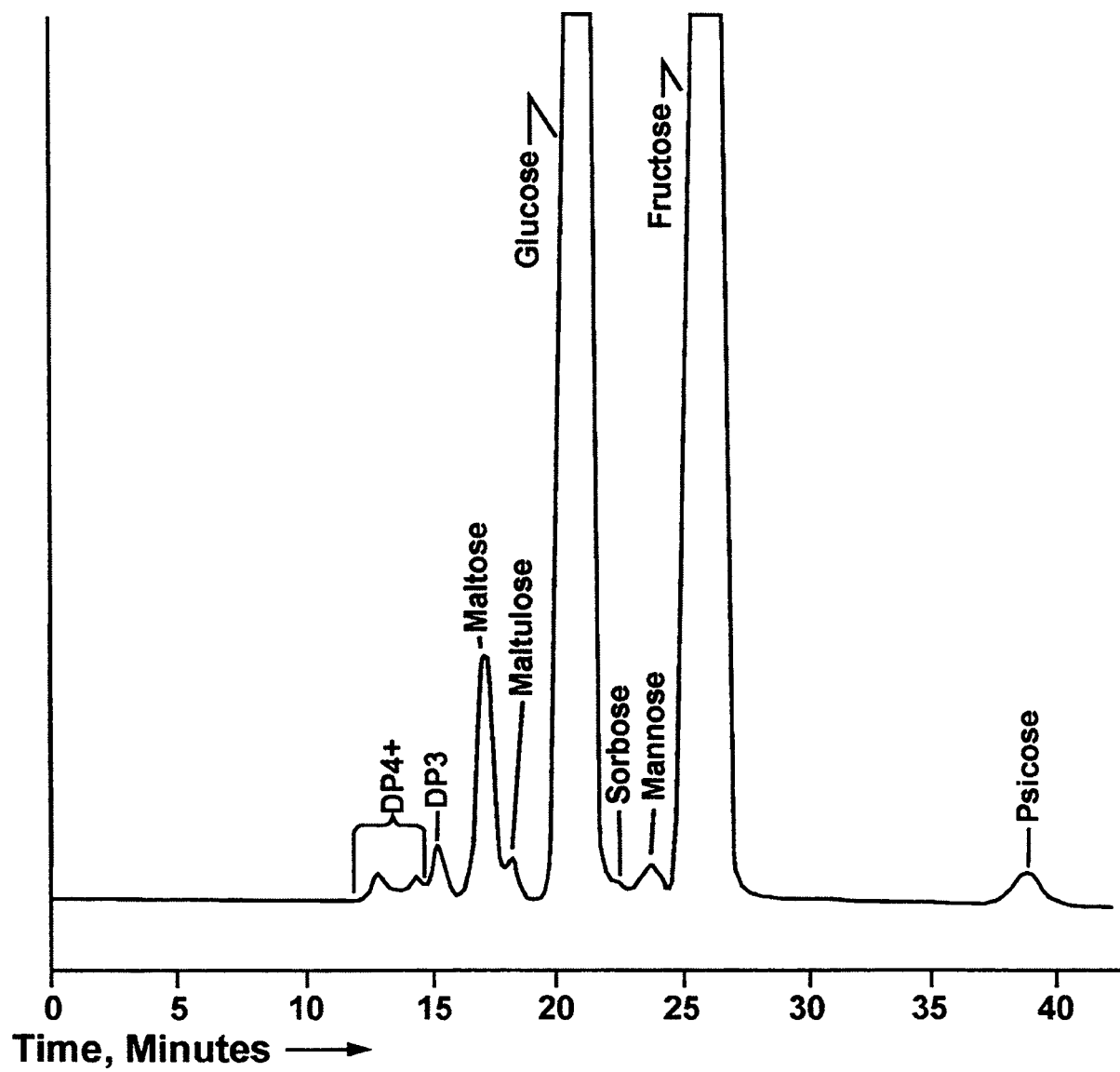


Figure 2

Typical Chromatogram