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# Determination of Lactose in Lactose-Free Milk Products by High-Performance Anion-Exchange Chromatography with Pulsed Amperometric Detection

## INTRODUCTION

Lactose is the major disaccharide found in milk products and is catabolized into glucose and galactose by the enzyme lactase. Lactose-intolerant individuals have a lactase deficiency; therefore, lactose is not completely catabolized. While lactose intolerance is not a dangerous condition, its global prevalence has created a large market for lactose-free products. Commercially available lactose-free products are produced by breaking down lactose into glucose and galactose by enzymatic hydrolysis. However, the resulting milk products contain varying amounts of residual lactose. This has created the need for simple, reliable, and accurate analytical methods to quantify lactose.

Milk changes structurally and chemically when heat-treated, but the extent of the change depends on the temperature and duration of the heating. Lactulose is a disaccharide containing galactose and fructose that is not naturally found in raw milk but is formed during the heat treatment of milk by the isomerization of lactose. Lactulose levels in milk can be used to determine the method that was used to sterilize the milk. The average lactulose content when using in-container sterilization is 744 mg/L, but only 3.5 mg/L in milk treated by low-temperature pasteurization methods.[1]

Currently available analytical methods for the detection of lactose include mid-infrared detection, fluorometry, photometric methods, polarimetry, gravimetric detection, differential pH techniques, and enzymatic assays.[2–5] These methods are time consuming because of extensive sample preparation. They cannot differentiate individual carbohydrates, and polarimetry measurements have interferences from other optically active components.

The Association of Official Analytical Chemists (AOAC) Method 984.15 uses enzymatic hydrolysis of lactose to glucose and galactose at pH 6.6 by β-galactosidase. This method is time consuming, however, and needs extensive reagent preparations. The reported detection limits of this assay may not allow for the determination of lactose in lactose-free samples.[6]

The work shown here describes a sensitive and accurate method to determine lactose and lactulose in dairy products, including lactose-free products, using high-performance anion-exchange chromatography with pulsed amperometric detection (HPAE-PAD) in six different commercial products. HPAE-PAD is a widely used technique for the determination of monosaccharides, disaccharides, oligosaccharides, smaller polysaccharides, sialic acids, and other sugar acids. Being a direct-detection technique, HPAE-PAD eliminates errors associated with analyte derivatization. The CarboPac® PA20 column in combination with PAD provides high-resolution separations of small and larger carbohydrates with sensitive detection. The method described here was used to determine low levels of lactose in several commercially available products, four of which were lactose-free. Lactose-free Gouda and Havarti cheeses had no detectable lactose, whereas lactose-free cottage cheese and lactose-free milk had 2.17 mg/mL and 0.6 mg/mL lactose, respectively.

## EQUIPMENT

Dionex ICS-3000 system including:

SP Single Pump (P/N 061707) or DP Dual Pump (P/N 061712) with degas option

DC Detector compartment (P/N 061767) single- or dual-temperature zone

Electrochemical detector (P/N 061719)

Disposable Au on PTFE working electrode, pack of 6 (with 2 mil gaskets included) (P/N 066480)

pH-Ag/AgCl reference electrode (P/N 061879)

AS Autosampler (P/N 061289) with cooling tray option (recommended)

Chromeleon® Chromatography Data System software

## CONSUMABLES

CarboPac PA20 Analytical Column, 3 × 150 mm (Dionex P/N 060142)

CarboPac PA20 Guard Column, 3 × 30 mm (Dionex P/N 060144)

OnGuard® IIA, 2.5 cc cartridge (Dionex P/N 057092)

Syringe filters (Gelman IC Acrodisc® 0.2 µm, P/N 4483)

Disposable filtration units, 0.20 µm nylon membrane (Nalgene®, P/N 164-0020)

Centrifuge equipped with a ten-place, aluminum fixed-angle rotor (Beckman)

Spinchron R, GS-6R Series (Beckman Coulter, P/N 358702) or equivalent

## REAGENTS AND STANDARDS

Reagent-grade water, Type I, 18 MΩ-cm resistance or better, filtered through a 0.2 µm filter immediately before use

Sodium acetate, anhydrous (Fluka P/N 71183)

Sodium hydroxide, 50% (Fischer P/N SS254-500)

Potassium hexacyanoferrate(III), ACS reagent, ≥ 99%, powder (Sigma-Aldrich P/N 393517)

Zinc sulfate, monohydrate (Sigma-Aldrich P/N 96495)

α–Lactose, monohydrate (Sigma P/N L-3625)

β–D-Glucose (Sigma-Aldrich P/N G-5250)

D–Galactose (Sigma-Aldrich P/N G-0625)

Lactulose, 4-O-β Galactopyranosyl-D fructofuranose (Sigma-Aldrich P/N L-7877)

Sucrose, α–D-Glucopyranosyl-β-D-fructofuranoside (Sigma-Aldrich P/N S-9378)

Nitrogen; 4.8-grade, 99.998%

## SAMPLES

Low-fat cottage cheese
Lactose-free Havarti cheese
Lactose-free Gouda cheese
Light nonfat yogurt
Whole milk
Lactose-free 1% milk

## CONDITIONS

Columns: CarboPac PA20 Analytical Column, 3 × 150 mm (Dionex P/N 060142)
CarboPac PA20 Guard Column, 3 × 30 mm (Dionex P/N 060144)

Flow Rate: 0.4 mL/min

Inj. Volume: 10 µL

Tray Temp: 4 ºC

Detection: Integrated pulsed amperometry, Au on PTFE disposable or conventional Au working electrodes

Waveform: Carbohydrate (standard quad)

Background: <20 nC

Noise: 30 to 80 pC

Temperature: 30 ºC

Eluents: A) Deionized water
B) 200 mM NaOH
C) 200 mM NaOH, 100 mM sodium acetate
D) 200 mM NaOH, 1 M sodium acetate

| Time (s) | Potential (V) | Gain Region* | Ramp* | Integra-tion |
|---|---|---|---|---|
| 0.00 | +0.1 | Off | On | Off |
| 0.20 | +0.1 | On | On | On |
| 0.40 | +0.1 | Off | On | Off |
| 0.41 | –2.0 | Off | On | Off |
| 0.42 | –2.0 | Off | On | Off |
| 0.43 | +0.6 | Off | On | Off |
| 0.44 | –0.1 | Off | On | Off |
| 0.50 | –0.1 | Off | On | Off |

*Settings required when using the ICS-3000 or ICS-5000 but not for older Dionex systems.

Gradient Conditions:

| Time (min) | Flow (mL/min) | % A | % B | % C | % D |
|---|---|---|---|---|---|
| 0.0 | 0.40 | 94.0 | 6.0 | 0.0 | 0.0 |
| 10.0 | 0.40 | 94.0 | 6.0 | 0.0 | 0.0 |
| 20.0 | 0.40 | 90.0 | 7.5 | 2.5 | 0.0 |
| 25.0 | 0.40 | 90.0 | 7.5 | 2.5 | 0.0 |
| 31.0 | 0.40 | 92.5 | 7.5 | 0.0 | 0.0 |
| 33.0 | 0.40 | 0.0 | 25.0 | 0.0 | 75.0 |
| 43.0 | 0.40 | 0.0 | 25.0 | 0.0 | 75.0 |
| 43.1 | 0.40 | 0.0 | 100.0 | 0.0 | 0.0 |
| 49.0 | 0.40 | 0.0 | 100.0 | 0.0 | 0.0 |
| 49.1 | 0.40 | 94.0 | 6.0 | 0.0 | 0.0 |
| 65.0 | 0.40 | 94.0 | 6.0 | 0.0 | 0.0 |

## *PREPARATION OF SOLUTIONS AND REAGENTS*

### 200 mM NaOH

Transfer 10.4 mL of 50% sodium hydroxide into a polypropylene 1 L volumetric flask containing approximately 800 mL degassed and filtered deionized water using a plastic serological pipette. Mix by inverting the volumetric flask and bring to volume with degassed and filtered deionized water. Refer to Dionex TN 71 for a more detailed discussion on proper preparation, storage and use of eluents for HPAE-PAD.[7] Keep the eluent blanketed under 5 to 8 psi of nitrogen (or any other inert gas) at all times to reduce carbonate contamination.

### 200 mM NaOH, 100 mM Sodium Acetate

Dissolve 8.204 g high-purity anhydrous sodium acetate into approximately 800 mL deionized water. Vacuum filter the solution through a 0.20 µm filter to remove any particulates. Transfer the filtered solution into a 1 L plastic volumetric flask, and add 10.4 mL of 50% sodium hydroxide using a plastic serological pipette. Bring to volume with degassed and filtered deionized water. Keep the eluent blanketed under 5 to 8 psi of nitrogen (or any other inert gas) at all times to reduce carbonate contamination.

### 200 mM NaOH, 1 M Sodium Acetate

Dissolve 82.04 g high-purity anhydrous sodium acetate into approximately 800 mL of deionized water. Vacuum filter the solution through a 0.20 µm filter to remove any particulates. Transfer the filtered solution into a 1 L plastic volumetric flask and add 10.4 mL of 50% sodium hydroxide using a plastic serological pipette. Bring to volume with degassed and filtered deionized water. Keep the eluent blanketed under 5 to 8 psi of nitrogen (or any other inert gas) at all times to reduce carbonate contamination.

### Carrez I Solution

Dissolve 15.0 g potassium hexacyanoferrate(III) in 75 mL DI water and filter through a 0.20 µm filter. Transfer to a 100 mL volumetric flask and bring to volume.

### Carrez II Solution

Dissolve 30.0 g zinc sulfate monohydrate in 75 mL DI water and filter through a 0.20 µm filter. Transfer to a 100 mL volumetric flask and bring to volume.[8]

### Standards

All standard concentrates can be stored for up to 6 months at -40 °C. Diluted intermediate standards are stable for 3 months at -40 °C, and working and mixed standards are stable for two weeks at 2 to 4 °C.

### 1000 mg/L Standard Concentrates

Prepare the stock standard solution by dissolving the appropriate amount of carbohydrate (Table 1) in approximately 75 mL DI water and diluting to 100 mL in a volumetric flask. Store the stock solution in a high-density polyethylene or polypropylene bottle at 4 °C.

**Table 1. Amounts for the Preparation of Standard Concentrates (100 mL)**

| Carbohydrate | Weight (g) |
|---|---|
| α–Lactose | 0.360 |
| β–D-Glucose | 0.180 |
| D–Galactose | 0.182 |
| Lactulose | 0.342 |
| Sucrose | 0.342 |

### Working Standards and Standards for Method Linearity

To prepare working standards, use a calibrated pipette to deliver the appropriate volume of 1000 mg/L stock standard into a volumetric flask and dilute to volume with DI water. For method linearity studies, the following standards of lactose and lactulose were used: 100, 50, 25, 15, 10, 7.5, 5, 3, 2, 1, 0.5, and 0.25 mg/L. The exception was the linearity studies for lactulose for which the minimum concentration was 0.5 mg/L.

### Mixed Standards

To prepare mixed carbohydrate standards, combine appropriate volumes of individual stock carbohydrate standards into a volumetric flask and dilute to volume with DI water.

## SAMPLE PREPARATION

Weigh 1 g of sample and add 10 mL DI water to the sample. Add 200 µL Carrez I solution and 200 µL Carrez II solution to the mixture, shaking after each addition. Transfer the mixture to a 100 mL volumetric flask and bring the volume to 100 mL. Centrifuge a portion of this sample at 3000 RPM. Aspirate the supernatant and filter through a 0.20 µm filter. Prepare an OnGuard IIA, 2.5 cc cartridge by flushing it with 15 mL DI water at a flow rate of less than 2 mL/min, then discarding the effluent. Load 8 mL of sample, discard the first 6 mL into a waste container, and collect the next 2 mL for analysis. Samples can be stored at -4 ºC for up to 2 weeks. Filter the sample through an IC-grade syringe filter prior to injection.

## RESULTS AND DISCUSSION

### Chromatography and Interference Studies

To optimize the separation of lactose and lactulose in the presence of expected sample carbohydrates, a mixed carbohydrate standard was prepared. Figure 1 shows a chromatogram of a mixed carbohydrate standard with an optimized gradient for the separation of lactose and lactulose. The retention times of galactose, glucose, sucrose, lactose, and lactulose are 9.63, 10.65, 13.79, 22.98, and 24.36 min, respectively. All the carbohydrates are well separated from each other, including lactose and lactulose.

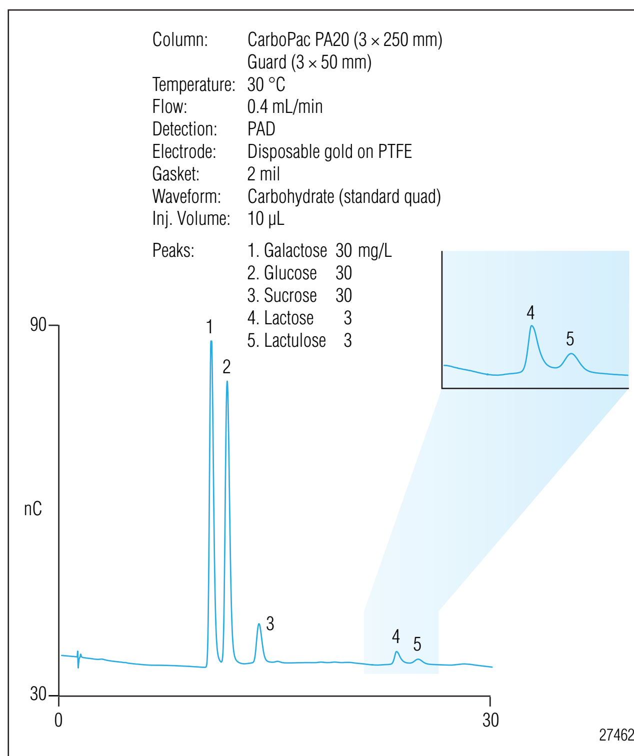


*Figure 1. Separation of a mixed-carbohydrate standard, including lactose and lactulose.*

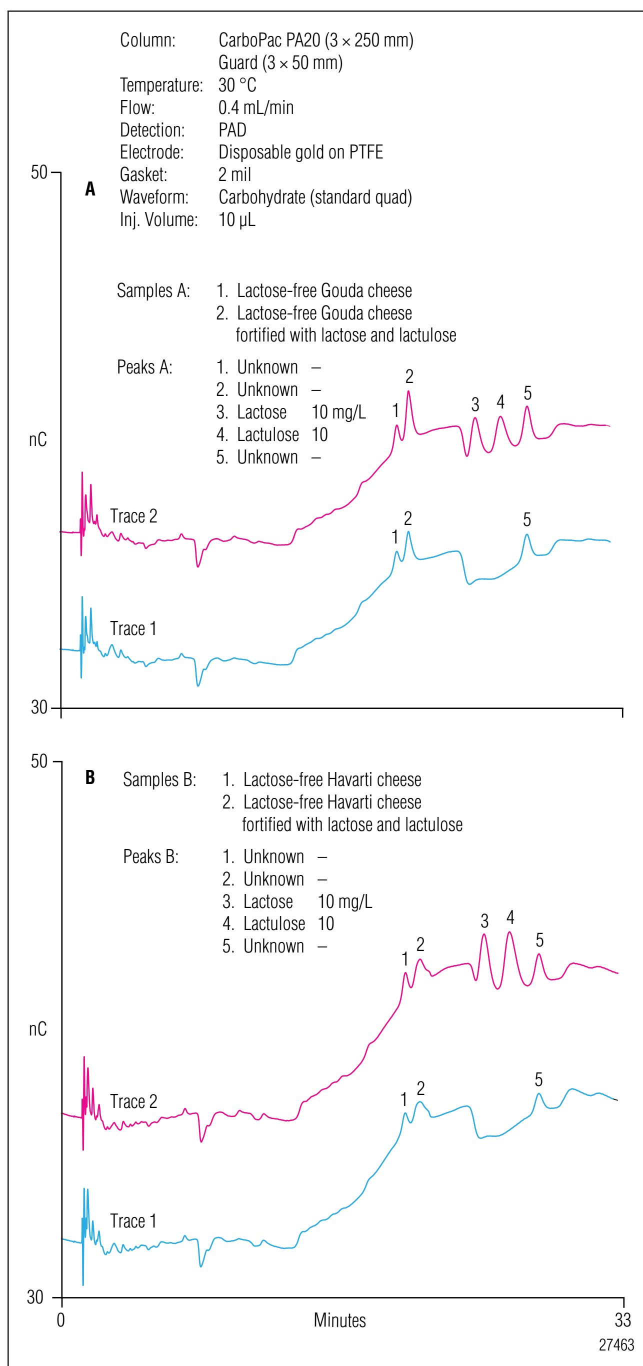


*Figure 2. A) Separation of carbohydrates in fortified and unfortified lactose-free Gouda cheese samples. B) Separation of carbohydrates in fortified and unfortified lactose-free Havarti cheese samples.*

## Preliminary Sample Analysis

The optimized separation was applied to two samples matrices, lactose-free Gouda cheese and lactose-free Havarti cheese. Figure 2A shows overlayed chromatograms of fortified and unfortified Gouda cheese. Trace 1 shows a separation of unfortified cheese with no detectable lactose. Trace 2 shows the separation of a Gouda cheese sample fortified with 10 mg/L each of lactose and lactulose. This chromatogram shows that lactose and lactulose are well separated from each other and matrix-related interferants.

Figure 2B shows overlayed chromatograms of fortified and unfortified Havarti cheese. Trace 1 shows a separation of unfortified Havarti cheese with no lactose detected. Trace 2 shows the separation of an Havarti cheese sample fortified with 10 mg/L each of lactose and lactulose. The chromatography is similar to Figure 2A.

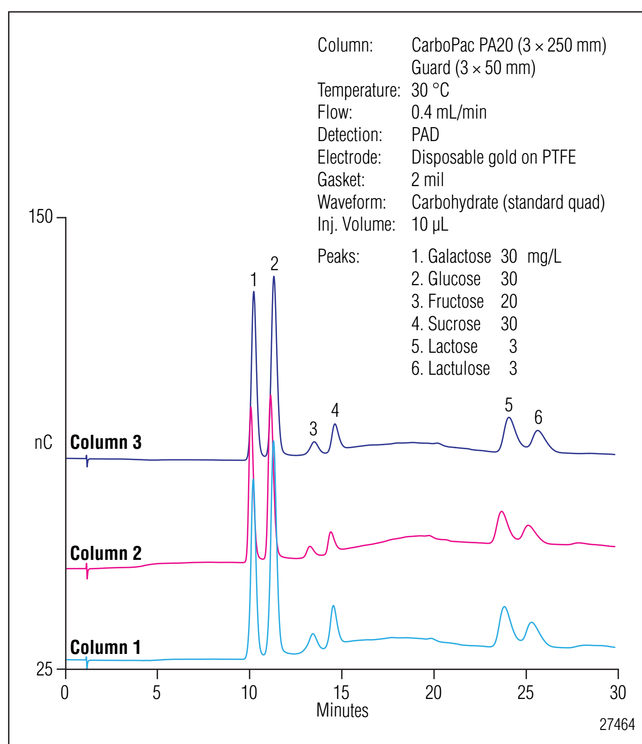


*Figure 3. Overlay of the lactose and lactulose separation on columns from three different lots.*

| Table 2. Intraday Reproducibility | | | | |
|---|---|---|---|---|
| **Analyte** | **Conc. (mg/L)** | **RSD** | | |
| | | **Ret. Time** | **Peak Area** | **Peak Height** |
| Galactose | 30 | 0.15 | 1.03 | 1.01 |
| Glucose | 30 | 0.14 | 1.30 | 1.08 |
| Sucrose | 30 | 0.16 | 1.51 | 1.37 |
| Lactose | 3 | 0.13 | 3.70 | 5.74 |
| Lactulose | 3 | 0.12 | 5.06 | 5.42 |

n = 30 injections

### Column-to-Column Reproducibility

Due to the close elution of lactose and lactulose, the method ruggedness was evaluated by checking the separation on three columns from three different lots. Figure 3 shows that lactose and lactulose are well separated on each column.

### Short-Term Reproducibility

Table 2 shows intraday reproducibility measured by making 30 consecutive injections of a mixed-carbohydrate standard containing 30 mg/L each of galactose, glucose, and sucrose; 3 mg/L each of lactose and lactulose; and 20 mg/L fructose. The method exhibited good short-term reproducibility; the intraday retention time RSDs ranged from 0.12 for lactulose to 0.16 for sucrose, and the peak area RSDs ranged from 1.03 for galactose to 5.07 for lactulose.

| Table 3. Linearity and MDL for Lactose and Lactulose | | | | |
|---|---|---|---|---|
| **Carbohydrate** | **Range mg/L** | **$r^2$** | **MDL Standard (mg/L)** | ***Calculated MDL (mg/L)** |
| Lactose | 0.25–100 | 0.9966 | 0.5 | 0.12 |
| Lactulose | 0.5–100 | 0.9942 | 1.0 | 0.23 |

*The method detection limits (MDL) for lactose and lactulose were determined by making seven injections of a low-level solution fortified with lactose and lactulose at 3 to 5 times the estimated MDL.

### Determination of Linearity for Lactose and Lactulose

Calibration standards were prepared in DI water. Table 3 summarizes the calibration data for a calibration curve obtained by injecting calibration standards between 0.25 to 100 mg/L lactose. Table 3 also summarizes the calibration data for lactulose using the same calibration standards with the exception of the 0.25 mg/L standard. The calibration curve for both compounds was linear with a correlation coefficient ($r^2$) of 0.9966 for lactose and 0.9942 for lactulose, respectively.

### MDL for Lactose and Lactulose

The MDL is defined as the minimum concentration of an analyte that can be identified, measured, and reported with 99% confidence that the analyte concentration is greater than zero. It is basically a measure of the precision of preparing and analyzing low-level standards according to the method. The MDLs for lactose and lactulose were determined by making seven injections of a low-level solution fortified with lactose and lactulose at 3 to 5 times the estimated MDL. MDLs were calculated using the calibration curve.

The calculated MDLs in DI water obtained by this method are 0.12 mg/L for lactose and 0.23 mg/L for lactulose. Table 3 summarizes the data for this determination.

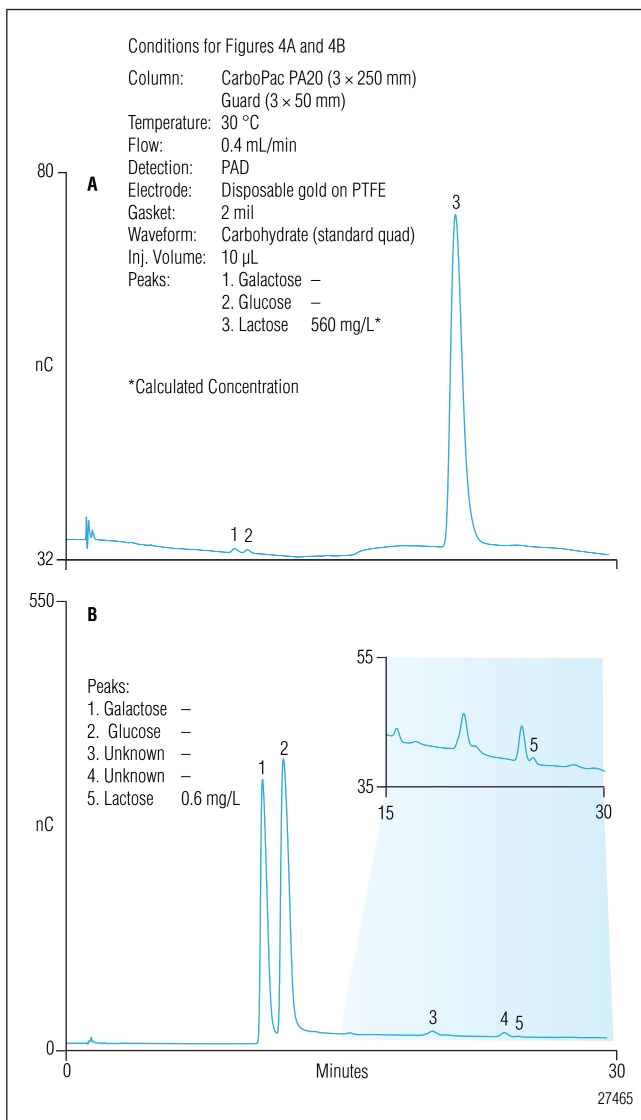


*Figure 4. A bi-panel showing the following chromatograms: A) separation of carbohydrates in whole milk, and B) separation of carbohydrates in lactose-free low-fat milk.*

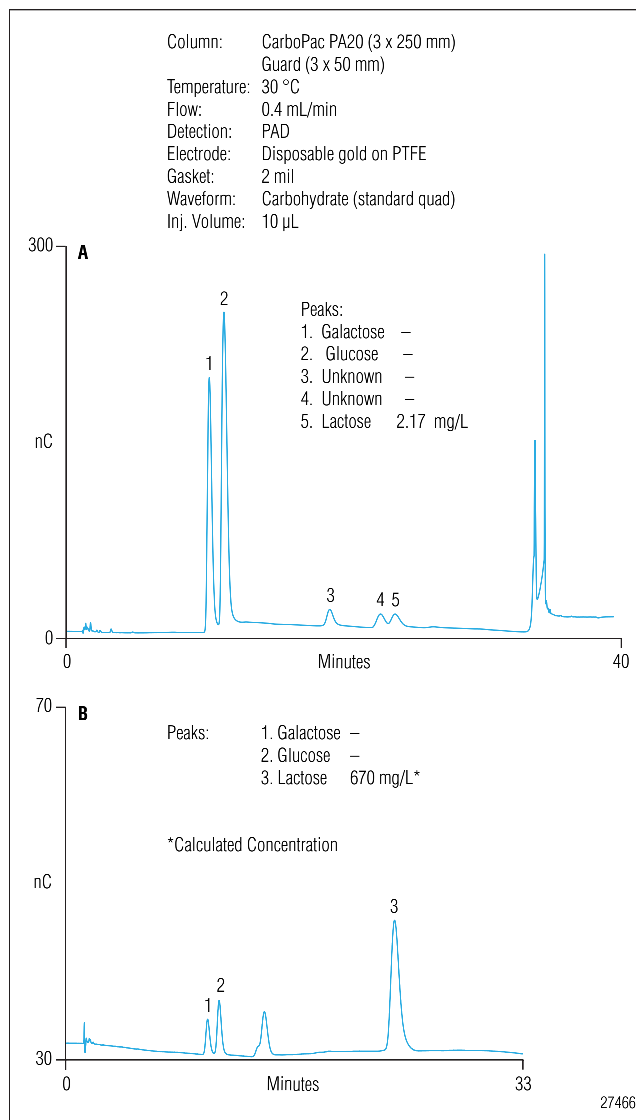


*Figure 5. A bi-panel showing the following chromatograms: A) separation of carbohydrates in lactose-free low-fat cottage cheese, and B) separation of carbohydrates in 1:20 diluted low-fat yogurt.*

## Sample Analysis

Several milk-based products were evaluated for their lactose and lactulose content. Figure 4A shows the separation of carbohydrates in whole milk. The prepared milk samples were diluted 1:10 to prevent overloading with lactose. The diluted milk sample showed some galactose and glucose and large amounts of lactose. Figure 4B shows the separation of carbohydrates in lactose-free milk. The chromatogram shows that lactose-free milk contains high concentrations of galactose and glucose and trace amounts of lactose (0.6 mg/L, 0.00006%). This product showed the lowest detected lactose concentration among the products evaluated.

Figure 5A shows the separation of carbohydrates in lactose-free cottage cheese. This sample contained high concentrations of galactose and glucose, 21.7 mg/L (0.00217%) of lactose, and unknown peaks. Figure 5B shows the separation of carbohydrates in low-fat yogurt.

| Table 4. Recovery of Lactose and Lactulose in Various Matrices | | | |
|---|---|---|---|
| Matrix | Amount Added (mg/L) | Lactose Recovery (%) n=3 | Lactulose Recovery (%) n=3 |
| Whole milk (1:10 diluted) | 10 | 85.3 | 98.1 |
| Lactose-free low-fat milk | 10 | 97.6 | 94.5 |
| Lactose-free Gouda cheese | 10 | 90.1 | 100.8 |
| Lactose-free Havarti cheese | 10 | 99.7 | 93.2 |
| Lactose-free cottage cheese | 10 | 102.0 | 86.0 |
| Low-fat yogurt (1:20 diluted) | 10 | 89.9 | 97.0 |

The chromatogram shows that low-fat yogurt contains galactose, glucose, and 33.5 mg/L (0.00335%) lactose.

A duplicate of each of the samples was fortified with known amounts of lactose and lactulose prior to sample preparation. Recoveries were calculated following analysis of both native and spiked samples. Recoveries of lactose and lactulose for all matrices were 86 to 100% (Table 4).

## CONCLUSION

This work describes a sensitive and accurate method to extract, separate, and quantify lactose and lactulose in milk-based products. The method uses a CarboPac PA20 column with PAD to quantify lactose and lactulose in a separation time of less than 30 min. The use of disposable gold electrodes provides the benefit of high electrode-to-electrode reproducibility and rapid equilibration upon installation.

## PRECAUTIONS

Potassium hexacyanoferrate is a red crystalline solid that may be harmful. It may cause respiratory tract irritation if inhaled. In addition, it can be harmful if absorbed through the skin or swallowed, and it can cause irritation to the eyes. Consult the potassium hexacyanoferrate MSDS for additional information. Potassium hexacyanoferrate should be stored tightly closed in a dry and well-ventilated place and should not be stored near acids. Potassium hexacyanoferrate should not be disposed into drains. Contact a licensed waste disposal organization to ensure all disposals are in accordance with existing federal, state, and local environmental regulations.

The reference electrode must be hydrated at all times and should not be allowed to dry out, especially when the cell is on. Reduce the eluent flow to 0.25 mL/min if you anticipate not using the system. For long-term storage of more than a week, disassemble the cell, remove the reference electrode, and store it in saturated KCl.

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**Dionex Corporation**
1228 Titan Way
P.O. Box 3603
Sunnyvale, CA
94088-3603
(408) 737-0700

**North America**
U.S./Canada (847) 295-7500

**South America**
Brazil (55) 11 3731 5140

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Australia (61) 2 9420 5233 China (852) 2428 3282 India (91) 22 2764 2735
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