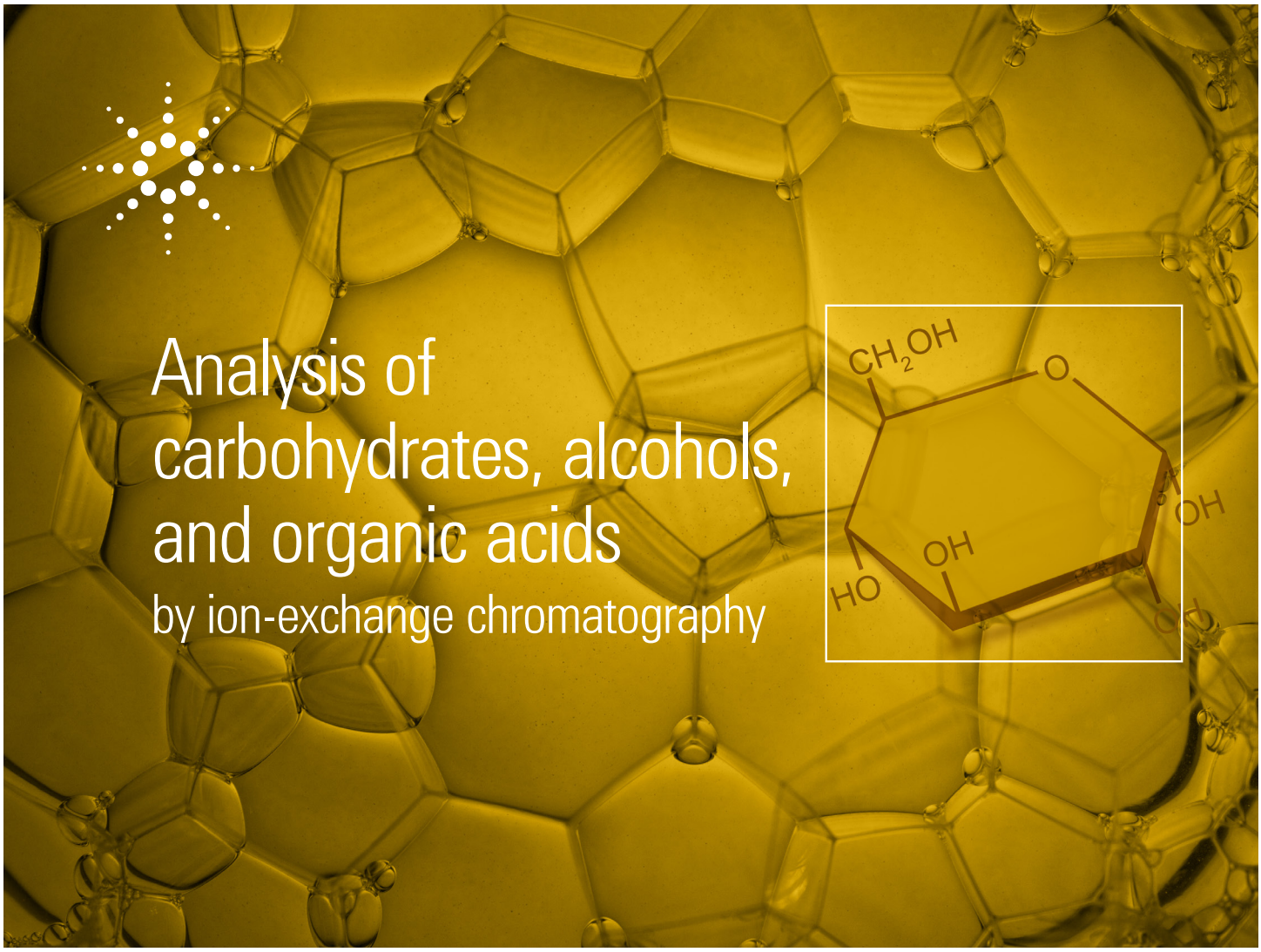


# Analysis of carbohydrates, alcohols, and organic acids

## by ion-exchange chromatography

## Agilent Hi-Plex Columns Applications Compendium

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The Measure of Confidence

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# Table of Contents

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**www.agilent.com/chem/hi-plex**

# Introduction

Carbohydrates, alcohols, and organic acids are important in the manufacture of many foods, pharmaceuticals, and biofuels. This applications compendium describes some of the uses of Agilent Hi-Plex columns and systems for the ion-exchange chromatography of these valuable compounds.

## Agilent Hi-Plex columns – a comprehensive range of ligand-counter ions for optimum selectivity and resolution

Hi-Plex columns deliver improved efficiency, lower operating pressures, and longer column lifetimes from monodispersed materials. With a range of ligand-counter ions for optimum selectivity, along with resolution and materials matched to the USP definitions of media types L17, L19, L34, and L58, the Hi-Plex range is ideal for isocratic separations using water or dilute acid as the mobile phase. This simplifies your HPLC system requirements and eliminates the use of potentially hazardous organic solvents.

Hi-Plex separates carbohydrates and alcohols through ligand exchange and organic acids via ion exchange. These are the preferred separation mechanisms for the analysis of simple sugars, alcohols, oligosaccharides, and organic acids in foods and pharmaceuticals.

The range comprises a 4% crosslinked resin for the analysis of oligosaccharides and an 8% crosslinked resin, with lower exclusion limit, for mono-, di-, and trisaccharide analyses.

For carbohydrate and alcohol investigations, Hi-Plex columns use isocratic conditions with water as the mobile phase and temperature as the main variable for control of resolution. The exception is the Hi-Plex Na (Octo), which is used with sodium hydroxide mobile phases when pulsed amperometric detection (PAD) is employed.

## Typical operating conditions for Hi-Plex columns

| Column Type | Temperature | Flow Rate | Eluent |
|---|---|---|---|
| Hi-Plex Ca | 80 - 90 °C | 0.6 mL/min | Water |
| Hi-Plex Ca USP L19 | 80 - 90 °C | 0.3 mL/min | Water |
| Hi-Plex Pb | 70 - 90 °C | 0.6 mL/min | Water |
| Hi-Plex H for carbohydrates | 60 - 70 °C | 0.6 mL/min | Water |
| Hi-Plex H for organic acids | 40 - 60 °C | 0.6 mL/min | Dilute acid |
| Hi-Plex Ca (Duo) | 80 - 90 °C | 0.6 mL/min | Water |
| Hi-Plex K | 80 - 90 °C | 0.6 mL/min | Water |
| Hi-Plex Na (Octo) | 80 - 90 °C | 0.6 mL/min | Water, sodium hydroxide |
| Hi-Plex Na | 80 - 90 °C | 0.3 mL/min | Water |

Typically, Agilent Hi-Plex columns use isocratic conditions with water as the mobile phase and temperature as the main variable for resolution control. The only exceptions are Hi-Plex Na (Octo) columns, used with sodium hydroxide and pulsed amperometric detection (PAD), and Hi-Plex H columns, used with dilute acid to analyze organic acids (see above). Detailed operating conditions can be found in the data sheet supplied with all columns.

# Introduction

## Agilent Hi-Plex column selection guide

Pharmacopeia methods specify the HPLC media and column dimensions that should be used for specific applications. The Agilent Hi-Plex column portfolio includes four materials that comply with USP definitions:

- **Media type L17** (Hi-Plex H): Strong cation-exchange resin consisting of sulfonated, crosslinked styrene-divinylbenzene copolymer in hydrogen form, 7 to 11 µm in diameter
- **Media type L19** (Hi-Plex Ca and Hi-Plex Ca [Duo]): Strong cation-exchange resin consisting of sulfonated, crosslinked styrene-divinylbenzene copolymer in calcium form, 9 µm in diameter
- **Media type L34** (Hi-Plex Pb): Strong cation-exchange resin consisting of sulfonated, crosslinked styrene-divinylbenzene copolymer in lead form, 9 µm in diameter
- **Media type L58** (Hi-Plex Na and Hi-Plex Na [Octo]): Strong cation-exchange resin consisting of sulfonated, crosslinked styrene-divinylbenzene copolymer in sodium form, 6 to 30 µm in diameter

In addition to standard column sizes, the media is also packed in specific column dimensions for different USP methods, including sugar alcohol analysis. For some applications, the choice of media will depend on the carbohydrate composition and matrix of the sample being analyzed.

| Application Areas | Recommended Column(s) |
|---|---|
| USP methods specifying L17 media | Hi-Plex H |
| USP methods specifying L19 media | Hi-Plex Ca, Hi-Plex Ca (Duo) |
| USP methods specifying L34 media | Hi-Plex Pb |
| USP methods specifying L58 media | Hi-Plex Na, Hi-Plex Na (Octo) |
| Mono- and disaccharides | Hi-Plex Ca, Hi-Plex Pb, Hi-Plex H, Hi-Plex Na (Octo) |
| Anomer separations | Hi-Plex Ca |
| Organic acids | Hi-Plex H |
| Alcohols | Hi-Plex Ca, Hi-Plex K, Hi-Plex H, Hi-Plex Pb |
| Adulteration of food and beverages | Hi-Plex Ca, Hi-Plex Pb |
| Food additives | Hi-Plex Ca, Hi-Plex Pb |
| Dairy products | Hi-Plex Ca, Hi-Plex H |
| Sweetened dairy products | Hi-Plex Pb |
| Confectionery | Hi-Plex Ca, Hi-Plex Pb |
| Fruit juice | Hi-Plex Ca |
| Wine | Hi-Plex H |
| Wood pulp hydrolysates (cellulose/hemicellulose) | Hi-Plex Pb |
| Fermentation monitoring | Hi-Plex H |
| Oligosaccharides | Hi-Plex Na |
| Samples with high salt content (molasses) | Hi-Plex Na (Octo) |
| Oligosaccharides < Dp 5 with monosaccharides | Hi-Plex Ca (Duo) |
| Corn syrups | Hi-Plex Na |

# System Solutions

## Take your carbohydrate analysis to the next level

### Agilent 1260 Infinity LC

*Infinitely more confident.* The **new Agilent 1260 Infinity LC** sets higher standards in performance and value giving you more confidence in your results.

The 600 bar power range combines with 80 Hz UV detector speeds and up to ten times higher sensitivity. That's true UHPLC performance!

The modular design of the 1260 Infinity LC and the wide range of solvent delivery, injection, and detection options allow you to configure a system that exactly matches your needs for chromatographic performance and flexibility.

**www.agilent.com/chem/infinity**

### Agilent 1260 Infinity RID

The **Agilent 1260 Infinity refractive index detector (RID)** is the ideal detector for fast and reliable LC results in the routine analysis of non-UV absorbing substances, such as carbohydrates, lipids, and polymers. Countercurrent heat exchangers keep the optical unit and the flow cells at constant temperature to achieve unmatched stability for reproducible results. The minimum baseline noise of the RID maximizes signal-to-noise ratio, and low limits of detection allow you to inject small amounts of sample.

**www.agilent.com/chem/1260ri**

### Agilent 385-ELSD

**Evaporating light scattering detection (ELSD)** offers many advantages for carbohydrate analysis and is featured in many of our Hi-Plex Application Notes. The Agilent 385-ELSD is the only ELSD that delivers subambient operation, for unrivaled detection of thermally labile analytes that other ELSDs miss.

**www.agilent.com/chem/elsd**

## Agilent HPLC and UHPLC supplies and sample prep products **ensure accurate measurements** right from the start

Vials, caps, and other small LC system components can contribute to big problems, such as injector damage, ghost peaks, and analyte degradation. Agilent supplies and sample preparation products are engineered with the same reliability and reproducibility you expect from Agilent instruments and columns. They help you keep your system operating at peak performance with the highest possible uptime.

# Analysis of Aliphatic Alcohols by Ligand-Exchange Chromatography

Application Note

Chemical


## Author

Stephen Ball

Agilent Technologies, Inc.


## Introduction

This application note demonstrates how an Agilent Hi-Plex H column can be used to separate aliphatic alcohols.

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## Materials and Reagents

| | |
|---|---|
| Column | Agilent Hi-Plex H (8% crosslinked), 7.7 × 300 mm, 8 µm (p/n PL1170-6830) |
| Mobile phase | 100% DI $H_2O$ |
| Flow rate | 0.6 mL/min |
| Temperature | 40 °C |
| Detector | RI |

## Conclusion

Using only pure HPLC-grade water as eluent, the Agilent Hi-Plex H column is capable of separating a range of aliphatic alcohols. In addition to those shown in Figure 1, it may also be possible to separate a much wider range of this type of compound. Molecular weight and degree of branching are critical factors in determining the amount of retention on a Hi-Plex H column.

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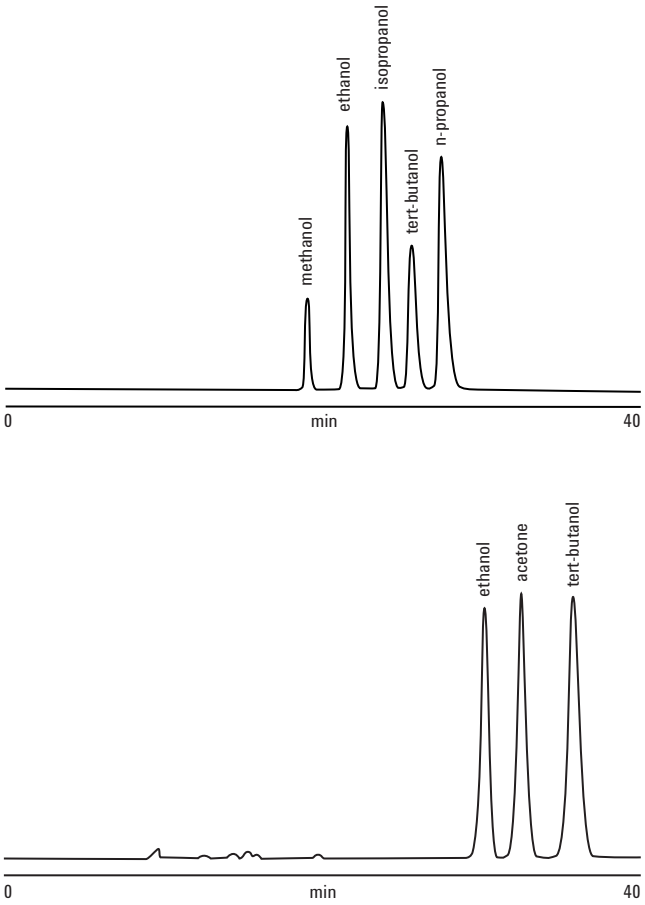


*Figure 1. Separation of different aliphatic compounds on an Agilent Hi-Plex H column.*

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# Analysis of Byproducts in Fermentation Liquids Using an Agilent Hi-Plex H Column

Application Note

Food and Beverage


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## Introduction

Biomass fermentation has grown in importance because diverse products such as fuel, lubricants, and chemicals can be derived. One option for this use of biomass is the fermentation of xylose from hemicelluloses, to xylitol, a sugar substitute. For the HPLC analysis of fermentation liquids, the US NREL Biomass Program method *Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples* can be applied.


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## Materials and Methods

Two fermentation samples were analyzed. The first was obtained by a hydrothermal digestion of straw (as an example of biomass) that destroys the hemicelluloses and frees the xylose. Following partial evaporation of water, the second sample was obtained after fermentation of xylose to xylitol.

### Conditions

| | |
|---|---|
| Column | Agilent Hi-Plex H, 7.7 × 300 mm, 8 µm (p/n PL1170-6830) |
| Mobile phase | 0.005 M $H_2SO_4$ |
| Gradient | Isocratic |
| Flow rate | 0.7 mL/min |
| Injection volume | 20 µL |
| Sample concentration | Xylose ~ 8 g/L |
| | Glucose ~ 1.5 g/L |
| | Xylitol ~ 13 g/L |
| | Furfural 10 ~ 500 mg/L |
| | Hydroxymethylfurfural ~ 100 mg/L |
| | Acetic acid ~ 1000 mg/L |
| | Ethanol ~ 2000 mg/L |
| | Lactic acid ~ 2500 mg/L |
| Temperature | 60 °C |
| Pressure | 4.6 MPa (46 bar, 670 psi) |
| Detector | RI (55 °C) |

## Results

After hydrothermal digestion, a large quantity of xylose is present in solution, as expected (Figure 1). Figure 2 shows that further fermentation of the sample converts a large quantity of this xylose into xylitol and gives a very large RI response for this sugar alcohol.

## Conclusion

The Agilent Hi-Plex H column is specially suited for the analysis of byproducts and degradation products (acids, alcohols, furfural, hydroxymethylfurfural), such as those produced by biomass fermentation. The column is recommended for use with samples that contain high levels of organic acids or for simultaneous analysis of these acids and sugars, using sulfuric acid as the mobile phase.

## For More Information

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*Peak identification for Figures 1 and 2*

1 Glucose
2 Xylose
3 Arabinose
4 Xylitol
5 Lactic acid
6 Glycerol
7 Acetic acid
8 Ethanol
9 Hydroxymethylfurfural (HMF)
10 Furfural

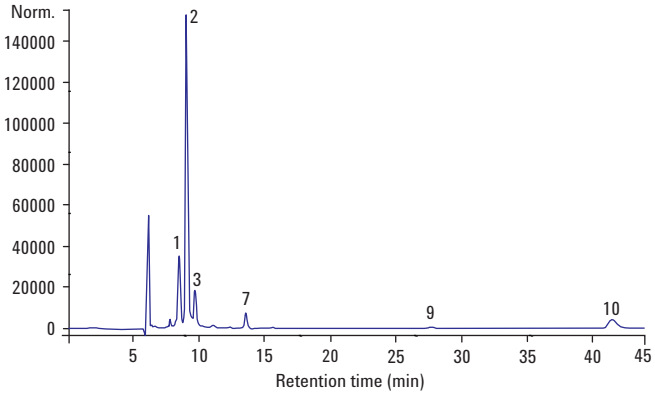


*Figure 1. Analysis of a sample of straw after hydrothermal digestion using an Agilent Hi-Plex H column.*

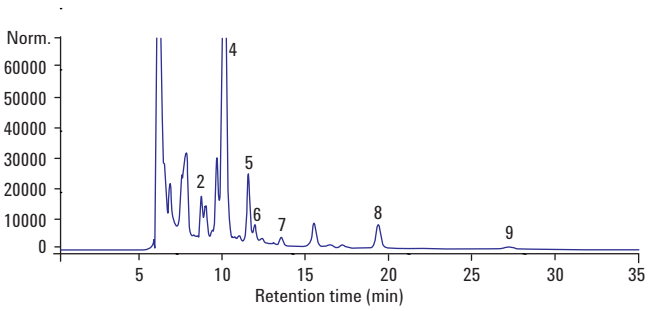


*Figure 2. Components of a straw sample after fermentation of xylose to xylitol.*

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# Fast Analysis of Carbohydrates in Chocolate Using Ligand-Exchange Chromatography with ELSD

Application Note

Food


## Author

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## Introduction

Methods of detection for carbohydrates are severely limited because they do not normally possess chromophores or fluorophores. Detection can sometimes be accomplished in the low UV range, 190-200 nm, but unless high-purity eluents are used and extensive sample preparation employed, excessive interference from other compounds may occur.

The refractive index (RI) detector is routinely used, but RI is relatively insensitive, relying on a refractive index difference between solute and eluent. Where increased sensitivity is required, a pulsed amperometric detector (PAD) is employed, but for uniform response, the carbohydrate must be in a high pH environment.

A better detector for the analysis of carbohydrates is the Agilent evaporative light scattering detector (ELSD). When the Agilent ELSD is used in combination with Agilent Hi-Plex ligand-exchange columns, rapid isocratic separations of mono-, di-, and oligosaccharides are achieved. The Agilent ELSD does not require the solutes of interest to have any particular optical properties. The principle of operation is a three-stage process; the first stage involves the nebulization of the eluent, the second the evaporation of the solvent to leave solute particles, and the third, the detection of the light scattered by the solid solute particles as they pass through the light beam. The only requirement for using the Agilent ELSD is that the eluent be more volatile than the solutes.


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When using Agilent Hi-Plex columns for the analysis of carbohydrates, water (with no buffer or added salt) is used as the eluent. This is an ideal application for the Agilent ELSD because neutral carbohydrates have little UV activity. Sugars may be detected with the ELSD and a Hi-Plex column that has strong cation-exchange resins available in differing ionic forms.

The sulfonated column resin gives a fundamental improvement in performance and overcomes the problems of low efficiencies and high backpressures encountered with soft gels. The separation mechanism is achieved initially by size exclusion, with larger oligosaccharides eluting before smaller monosaccharides, and then by ligand-exchange interaction of the numerous hydroxyl groups on the sugar molecules with the metal ion associated with the resin. Hi-Plex columns are used at elevated temperature with isocratic eluents.

Chocolate is produced in three distinct forms: dark chocolate, milk chocolate, and white chocolate. The predominant sugar in the three varieties is the disaccharide sucrose. However, the milk sugar, lactose, will also be present in milk and white chocolate. The amount of lactose present will be indicative of the amount of milk solids used in the production process. As both sucrose and lactose are disaccharides, the Hi-Plex Pb column is the preferred choice for the analysis and quantification of these two components.

Hi-Plex resins are available in 8% crosslinked calcium forms for the analysis of mono- and disaccharides and in hydrogen (acid) forms for the analysis of sugar alcohols and organic acids. Also available is a 4% crosslinked sodium form for the separation of high molecular weight oligosaccharides, such as corn syrups, to Dp 9.

## Materials and Reagents

### Instrumentation

| | |
|---|---|
| Column | Agilent Hi-Plex Pb, 7.7 × 300 mm, 8 µm (p/n PL1170-6820) |
| Detector | Agilent ELSD |

### Sample Preparation

Aqueous solutions were prepared at a concentration of 100 mg chocolate/mL, and 2 µL injection volumes were used for the quantitation.

## Results and Discussion

Table 1 summarizes the quantitation of the two disaccharides, sucrose and lactose. Sucrose is present in all four samples, with the plain chocolate having the highest level. Lactose, the milk sugar, can be seen in the other three samples. Differences in the sucrose and lactose content of the two milk chocolate samples from different manufacturers are evident.

The disaccharide composition of four commercial chocolate samples is shown in Figure 1.

*Table 1. Disaccharide Content of Commercial Chocolate Samples Expressed as a Percentage by Weight of Chocolate*

| Carbohydrate | Milk sample 1 | Milk sample 2 | Plain | White |
|---|---|---|---|---|
| Lactose | 7 | 17 | nd | 9 |
| Sucrose | 30 | 41 | 69 | 42 |
| Total | 37 | 58 | 69 | 51 |

nd - not detected

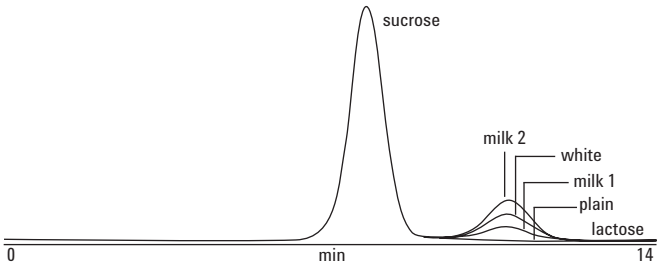


*Figure 1. HPLC chromatograms of four commercial samples of chocolate, normalized to the height of the sucrose peak.*

## Conclusion

The composition of chocolate and levels of added milk solids in milk and white chocolate are readily achieved, using water as the mobile phase with an Agilent Hi-Plex Pb column and the Agilent ELSD.

This system avoids the use, high cost, and disposal implications of toxic acetonitrile when separations are performed on amino silica columns. In addition, the Hi-Plex columns stay active in the presence of sugar molecules. Together with fast dissolution, this benefit results in long lifetimes as compared to amino silica columns.

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Agilent Technologies

# Agilent Hi-Plex Columns for Carbohydrates, Alcohols, and Acids

## Application Note

Food and Pharmaceutical


## Authors

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## Introduction

Agilent Hi-Plex columns are ion-exchange ligand-exchange columns used predominantly for the separation of carbohydrates and organic acids. These columns use the preferred separation mechanism for the analysis of simple sugars, alcohols, oligosaccharides, and organic acids in foodstuffs, but can also be used for the separation of other compounds.

The range comprises a 4% crosslinked resin for the analysis of oligosaccharides and an 8% crosslinked resin, with lower exclusion limit, for mono-, di-, and trisaccharide analysis. For carbohydrate and alcohol investigations, Hi-Plex columns use isocratic conditions with water as the eluent and temperature as the main variable for control of resolution. The exception is Agilent Hi-Plex Na (Octo), which is used with sodium hydroxide eluents when pulsed amperometric detection is employed.

In these examples, we use Agilent Hi-Plex H, Agilent Hi-Plex Ca, as well as Agilent Hi-Plex Ca (Duo) for the analysis of organic acids, sugars, and sugar alcohols.


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## Organic Acids on Hi-Plex H

Where samples contain organic acids, whether or not in the presence of neutral mono- and disaccharides, Agilent Hi-Plex H is the preferred option. The column is run using water as the eluent for the analysis of sugars and organic acids or, more commonly, dilute acid for separation of organic acids. Refractive index (Figure 1 and Table 1, Figure 2 and Table 2) or UV detection (Figure 3 and Table 3) is used.

### Conditions

| | |
|---|---|
| Sample | Sugars and organic acids |
| Column | Agilent Hi-Plex H, 7.7 × 300 mm, 8 µm (p/n PL1170-6830) |
| Mobile phase | 100% 0.0085 M $H_2SO_4$ |
| Flow rate | 0.4 mL/min |
| Injection volume | 20 µL |
| Temperature | 65 °C |
| Detector | RI |

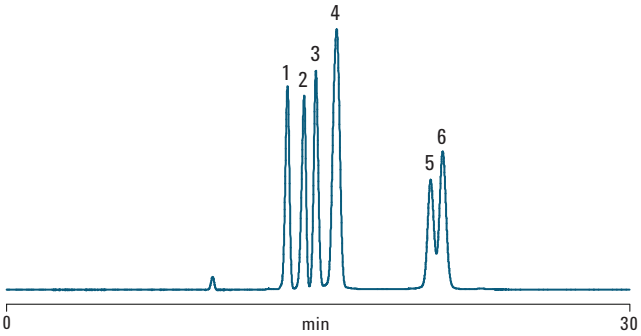


*Figure 1. Separation of monosaccharides, organic acids, and glycerol on an Agilent Hi-Plex H column with RI detection.*

### Conditions

| | |
|---|---|
| Sample | Organic acids |
| Column | Agilent Hi-Plex H, 7.7 × 300 mm, 8 µm (p/n PL1170-6830) |
| Mobile phase | 0.1 M $H_2SO_4$ |
| Flow rate | 0.6 mL/min |
| Temperature | 50 °C |
| Detector | RI |

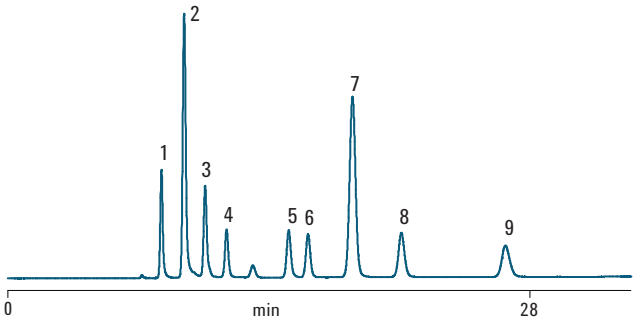


*Figure 2. Separation of nine organic acids on an Agilent Hi-Plex H column with RI detection.*

*Table 1. Peak Data from the Analysis of Monosaccharides, Organic Acids, and Glycerol on an Agilent Hi-Plex H Column Using RI Detection*

| Peak number | Analyte | As. USP | 10% Asymmetry | Plate count | Plates/m |
|---|---|---|---|---|---|
| 1 | Citric acid | 0.92 | 0.92 | 21207 | 70691 |
| 2 | Tartaric acid | 0.84 | 0.85 | 21475 | 71583 |
| 3 | Glucose | 1.04 | 1.04 | 21805 | 72684 |
| 4 | Malic acid, fructose | 0.93 | 0.93 | 10012 | 33372 |
| 5 | Lactic acid | 0.89 | 0.96 | 19685 | 65618 |
| 6 | Glycerol | 1.16 | 1.06 | 19070 | 63566 |

*Table 2. Peak Data from the Separation of Organic Acids on an Agilent Hi-Plex H Column Using RI Detection*

| Peak number | Analyte | As. USP | 10% Asymmetry | Plate count | Plates/m |
|---|---|---|---|---|---|
| 1 | Oxalic acid | 1.23 | 1.12 | 19471 | 64904 |
| 2 | *cis*-Aconitic acid | 1.29 | 1.15 | 16122 | 53741 |
| 3 | Tartaric acid | 1.30 | 1.24 | 19272 | 64240 |
| 4 | Malic acid | 1.10 | 1.07 | 20153 | 67176 |
| 5 | Lactic acid | 1.16 | 1.10 | 21469 | 71563 |
| 6 | Formic acid | 1.08 | 1.05 | 22118 | 73726 |
| 7 | Fumaric acid | 1.05 | 1.03 | 15751 | 52504 |
| 8 | Propionic acid | 1.12 | 1.09 | 20492 | 68305 |
| 9 | Butyric acid | 1.15 | 1.13 | 18181 | 60603 |

## Conditions

| | |
|---|---|
| Sample | Organic acids |
| Column | Agilent Hi-Plex H, 7.7 × 300 mm, 8 µm (p/n PL1170-6830) |
| Mobile phase | 100% 0.01 M $H_2SO_4$ |
| Flow rate | 0.6 mL/min |
| Injection volume | 20 µL |
| Temperature | 50 °C |
| Detector | UV, 210 nm |

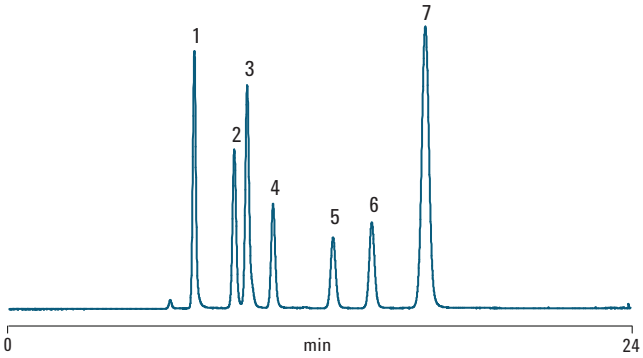


*Figure 3. Separation of seven organic acids on an Agilent Hi-Plex H column with UV detection.*

*Table 3. Peak Data from the Separation of Organic Acids on an Agilent Hi-Plex H Column Using UV Detection*

| Peak number | Analyte | As. USP | 10% Asymmetry | Plate count | Plates/m |
|---|---|---|---|---|---|
| 1 | Oxalic acid | 1.13 | 1.02 | 17164 | 57212 |
| 2 | Citric acid | 1.11 | 1.07 | 17588 | 58626 |
| 3 | Tartaric acid | 1.30 | 1.23 | 19251 | 64170 |
| 4 | Malic acid | 1.11 | 1.07 | 20170 | 67233 |
| 5 | Succinic acid | 1.08 | 1.06 | 19705 | 65684 |
| 6 | Formic acid | 1.07 | 1.05 | 21991 | 73302 |
| 7 | Fumaric acid | 1.05 | 1.03 | 15139 | 50464 |

## Sugars and Sugar Alcohols on Hi-Plex Ca

Agilent Hi-Plex Ca is recommended for the analysis of samples containing the sweetening sugars (glucose, fructose, and sucrose) and the sugar alcohols (mannitol and sorbitol) (Figure 4). The 4.0 × 250 mm column is referenced in the USP method that specifies L19 media for sugar alcohols analysis.

## Conditions

| | |
|---|---|
| Sample | Sugars and sugar alcohols |
| Column | Agilent Hi-Plex Ca, 7.7 × 300 mm, 8 µm (p/n PL1170-6810) |
| Sample size | 10 mg/mL |
| Mobile phase | 100% DI $H_2O$ |
| Flow rate | 0.6 mL/min |
| Injection volume | 10 µL |
| Temperature | 85 °C |
| Detector | RI |

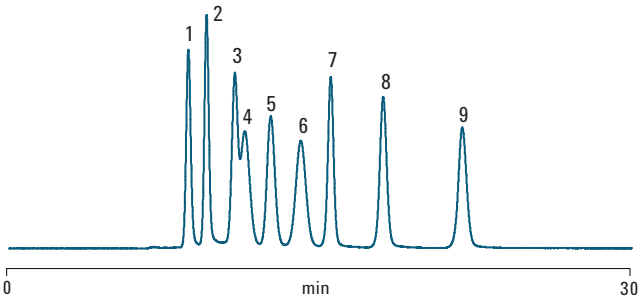


*Figure 4. Separation of a mixture of sugars and sugar alcohols on an Agilent Hi-Plex Ca column.*

*Table 4. Peak Data from the Separation of a Sugars and Sugar Alcohols Mix on an Agilent Hi-Plex Ca Column*

| Peak number | Analyte | As. USP | 10% Asymmetry | Plate count | Plates/m |
|---|---|---|---|---|---|
| 1 | Raffinose | 1.12 | 1.08 | 7138 | 23793 |
| 2 | Sucrose | 1.12 | 1.06 | 9389 | 31298 |
| 3 | Lactulose | 0.85 | 0.92 | 3858 | 12861 |
| 4 | Glucose | 1.79 | 1.59 | 2986 | 9955 |
| 5 | Galactose | 1.07 | 1.07 | 5008 | 16694 |
| 6 | Fructose | 1.01 | 1.01 | 3727 | 12423 |
| 7 | Ribitol | 1.00 | 1.00 | 14758 | 49194 |
| 8 | Mannitol | 1.04 | 1.04 | 13861 | 46204 |
| 9 | Sorbitol | 1.04 | 1.04 | 14170 | 47234 |

## Monosaccharide and Oligosaccharide Mixture on Hi-Plex Ca (Duo)

Agilent Hi-Plex Ca (Duo) is an 8% crosslinked material and therefore has a smaller pore size and less resolution for the larger oligomers. However, the Ca counter ion has improved ligand-exchange capability for monosaccharides, and so it is most suited for the analysis of samples containing both mono- and oligosaccharides (Figure 5).

## Conditions

| | |
|---|---|
| Sample | Sugars and sugar alcohols |
| Column | Agilent Hi-Plex Ca (Duo), 6.5 × 300 mm, 8 µm (p/n PL1F70-6850) |
| Sample size | 10 mg/mL |
| Mobile phase | 100% DI $H_2O$ |
| Flow rate | 0.4 mL/min |
| Injection volume | 10 µL |
| Temperature | 85 °C |
| Detector | RI |

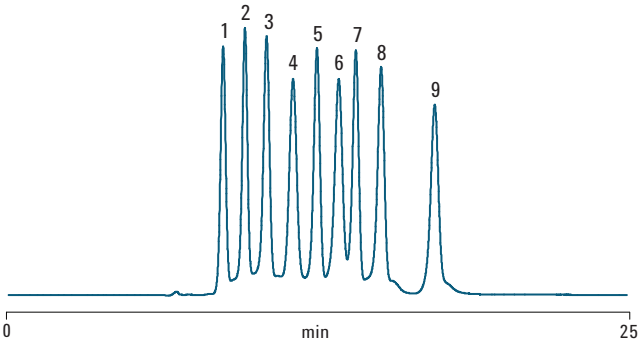


*Figure 5. Separation of a mixture of mono- and oligosaccharides on an Agilent Hi-Plex Ca (Duo) column.*

*Table 5. Peak Data from the Separation of a Sugars and Sugar Alcohols Mix on an Agilent Hi-Plex Ca (Duo) Column*

| Peak number | Analyte | As. USP | 10% Asymmetry | Plate count | Plates/m |
|---|---|---|---|---|---|
| 1 | Raffinose | 1.13 | 1.00 | 7827 | 26091 |
| 2 | Sucrose | 0.80 | 0.96 | 9363 | 31211 |
| 3 | Lactulose | 1.03 | 0.87 | 7895 | 26316 |
| 4 | Glucose | 0.99 | 0.97 | 6204 | 20680 |
| 5 | Galactose | 0.85 | 0.90 | 10869 | 36229 |
| 6 | Fructose | 0.82 | 0.88 | 7765 | 25884 |
| 7 | Ribitol | 1.02 | 0.87 | 13784 | 45948 |
| 8 | Mannitol | 0.95 | 0.87 | 13431 | 44771 |
| 9 | Sorbitol | 1.15 | 0.95 | 13807 | 46025 |

## Conclusion

Agilent Hi-Plex columns deliver improved efficiency, lower operating pressures, and longer column lifetimes from monodispersed materials. With a range of ligand counter ions for optimum selectivity and with resolution and materials matched to the USP definitions of media types L17, L19, L34, and L58, the Hi-Plex range is ideal for isocratic separations using water or dilute acid as the eluent. This simplifies system requirements for HPLC and eliminates the use of potentially hazardous organic solvents.

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Agilent Technologies

# Agilent Hi-Plex Columns for Fingerprinting Organic Acids in Wine

## Application Note

Food and Beverage


## Author

Stephen Ball

Agilent Technologies, Inc.


## Introduction

The Agilent Hi-Plex H is a high-performance ligand-exchange chromatography column. The column is based on polystyrene/divinylbenzene with an 8% crosslinking and hydrogen counter ion. Typically used for the analysis of sugars, sugar alcohols, and organic acids, its monodisperse sulfonated packing gives improved column efficiency, lower column pressure, and assured batch-to-batch reproducibility.

The superior separation ability of Hi-Plex H is demonstrated in the quantitative analysis of organic acids in four different samples of wine: red, white, rosé, and dessert wine. This type of analysis is important for wine quality control because the classes and content of organic acids give a characteristic taste to the finished product. Acetic acid, lactic acid, succinic acid, malic acid, citric acid, and tartaric acid are the main organic acids in wine.

The use of a ligand-exchange chromatography column such as Hi-Plex H significantly reduces the need for complicated sample preparation (typically involving elution through an ion-exchange resin bed), as retention is brought about not only by ion exchange, but also by ion exclusion and partitioning on this type of column.


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## Materials and Reagents

### Sample Preparation

In order to obtain a complete refractive index (RI) profile, each wine was directly injected onto the column without any sample pretreatment. The exceptions to this being the Inniskillin Eiswein, which was diluted by a factor of five with HPLC-grade water, and the Marsala wine, which was diluted by a factor of three with water. Injection volume was 20 µL.

### Conditions

| | |
|---|---|
| Column | Agilent Hi-Plex H, 7.7 × 300 mm, 8 µm (p/n PL1170-6830) |
| Mobile phase | 0.004 M $H_2SO_4$ |
| Flow rate | 0.4 mL/min |
| Temperature | 75 °C |
| Detector | RI |

## Results and Discussion

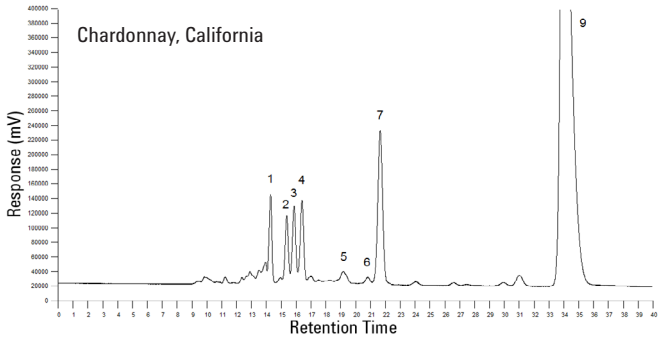
The results demonstrate that it is possible to distinguish between different types of wine (i.e., red, white, rosé, dessert) by HPLC analysis with the Hi-Plex H column. By using an RI detector, levels of organic acids and sugars can be quantified simultaneously.

The rosé and dessert wines all contain very high levels of malic acid and fructose (fruit sugar). In fact, the Inniskillin Eiswein and Masala wines, both dessert wines, contain up to five times as much sugar as ordinary rosé wine and 70 times as much sugar as the red and white wines. Eiswein (commonly called Ice Wine) is produced from grapes that have been frozen, causing some of the water to freeze out, leaving the sugars and other solids dissolved in the remaining juice. The resulting wine is therefore very sweet but has a great deal of balancing acidity, which also explains the high level of malic acid in the wine samples.

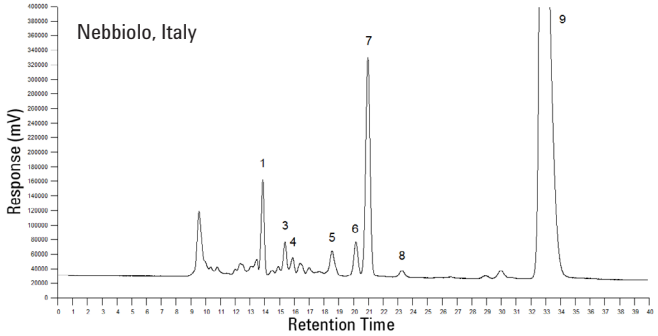
The chromatograms of the red and white wines look very different from those of the other wines, in that they have much lower levels of sugar but much higher levels of lactic acid and glycerol. Red wine is made from the must (pulp) of red or black grapes that undergo fermentation together with the grape skins, while white wine is usually made by fermenting juice pressed from white grapes. During the fermentation process, yeast converts most of the sugars in the grape juice into ethanol and carbon dioxide, which explains the low levels of glucose and fructose in the wine samples. Some wines also undergo malolactic fermentation, where bacteria convert malic acid into the milder lactic acid. All of these factors and the levels of sugars and organic acids produced by the various fermentation processes contribute to the different taste that each wine has and give each one a unique profile when analyzed by HPLC.

Some of the other peaks that appear in the chromatograms are likely to be from the tannins (bitter-tasting plant polyphenols) present in the skins and seeds of the grapes used in the fermentation process.

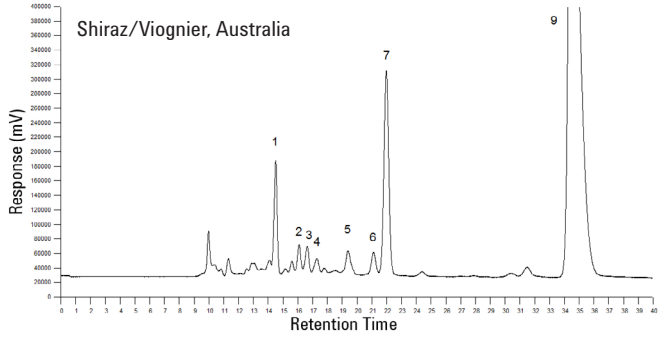
*Key*
*1). Tartaric acid, 2). Malic acid, 3). Glucose, 4). Fructose, 5). Succinic acid, 6). Lactic acid, 7). Glycerol, 8). Acetic acid, 9). Ethanol*



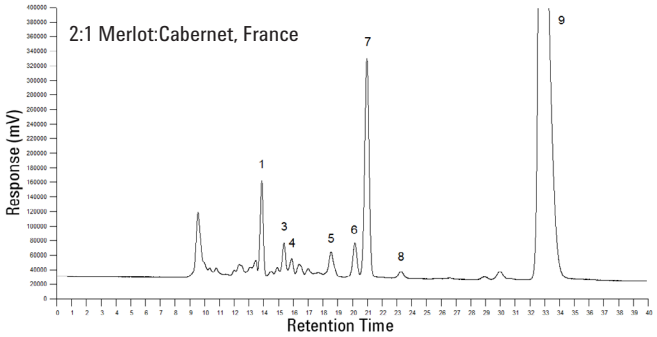

*White wine contains a wide variety of organic acids and sugars.*



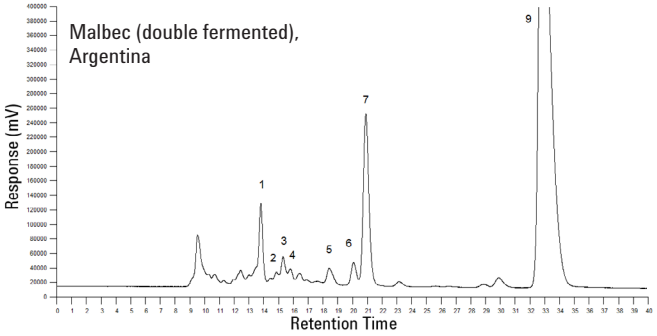

*The main constituents of this red wine are tartaric acid, succinic acid, and glycerol.*



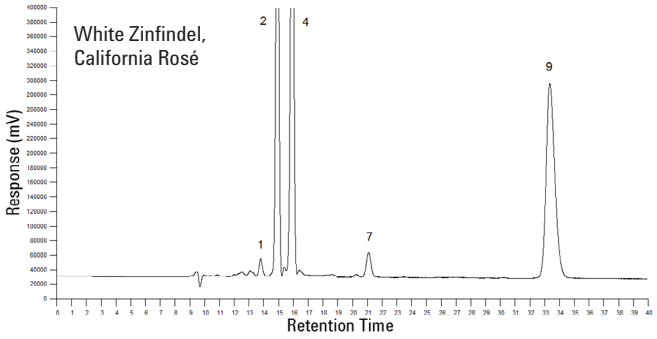

*This wine contains slightly higher levels of malic acid and fructose than the Nebbiolo.*



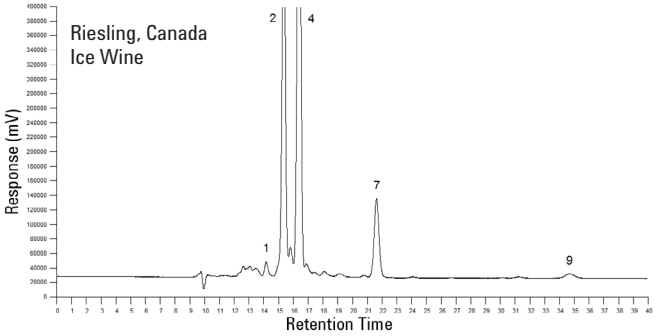

*As shown, this red wine contains a slightly higher level of lactic acid than the other wines.*



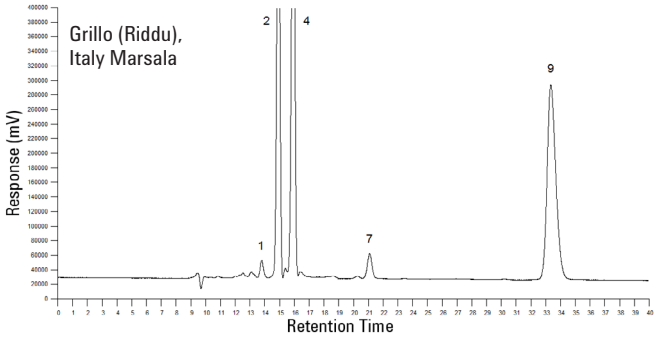

*This wine has another unique profile, with differing levels of organic acids and sugar.*




*Rosé wine contains fewer organic acids and a significantly higher fructose content.*




*This dessert wine contains very high levels of malic acid, fructose, and glycerol, but little else.*




*As expected, this dessert wine also contains high amounts of malic acid and fructose.*

## Conclusion

The analysis of wines demonstrates the use of Agilent Hi-Plex H columns to provide resolution of closely eluting compounds, enabling quantitation of each. These columns are ideal for the analysis of sugar alcohols and sugar molecules, using water as the mobile phase. Hi-Plex H is also the column of choice for the analysis of organic acids, using dilute mineral acid as eluent. By using the columns at higher operating temperatures, closely eluting compounds can be resolved.

## References

1. M.Y. Ding, H. Koizumi, and Y. Suzuki. (1995). Comparison of Three Chromatographic Systems for Determination of Organic Acids in Wine. *Analytical Sciences*. 11: 239-243.

2. A. Schneider, V. Gerbi, and M. Redoglia. (1987). A Rapid HPLC Method for Separation and Determination of Major Organic Acids in Grape Musts and Wines. *American Journal of Enology and Viticulture*. 38 (2), 151-155.

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Agilent Technologies

# Fruit Wine Analysis by HPLC

Application Note

Food and Beverage


## Author

Stephen Ball

Agilent Technologies, Inc.


## Introduction

Typically, wine is made by the fermentation of grapes that have been crushed to extract the juice. However, wine can also be made from other fruit juices. This is a cheaper method than using grapes and is therefore becoming more popular.

Most fruits and berries can produce wine, but few of them have the proportions of sugars, acids, tannins, yeast nutrients, and water to deliver a drinkable and stable product. The amounts of fermentable sugars may be low, or the acid content may be too high. Fruit wines may therefore be supplemented with sucrose, or sorbitol may be added as an artificial sweetener.

An Agilent Hi-Plex Ca column can be used to quantify the levels of sugars, artificial sweetener, and alcohols in fruit juice wine, which all contribute to their individual flavors.


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## Materials and Reagents

| | |
|---|---|
| Column | Agilent Hi-Plex Ca (8% crosslinked), 7.7 × 300 mm, 8 µm (p/n PL1170-6810) |
| Mobile phase | 100% DI $H_2O$ |
| Flow rate | 0.6 mL/min |
| Temperature | 85 °C |
| Detector | RI |

## Conclusion

The Agilent Hi-Plex Ca column gives very good separation of the main constituents of fruit juice wine, allowing them to be easily quantified. The Hi-Plex Ca column gives good resolution of the components shown. Even at high levels of ethanol, it does not overlap the other components. However, the Hi-Plex Ca column gives sufficient resolution between analytes to prevent this from occurring.

A potentially useful application of this HPLC procedure is in the quality control of traditional wine or in flavor studies of fruit wine made from a variety of different sources.

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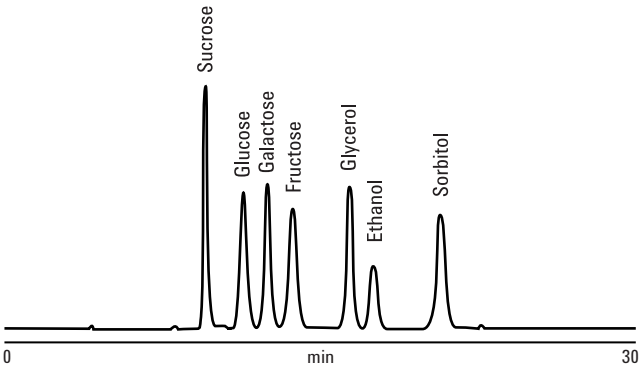


*Figure 1. Separation of compounds typically found in fruit wine on an Agilent Hi-Plex Ca column. Ten microliters of a 20 mg/mL solution were injected.*

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Agilent Technologies

# Glycol Analysis by HPLC

Application Note

Food


## Author

Stephen Ball

Agilent Technologies, Inc.


## Introduction

This application note demonstrates the use of an Agilent Hi-Plex Ca column for the separation of ethylene glycol and trimethylene glycol (or 1,3-propanediol).

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## Materials and Reagents

| | |
|---|---|
| Column | Agilent Hi-Plex Ca (8% crosslinked), 7.7 × 300 mm, 8 µm (p/n PL1170-6810) |
| Mobile phase | 100% DI $H_2O$ |
| Flow rate | 0.6 mL/min |
| Temperature | 85 °C |
| Detector | RI |

## Results

The Agilent Hi-Plex Ca column gives very good separation of ethylene glycol and trimethylene glycol.

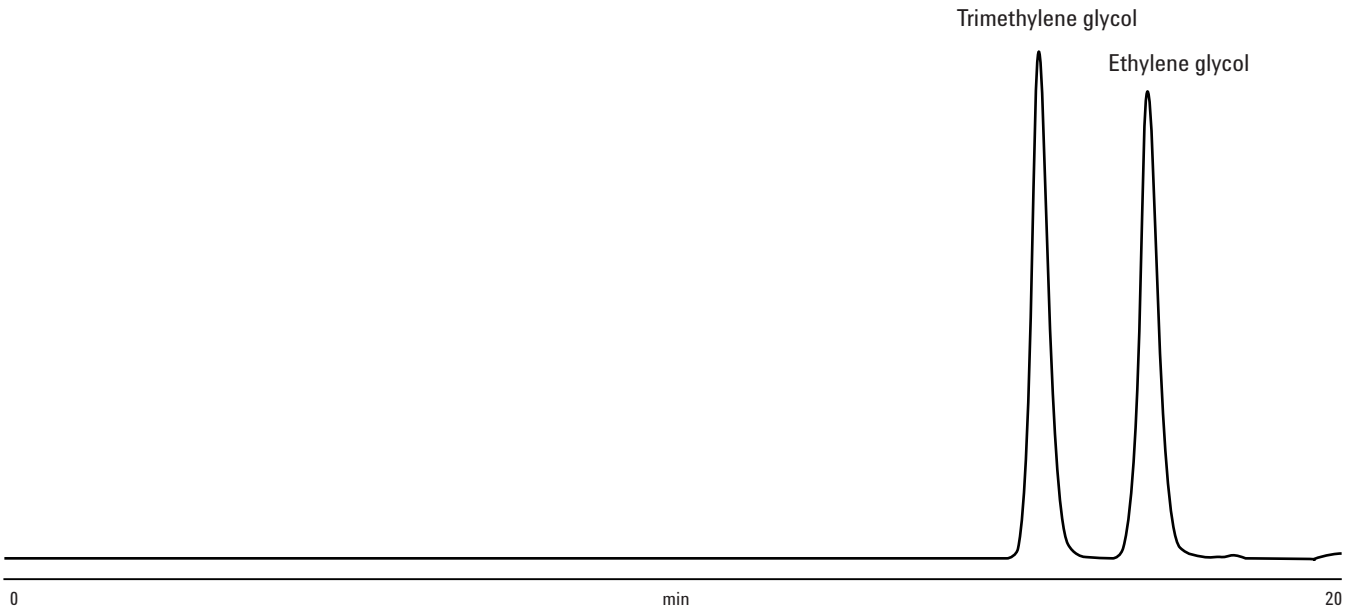


*Figure 1. Separation of glycols on an Agilent Hi-Plex Ca column. Twenty microliters of a 10 mg/mL solution were injected.*

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Agilent Technologies

# HPLC Determination of Carbohydrates in Food and Drink

## Application Note

Food and Beverage


## Author

Linda Lloyd

Agilent Technologies, Inc.


## Introduction

The separation, identification, and quantification of simple sugars can be readily achieved using chromatography. High-performance liquid chromatography (HPLC) is perhaps the simplest technique, often requiring little in the way of sample preparation, particularly with liquids. Sugars may be detected with a differential refractive index (RI) detector, provided isocratic elution is used. This is the case with Agilent Hi-Plex resins. These strong cation-exchange resins are available in different ionic forms. The sulfonated resin gives a fundamental improvement in performance and overcomes the problems of low efficiencies and high backpressures encountered with soft gels. The separation mechanism is achieved initially by size exclusion, with larger oligosaccharides eluting before smaller monosaccharides, and then by ligand-exchange interaction of the numerous hydroxyl groups on the sugar molecules with the metal ion associated with the resin.

Hi-Plex resins are available in 8% crosslinked calcium and lead forms for the analysis of mono- and disaccharides and in hydrogen (acid) forms for the analysis of sugar alcohols and organic acids. Also available is a 4% crosslinked sodium form for the separation of high molecular weight oligosaccharides, such as corn syrups, to Dp 14. Separations of sugars with Agilent Hi-Plex columns and water eluents are easily achieved, avoiding the need for toxic acetonitrile.


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## Experimental

### Instrumentation

Column Agilent Hi-Plex Ca, 7.7 × 300 mm, 8 µm (p/n PL1170-6810)

Detector RI

### Materials and Reagents

Mobile phase 100% DI $H_2O$

### Conditions

Flow rate 0.6 mL/min

Temperature 85 °C

## Results and Discussion

Results for analyses of unadulterated orange, pineapple, apple, and tomato juices are shown in Figures 1-4. The ratios of the different sugars are clearly expressed.

## Conclusion

The separation of sucrose, glucose, and fructose in fruit juices is readily achieved using water as the mobile phase and an Agilent Hi-Plex Ca column at 85 °C. This avoids the use, high cost, and disposal implications of toxic acetonitrile when separations are performed on amino silica columns. In addition, Hi-Plex stays active in the presence of sugar molecules. Together with fast dissolution, this benefit results in long lifetimes compared to amino silica columns.

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*Key (all figures)*

*1. Sucrose*
*2. Glucose*
*3. Fructose*

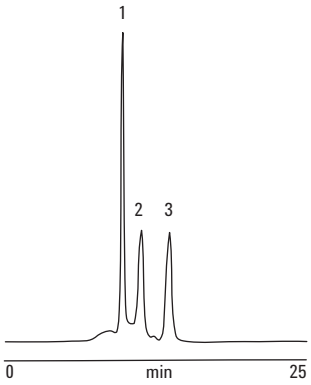


*Figure 1. HPLC of sugars in orange juice.*

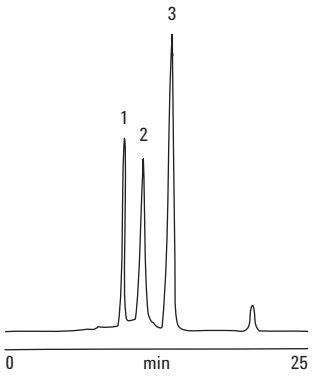


*Figure 2. HPLC of sugars in apple juice.*

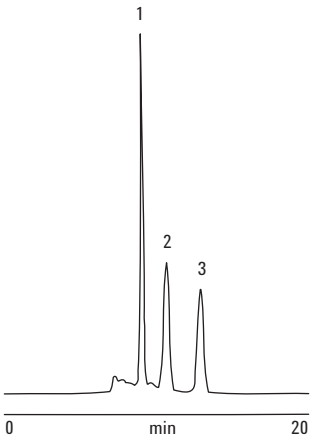


*Figure 3. HPLC of sugars in pineapple juice.*


*Figure 4. HPLC of sugars in tomato juice.*

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Agilent Technologies

# HPLC of Aloe Juice Using Evaporative Light Scattering Detection

## Application Note

Food and Pharmaceutical


## Author

Stephen Bullock

Agilent Technologies, Inc.


## Introduction

Aloe vera (syn. *Aloe barbadensis* Mill.) has been used as a medicinal plant for some 5,000 years. However, it is only in the last 20 to 30 years that information about this "miracle" plant and its possible healing power has reached a wider public. Aloe vera has been suggested as an ideal moisturizer and healing aid when applied topically on burns, sunburn, and various skin conditions. Many people also drink the juice in expectation of a balanced, healthy lifestyle or as an alternative to non-natural health supplements. In addition, the juice is consumed to aid the healing of specific illnesses or conditions.

Aloe vera juice contains over 200 active ingredients, of which the most prominent are vitamins, amino acids, minerals, phytonutrients, enzymes, and sugars (monosaccharides, disaccharides, and polysaccharides). Studies in the field of glycomics suggest that the monosaccharide content in aloe juice contributes significantly towards its anti-inflammatory activity. Normally, nonchromophoric sugar separations are performed using a refractive index (RI) detector, but RI commonly suffers from the problems of baseline instability and poor sensitivity. The Agilent 385-ELSD (evaporative light scattering detector) is a superior choice for this type of analysis.

Agilent Hi-Plex Ca columns contain a monodisperse sulfonated polystyrene incorporating 8% divinylbenzene with a calcium counter ion, and provide a separation based on a combination of both size exclusion and ligand-exchange chromatography.

To highlight the excellent resolving power of an Agilent 385-ELSD and Hi-Plex Ca system, aloe juice was analyzed, together with glucose and fructose (monosaccharides) and trehalose (disaccharide) standards. These sugars are commonly present in aloe juice.

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## Experimental

### Instrumentation

| | |
|---|---|
| Column | Agilent Hi-Plex Ca, 7.7 x 300 mm, 8 µm (p/n PL1170-6810) |
| Detector | Agilent 385-ELSD (neb = 50 °C, evap = 90 °C, gas = 1.6 SLM) |

### Materials and Reagents

| | |
|---|---|
| Mobile phase | 100% DI $H_2O$ |

### Sample Preparation

Glucose, fructose, and trehalose were dissolved in water to 1 mg/mL. Aloe juice was used as received.

### Conditions

Soft gel columns should be operated at elevated temperature to reduce operating pressure and permit the use of regular flow rates.

| | |
|---|---|
| Flow rate | 0.6 mL/min |
| Injection volume | 20 µL |
| Temperature | 80 °C |

## Results and Discussion

Figure 1 illustrates how well the three saccharide standards are resolved. Comparison of the chromatograms of the standards (lower trace) with that of the aloe juice sample (upper trace) confirms that all three saccharides are present in different quantities.

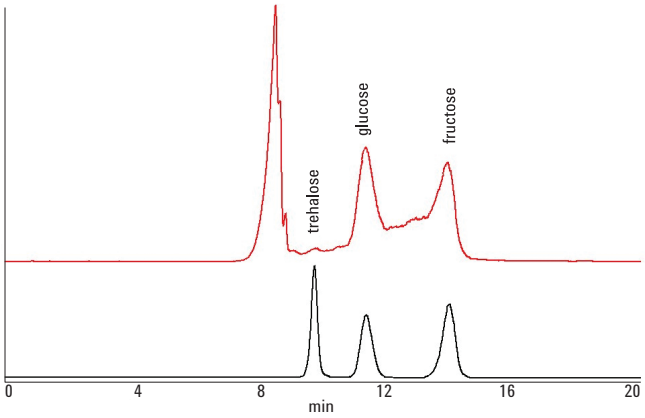


*Figure 1. Extremely stable baseline achieved by Agilent Hi-Plex Ca and Agilent 385-ELSD in profiling sugars in aloe vera juice.*

## Conclusion

An HPLC system comprising the Agilent 385-ELSD and Agilent Hi-Plex Ca column produced good separations and a very stable baseline in the analysis of aloe juice.

Hi-Plex columns are packed with sulfonated resin, giving a fundamental improvement in performance to overcome the problems of low efficiencies and high backpressures encountered with soft gels. The columns are available in calcium form for the analysis of carbohydrates in juices, to meet the growing demand for more detailed product information for labeling and control purposes.

The Agilent 385-ELSD surpasses other ELSDs for low-temperature HPLC applications with semivolatile compounds. Its innovative design represents the next generation of ELSD technology, providing optimum performance across a diverse range of HPLC applications. The unique gas control of the detector permits evaporation of high boiling solvents at very low temperatures. The instrument's novel design provides superior performance for the analysis of semivolatile compounds. Accurate determination of composition and content is assured using the Agilent 385-ELSD and Hi-Plex Ca columns.

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Agilent Technologies

# Analysis of the Isomeric Forms of Methyl-D-Glucopyranose

Application Note

Food


## Authors

Stephen Ball, Linda Lloyd

Agilent Technologies, Inc.


## Introduction

Agilent Hi-Plex ligand-exchange chromatography columns are commonly used for the analysis and separation of sugars and/or sugar alcohols. However, under the right conditions, these columns are also able to separate isomeric forms of simple sugars, such as methyl-D-glucopyranose shown here in this application note.

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## Conditions

| | |
|---|---|
| Column | Agilent Hi-Plex Ca, 7.7 × 300 mm, 8 µm (p/n PL1170-6810) |
| Sample | Methyl-alpha/beta-D-glucopyranose isomers |
| Sample size | 20 mg/mL |
| Mobile phase | 100% DI $H_2O$ |
| Flow rate | 0.6 mL/min |
| Injection volume | 20 µL |
| Temperature | 85 °C |
| Detector | RI |

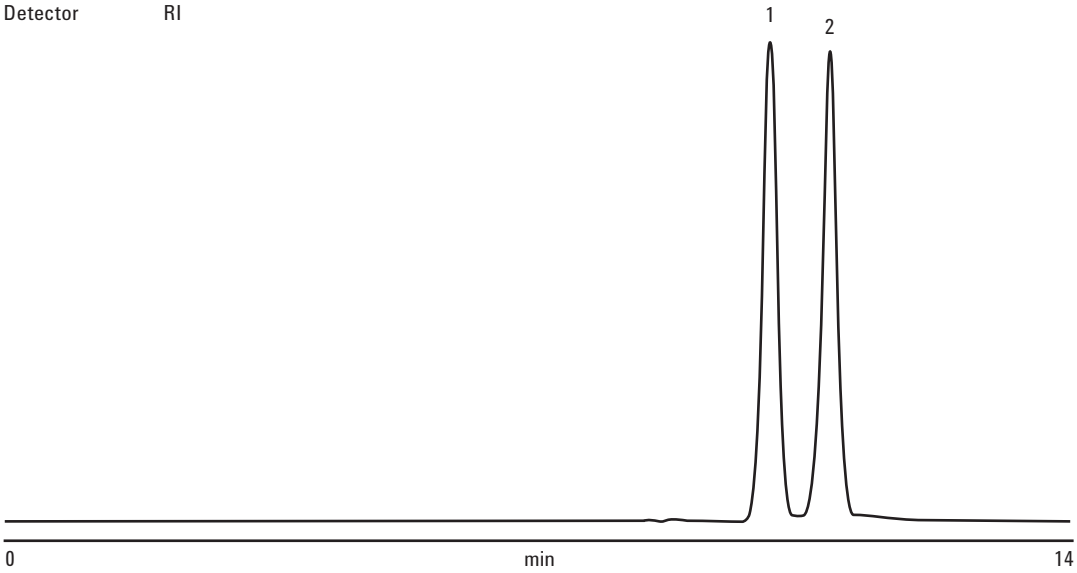


*Figure 1. Separation of methyl-alpha/beta-D-glucopyranose isomers using an Agilent Hi-Plex Ca, 8 µm column. See Table 1 for peak identification.*

*Table 1. Peak Identification for Figure 1*

| Peak | Name | Time (min) | Height (µV) | Area (%) | Width 50% (min) | As. USP | 10% Asymmetry | Res. HW | Plate counts | Plates/m |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Methyl-beta-D-glucopyranose | 10.75 | 707947.3 | 47.420 | 0.22 | 0.96 | 0.96 | 0.00 | 13722 | 45739 |
| 2 | Methyl-alpha-D-glucopyranose | 11.59 | 694379.7 | 52.580 | 0.23 | 0.96 | 0.97 | 2.19 | 13589 | 45296 |
| **Total** | | | **1402326.9** | **100.000** | | | | | | |

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Agilent Technologies

# Oligosaccharide Analysis on Agilent Hi-Plex Phases

Application Note

Food


## Authors

Stephen Ball, Linda Lloyd

Agilent Technologies, Inc.


## Introduction

Oligosaccharides are saccharide polymers containing usually three to ten component sugars. Some common oligosaccharides include fructooligosaccharides, found in many vegetables, and galactooligosaccharides, which also occur naturally. These compounds are only partially digestible by humans. However, glucose oligosaccharides, produced by the hydrolysis of starch, are a major energy source.

Oligosaccharides can be analyzed on two Agilent Hi-Plex phases, in order to determine the quantities of each different chain length in the sample. The Agilent Hi-Plex Na, with a crosslinking of 4% and a particle size of 10 µm, can separate oligosaccharides up to Dp 8. Alternatively, the Agilent Hi-Plex Ca (Duo), with an 8% crosslinking, can be used for the faster separation of oligosaccharides up to Dp 5.

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Because the Agilent Hi-Plex Na material has a crosslinking of 4%, it has the largest pore size of the entire range. This, in turn, allows the Hi-Plex Na to resolve the higher oligomers and gives definition in excess of Dp 8 for the oligomers of glucose, as shown in Figure 1.

## Conditions

| | |
|---|---|
| Column | Agilent Hi-Plex Na, 7.7 × 300 mm, 10 µm (p/n PL1171-6140) |
| Mobile phase | 100% DI $H_2O$ |
| Flow rate | 0.4 mL/min |
| Temperature | 85 °C |
| Detector | RI |

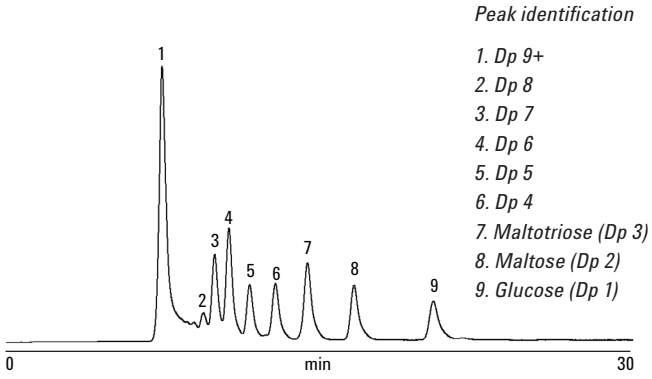


*Figure 1. Oligosaccharide separation up to Dp 8 using an Agilent Hi-Plex Na column.*

As the Hi-Plex Ca (Duo) is an 8% crosslinked material, its separation mechanism is predominantly size, but the higher crosslinked density reduces the number of oligomers that can be resolved, typically Dp 5 and below.

This material has improved mechanical strength relative to the softer Hi-Plex Na, and its calcium counter ion gives improved ligand-exchange capabilities, per Figure 2.

## Conditions

| | |
|---|---|
| Column | Agilent Hi-Plex Ca (Duo), 6.5 × 300 mm, 8 µm (p/n PL1F70-6850) |
| Mobile phase | 100% DI $H_2O$ |
| Flow rate | 0.5 mL/min |
| Temperature | 90 °C |
| Detector | RI |

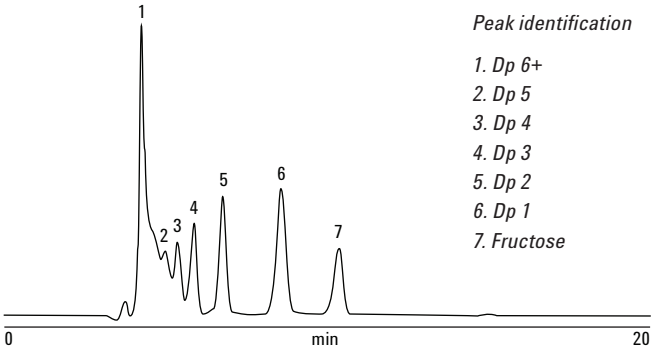


*Figure 2. Oligosaccharide separation up to Dp 5 using an Agilent Hi-Plex Ca (Duo) column.*

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SI-01673

Agilent Technologies

# Agilent Hi-Plex Columns for the Analysis of Organic Acids in Dairy Products

Application Note

Food


## Author

Stephen Ball

Agilent Technologies, Inc.


## Introduction

Quantitative determination of organic acids in dairy products is important in flavor studies, for nutritional reasons, and as an indicator of bacterial activity.

Here, we used an Agilent Hi-Plex H column to analyze the organic acid content of various dairy products.

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## Sample Preparation

For each dairy product, 0.125 g of sample was added to 125 µL of distilled water in a centrifuge tube, followed by 0.5 mL of HPLC-grade acetonitrile (to precipitate out proteins in the sample). After shaking for 1 minute, the sample was centrifuged at 13,000 rpm for 2 minutes. Ten µL of the resulting supernatant was then injected.

### Method

| | |
|---|---|
| Column | Agilent Hi-Plex H (hydrogen) (8% crosslinked), 7.7 x 300 mm, 8 µm (p/n PL1170-6830) |
| Mobile phase | 0.009 M $H_2SO_4$ |
| Flow rate | 0.7 mL/min |
| Temperature | 65 °C |
| Detector | Dual wavelength UV at 220 nm and 275 nm (for quantification of uric acid and formic acid) |

## Results

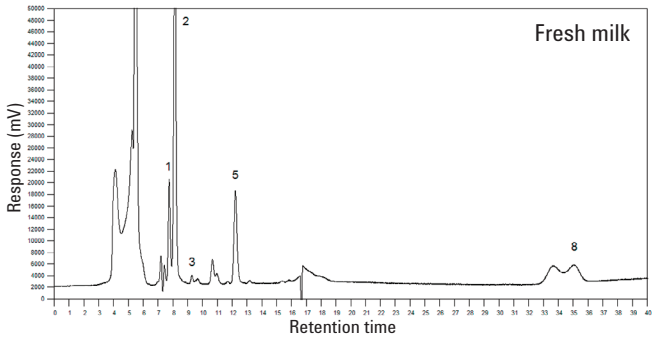
When analyzed, a different number of organic acids were present in each milk sample. The fresh milk sample contained only a few acids, while the sour milk contained each acid noted earlier, in addition to various unknown compounds. The initial solvent peaks for all chromatograms occur between 4 and 6 minutes and result from water, phosphates, and other unretained compounds. The negative peak occurring in all sample chromatograms at approximately 17 minutes is due to the acetonitrile denaturant/solvent.

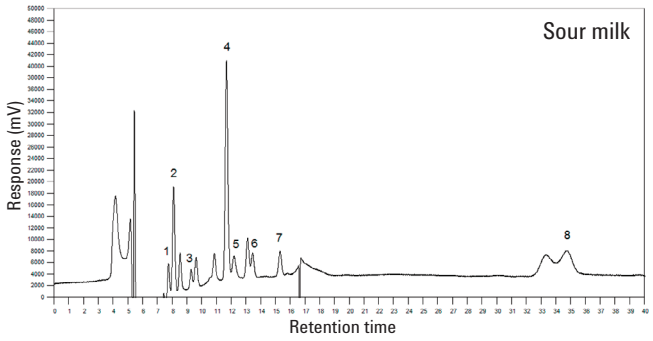
The main constituents of fresh milk include citric acid, orotic acid, uric acid, and hippuric acid, which would be expected as these are produced by the bovine metabolism. Cheese is manufactured by heating milk to a temperature that promotes the growth of lactic acid bacteria, which in turn leads to fermentation of lactose to lactic acid. As a result of this manufacturing process, the UV chromatograms for all three of the cheese samples show a distinct lactic acid peak. In addition, the cheese samples also give responses for pyruvic acid and propionic acid, which further proves that some form of bacterial action has taken place. It is worth noting, however, that blue cheese, despite containing a large amount of mold, contains the least amount of lactic acid.

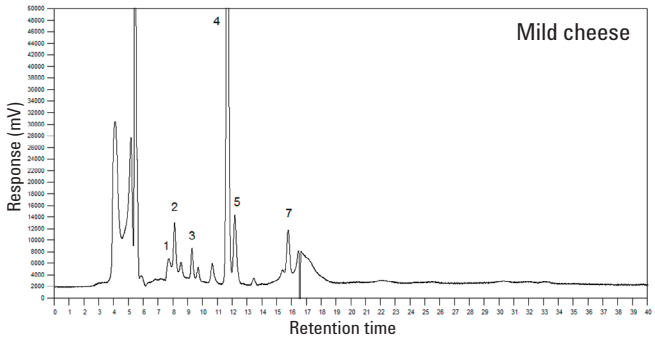
Yogurt is made in a similar way to cheese, as fermentation of the milk sugar (lactose) produces lactic acid, which acts on milk protein to give yogurt its texture and characteristic flavor. This is also reflected in the UV chromatograms for the two yogurt samples. Both samples contain live bacteria, which may account for the slightly higher levels of lactic and acetic acids.

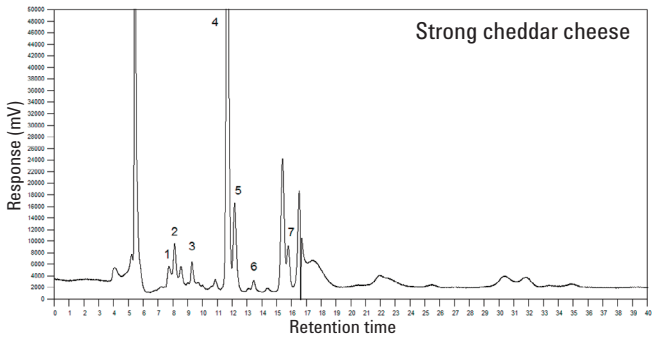
*Key*

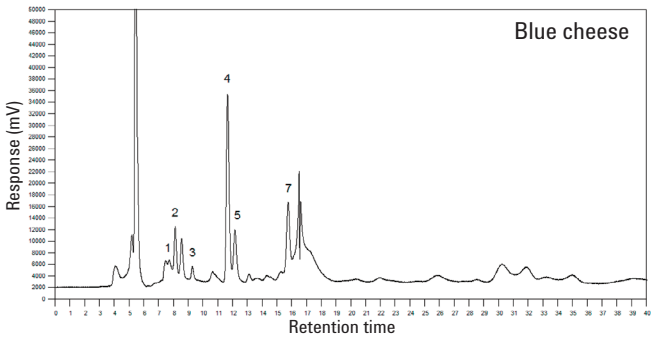
*1). Citric acid, 2). Crotic acid, 3). Pyruvic acid, 4). Lactic acid, 5). Uric acid + formic acid, 6). Acetic acid, 7). Propionic acid, 8). Hippuric acid*

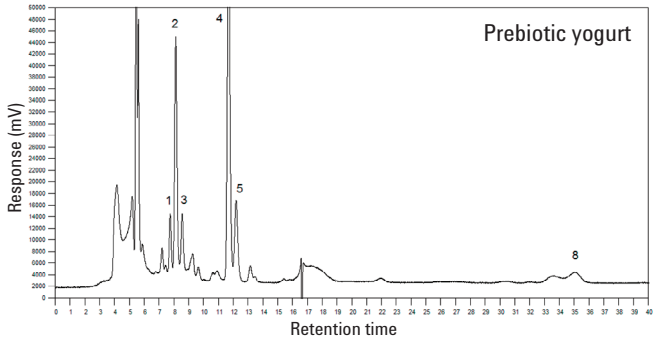


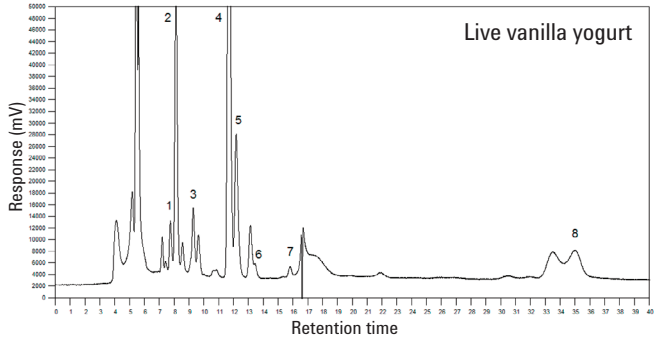



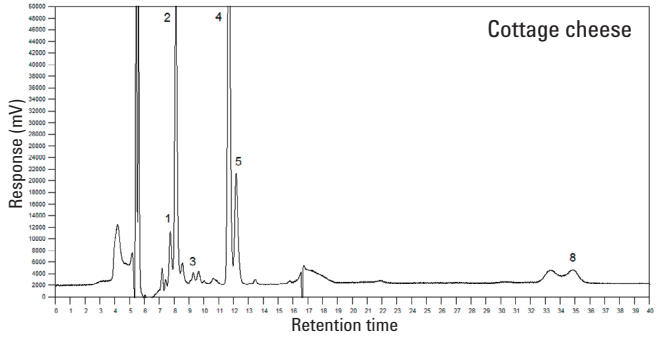






Cottage cheese is a mild white cheese made from the curds of soured skimmed milk and therefore contains a relatively high concentration of lactic acid. Fresh milk that has been allowed to go sour contains a large number of unknown compounds in addition to those expected from bacterial growth. These are likely to be some form of ammonia products that give this sample its distinctive smell.

## Conclusion

The Agilent Hi-Plex H column can be used to quantify the concentrations of a variety of organic acids in aqueous samples from dairy products. A potentially useful application of this HPLC procedure is to supply support data in microbiological studies by quantitating bacterial metabolites.

## Reference

1. "High Performance Liquid Chromatographic Determination of Organic Acids in Dairy Products." *Journal of Food Science*. Volume 46, issue 1, pages 52-57 (January 1981).

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# Organic Acids in Silage

## Application Note

Food and Environmental


## Author

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Germany


## Introduction


In addition to other factors, the concentration of the three fermentation acids lactic acid, acetic acid, and butyric acid is a criterion for the quality of silages. HPLC is the choice for this analysis, since volatile and nonvolatile acids can be determined together without prior derivatization. This application note shows the analysis of several specimens of silages (grass, whole plant, and corn) using an Agilent Hi-Plex H column.



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## Materials and Methods

The extraction of the acids is done according to EN 13037 (soil improvers and growing media determination of pH) by adding 1.25 L of water to 250 mL of silage and agitating for one hour. The sample was pretreated by filtration through a 0.45 µm membrane.

## Conditions

| | | |
|---|---|---|
| Column | Agilent Hi-Plex H, 7.7 x 300 mm, 8 µm (p/n PL1170-6830) | |
| Mobile phase | 0.005 M $H_2SO_4$ | |
| Gradient | Isocratic | |
| Flow rate | 0.7 mL/min | |
| Injection volume | 20 µL | |
| Sample concentration | Glucose | 50 – 1500 mg/L |
| | Succinic acid | 50 – 125 mg/L |
| | Lactic acid | 750 – 1000 mg/L |
| | Acetic acid | 200 – 450 mg/L |
| | Ethanol | 80 – 700 mg/L |
| Temperature | 60 °C | |
| Pressure | 4.6 MPa (46 bar, 670 psi) | |
| Detector | RI (55 °C) | |

## Results and Discussion

Figure 1 shows the analysis of grass silage, which has undergone a homofermentative process leading mostly to lactic acid and a small amount of ethanol. An example for a heterofermentative process is shown in Figure 2. Here the silage of corn yielded not only lactic acid, but also acetic acid and ethanol. Figure 3 shows the analysis of whole plant silage, which has undergone an untypical process, leaving a large amount of free sugars.

## Conclusion

Samples of silage from different crops were successfully separated by HPLC with an Agilent Hi-Plex H column.

Hi-Plex H is the column of choice for the analysis of organic acids in complex matrices, using dilute mineral acid as eluent. Hi-Plex columns are packed with sulfonated resin, giving a fundamental improvement in performance. They contain monodisperse sulfonated packing to overcome the problems of low efficiencies and high backpressures encountered with soft gels.

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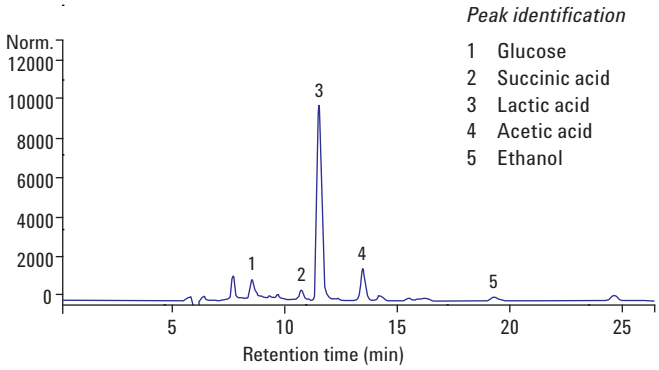


*Figure 1. Analysis of grass silage using an Agilent Hi-Plex H column.*

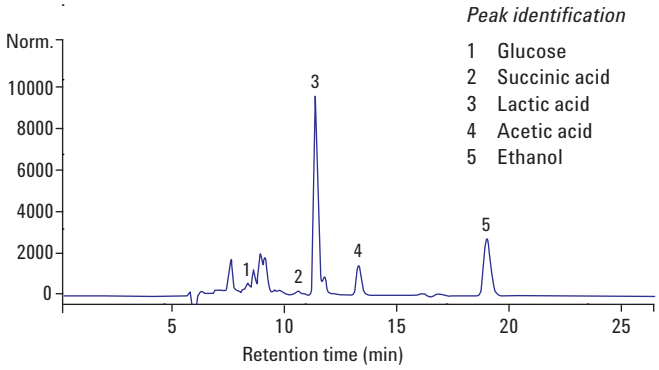


*Figure 2. Separation of corn silage using an Agilent Hi-Plex H column.*

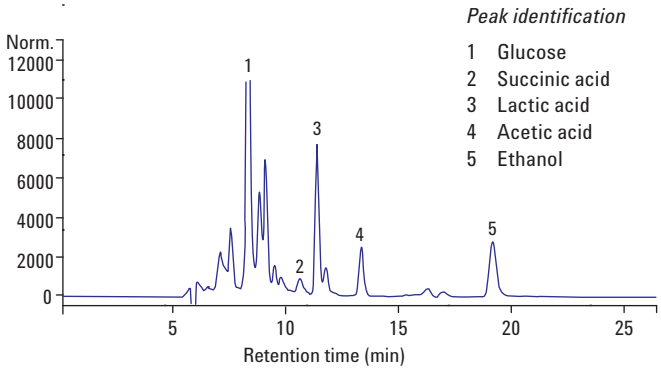


*Figure 3. Analysis of whole plant silage using an Agilent Hi-Plex H column.*

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Agilent Technologies

# Rapid Profiling of Saccharides Using HPLC with ELSD for Improved Precision

Application Note

Food


## Author

Stephen Bullock

Agilent Technologies, Inc.


## Introduction

Saccharides are of great importance in nature, and chemists and biochemists require sensitive and robust analytical methods for their identification and quantification.

These compounds do not possess a UV chromophore and are therefore not suited to UV detection. Normally, the nonchromophoric sugar separations would be performed using a refractive index (RI) detector. However, RI commonly suffers from baseline instability and poor sensitivity. Due to the nonvolatile nature of saccharides, evaporative light scattering detection (ELSD) using the Agilent ELSD, is better for this type of analysis, offering excellent baseline stability. There are a number of HPLC methods to quantify saccharides, with one of the most simple being the use of a calcium ligand-exchange column, Agilent Hi-Plex Ca, with water as the eluent.

The Hi-Plex Ca column contains a monodisperse sulphonated polystyrene incorporating 8% divinylbenzene with a calcium counter ion and provides a separation based on a combination of both size exclusion and ligand-exchange chromatography. These soft gel columns are operated at elevated temperature in order to reduce operating pressure and permit regular flow rates to be employed. Sensitivity in saccharide detection is achieved by using the Agilent ELSD. In addition to an increase in sensitivity, this detector also gives a more stable, drift-free baseline, improving the precision of the quantitation.


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## Experimental

### Instrumentation

| | |
|---|---|
| Column | Agilent Hi-Plex Ca, 7.7 × 300 mm, 8 µm (p/n PL1170-6810) |
| Detector | Agilent ELSD |

### Materials and Reagents

Mobile phase 100% DI $H_2O$

### Sample Preparation

Saccharides were dissolved in water at 1.0 mg/mL.

## Results and Discussion

Figure 1 shows that the five saccharide standards are well-resolved and the baseline is extremely stable.

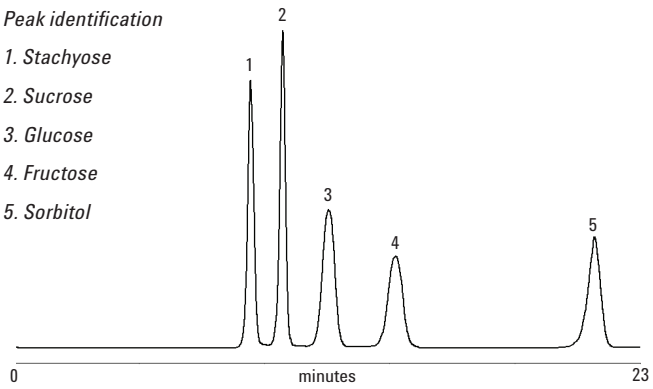


*Figure 1. Excellent separation to baseline of five saccharides by Agilent Hi-Plex Ca columns with the Agilent ELSD.*

## Conclusion

Combining the Agilent Hi-Plex Ca column with the Agilent ELSD provides an excellent solution for resolving saccharides. The sulfonated resin in Hi-Plex Ca offers a fundamental improvement in performance. Its monodisperse sulfonated packing overcomes problems of low efficiencies and high backpressures encountered with soft gels. The Agilent ELSD surpasses other ELSDs for low-temperature HPLC applications with semivolatile compounds. The Agilent ELSD's unique gas control permits evaporation of high boiling solvents at very low temperatures. For example, 100% water at a flow rate of 5 mL/min can be removed at 30 °C.

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SI-01220

Agilent Technologies

# Simple Analysis of Carbohydrates by HPLC Using Evaporative Light Scattering Detection

Application Note

Food


## Author

Stephen Bullock

Agilent Technologies, Inc.


## Introduction

The separation, identification, and quantification of simple sugars can be readily achieved using chromatography. High-performance liquid chromatography (HPLC) is perhaps the simplest technique, often requiring little in the way of sample preparation, particularly with liquids.

Sugars may be detected with the Agilent evaporative light scattering detector (ELSD) and an Agilent Hi-Plex column that has strong cation-exchange resins available in differing ionic forms. The sulfonated column resin gives a fundamental improvement in performance and overcomes the problems of low efficiencies and high backpressures encountered with soft gels. The separation mechanism is achieved initially by size exclusion, with larger oligosaccharides eluting before smaller monosaccharides, and then by ligand-exchange interaction of the numerous hydroxyl groups on the sugar molecules with the metal ion associated with the resin. Hi-Plex columns are used at elevated temperature with isocratic eluents.

As neutral carbohydrates have limited UV activity, the most commonly used detector with these columns is refractive index (RI). However, there are a number of issues related to the use of RI detectors, including baseline stability and sensitivity. A better method of detection is provided by evaporative light scattering detection. The Agilent ELSD does not require the solutes of interest to have any optical properties. The principle of operation is a three-stage process. The first stage involves the nebulization of the eluent; the second, the evaporation of the solvent to leave solute particles; and the third, the detection of the light scattered by the solid solute particles as they pass through the light beam. The only requirement for using the Agilent ELSD is that the eluent be more volatile than the solutes.

When using Agilent Hi-Plex columns for the analysis of carbohydrates, water (with no buffer or added salt) is used as the eluent, making this an ideal application for the Agilent ELSD because neutral carbohydrates have little UV activity.

Hi-Plex resins are available in 8% crosslinked calcium and lead forms for the analysis of mono- and disaccharides and in hydrogen (acid) forms for the analysis of sugar alcohols and organic acids. Also available is a 4% crosslinked sodium form for the separation of high molecular weight oligosaccharides, such as corn syrups, to Dp 9.

## Instrumentation

| | |
|---|---|
| Column | Agilent Hi-Plex Ca, 7.7 × 300 mm, 8 µm (p/n PL1170-6810) |
| Detector | Agilent ELSD |

## Materials and Reagents

Mobile phase 100% DI $H_2O$

## Results and Discussion

A separation of standard sugars – raffinose, lactose, glucose, galactose, and fructose – was obtained using the detection system (Figure 1). Calibration curves were produced for the six solutes in the test mixtures, as shown in Figure 2.

## Conclusion

The separation and detection of raffinose, lactose, glucose, galactose, and fructose are readily achieved using water as the mobile phase with an Agilent Hi-Plex Ca column and the Agilent ELSD. This system avoids the use, high cost, and disposal implications of toxic acetonitrile when separations are performed on amino silica columns. In addition, Hi-Plex stays active in the presence of sugar molecules. Together with fast dissolution, this benefit results in long lifetimes compared to amino silica columns.

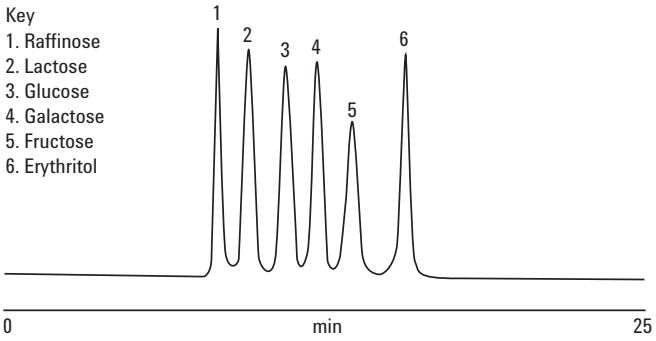


*Figure 1. Good separation of six simple sugars using the Agilent ELSD and an Agilent Hi-Plex Ca column.*

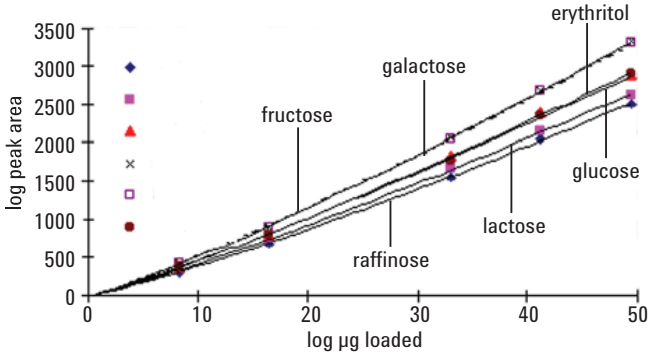


*Figure 2. Calibration curves of six sugars using the Agilent ELSD and an Agilent Hi-Plex Ca system.*

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Agilent Technologies

# Agilent Hi-Plex Columns for Sugar Separation: Effects of Temperature and Mobile Phase

Application Note

Food


## Author

Stephen Ball

Agilent Technologies, Inc.


## Introduction

The Agilent Hi-Plex H is a high-performance ligand-exchange chromatography column. The column is based on polystyrene/divinylbenzene with an 8% crosslinking and hydrogen counter ion. Typically used for the analysis of sugars, sugar alcohols, and organic acids, its monodisperse sulfonated packing gives improved column efficiency, lower column pressure, and assured batch-to-batch reproducibility.

This application note investigates the effect of temperature and mobile-phase acid concentration on the separation of sugars and organic acids in wine.

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## Materials and Reagents

### Sample Preparation

The seven compounds listed in Table 1 were weighed into the same vial in the quantities described and dissolved in 10 mL of 0.01 M $H_2SO_4$. Injection volume was 20 µL.

*Table 1. Compound Quantities*

| Constituent | Amount (g) |
|---|---|
| 1. Citric acid | 0.1010 |
| 2. Tartaric acid | 0.1032 |
| 3. Glucose | 0.1011 |
| 4. Malic acid | 0.1018 |
| 5. Fructose | 0.1011 |
| 6. Lactic acid | 0.1015 |
| 7. Glycerol | 0.1131 |

### Conditions

| | |
|---|---|
| Column | Agilent Hi-Plex H, 7.7 × 300 mm, 8 µm (p/n PL1170-6830) |
| Mobile phase | 0.01 M $H_2SO_4$ |
| Flow rate | 0.4 mL/min |
| Temperature | > 75 °C |
| Detector | RI |

## Results and Discussion

This mixture of sugars and organic acids is particularly difficult to analyze as several of the compounds elute very closely together and often simultaneously. However, these results show that as the temperature is increased, the minimum resolution of the separation gradually increases until all seven compounds are nearly separated.

At 35 °C, there are two pairs of co-eluted peaks, but by increasing the temperature to 55 °C, they have started to split into pairs of peaks. This increase in temperature, however, causes fructose and malic acid to become co-eluted. By increasing the temperature further to 75 °C, these two components begin to separate in the reverse order (Figure 1).

To gain complete separation of all seven compounds, this analysis would need to be run above 75 °C.

By varying the concentration of the sulfuric acid in the mobile phase, the selectivity of the column can be altered (Figure 2). At high concentrations, fructose and malic acid co-elute. Therefore, by reducing the mobile phase acid concentration, the minimum resolution of the separation can be improved. However, this reduction in mobile-phase acid strength does result in slightly fronted peak shapes for the organic acids. The best separation obtained consisted of a 0.003 M $H_2SO_4$, where malic acid elutes almost halfway between glucose and fructose.

By comparing these two sets of results, it can be seen that temperature is a more powerful tool in gaining complete separation of all seven compounds in this particular mixture, as resolution between them can be achieved while maintaining good peak shape.

*Peak identification*

1. Citric acid
2. Tartaric acid
3. Glucose
4. Malic acid
5. Fructose
6. Lactic acid
7. Glycerol

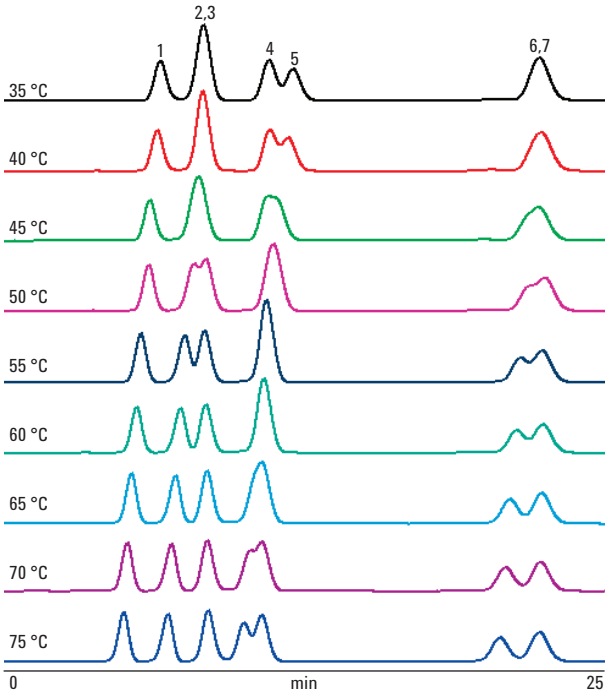


*Figure 1. Effect of temperature on the separation of sugars and organic acids on an Agilent Hi-Plex H column.*

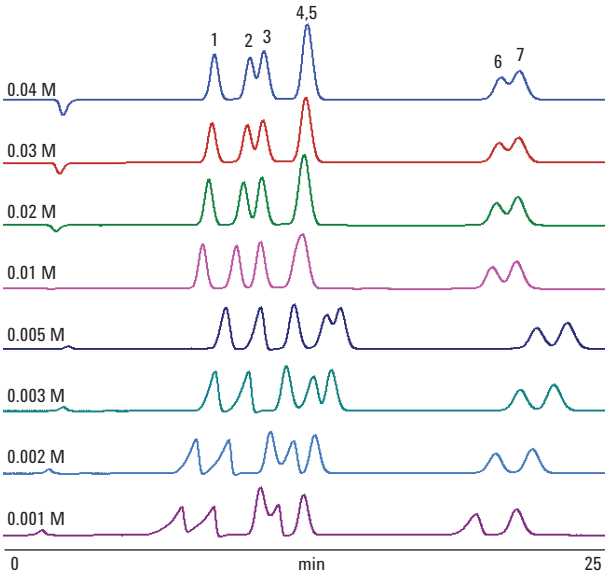


*Figure 2. Effect of acid concentration on the separation of sugars and organic acids on an Agilent Hi-Plex H column.*

## Conclusion

The analysis of wines demonstrates how Agilent Hi-Plex H columns provide optimum resolution of closely eluting compounds, enabling quantitation of each. These columns are ideal for the analysis of sugar alcohols and sugar molecules, using sulfuric acid as the mobile phase. The Hi-Plex H is also the column of choice for the analysis of organic acids, using dilute mineral acid as eluent. By using the columns at higher operating temperatures, closely eluting compounds can be resolved.

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Agilent Technologies

# Analysis of a Sugar, Organic Acid, and Ethanol Reference Sample

Application Note

Food


## Authors

Stephen Ball, Linda Lloyd

Agilent Technologies, Inc.


## Introduction

Food products commonly contain a mixture of sugars (of varying chain lengths), sugar alcohols, and organic acids. This application note shows how an Agilent Hi-Plex H ligand-exchange chromatography column and refractive index (RI) detector can be used to quantify levels of these components in a sample of food.

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## Conditions

| | |
|---|---|
| Column | Agilent Hi-Plex H, 7.7 × 300 mm, 8 µm (p/n PL1170-6830) |
| Sample | Sugars + acid mixture |
| Sample size | 10 mg/mL each |
| Mobile phase | 0.05 M $H_2SO_4$ |
| Flow rate | 0.5 mL/min |
| Injection volume | 20 µL |
| Temperature | Ambient |
| Detector | RI |

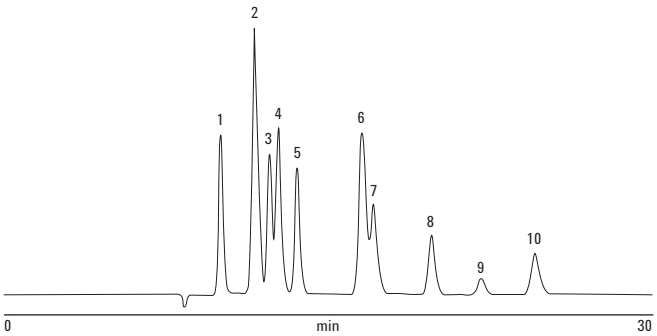


*Figure 1. Separation of a sugars and acids mixture using an Agilent Hi-Plex H 8 µm column. See Table 1 for peak identification.*

*Table 1. Peak Identification for Figure 1*

| Peak | Name | Time (min) | Height (µV) | Area (%) | Width 50% (min) | As. USP | 10% Asymmetry | Res. HW | Plate counts | Plates/m |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Sucrose | 10.04 | 193813.4 | 9.998 | 0.23 | 1.20 | 1.15 | 0.00 | 10870 | 36233 |
| 2 | Citric acid, glucose | 11.60 | 323837.5 | 21.448 | 0.30 | 1.24 | 1.18 | 3.48 | 8312 | 27708 |
| 3 | Tartaric acid | 12.28 | 170815.1 | 9.038 | 0.26 | 0.87 | 0.91 | 1.46 | 12812 | 42705 |
| 4 | Fructose | 12.70 | 203381.4 | 12.313 | 0.28 | 1.29 | 1.15 | 0.91 | 11430 | 38099 |
| 5 | Malic acid | 13.55 | 154736.4 | 9.548 | 0.27 | 1.14 | 1.12 | 1.85 | 14375 | 47918 |
| 6 | Lactic acid, glycerol | 16.53 | 196730.6 | 16.474 | 0.41 | 0.98 | 1.05 | 5.22 | 9104 | 30346 |
| 7 | Succinic acid | 17.07 | 110886.3 | 8.846 | 0.40 | 1.66 | 1.47 | 0.78 | 10298 | 34325 |
| 8 | Acetic acid | 19.72 | 73828.0 | 5.480 | 0.33 | 1.17 | 1.16 | 4.28 | 19217 | 64056 |
| 9 | Methanol | 22.00 | 19729.7 | 1.724 | 0.36 | 1.25 | 1.14 | 3.88 | 20778 | 69259 |
| 10 | Ethanol | 24.47 | 50557.3 | 5.131 | 0.42 | 1.25 | 1.20 | 3.75 | 19042 | 63475 |
| **Total** | | | **1498315.5** | **100.000** | | | | | | |

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Agilent Technologies

# Agilent Hi-Plex Columns for Superior Resolution of Organic Acids in Wine

Application Note

Food and Beverage


## Author

Stephen Ball

Agilent Technologies, Inc.


## Introduction

The Agilent Hi-Plex H is a high-performance ligand-exchange chromatography column based on polystyrene/divinylbenzene (PS/DVB) with an 8% crosslinking and hydrogen counter ion. Typically used for the analysis of sugars, sugar alcohols, and organic acids, its monodisperse sulfonated packing gives improved column efficiency, lower column pressure, and assured batch-to-batch reproducibility. The superior separation ability of Hi-Plex H is demonstrated by comparison with a column from another manufacturer, in the analysis and quantification of the organic acids in wine. The quantitative assessment of organic acids is important for the quality control of wine, because the classes and content of organic acids give a characteristic taste to the end product.

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## Materials and Reagents

### Sample Preparation

Acetic acid, lactic acid, succinic acid, malic acid, citric acid, and tartaric acid are the main organic acids in wine. A standard solution was made up containing these organic acids, along with glucose, fructose, and ethanol. The ten compounds were weighed into the same vial, as shown in Table 1, and dissolved in 10 mL of 0.009 M $H_2SO_4$. Injection volume was 20 µL.

*Table 1. Weight of Ten Compounds*

| Standard constituent | Amount (g) |
|---|---|
| 1. Citric acid | 0.0141 |
| 2. Tartaric acid | 0.0283 |
| 3. Glucose | 0.0120 |
| 4. Malic acid | 0.0352 |
| 5. Fructose | 0.0543 |
| 6. Succinic acid | 0.0201 |
| 7. Lactic acid | 0.0193 |
| 8. Glycerol | 0.0969 |
| 9. Acetic acid | 0.0450 |
| 10. Ethanol | 0.2127 |

Both the Shiraz wines analyzed in this study, one rosé and one red, were directly injected without sample pretreatment to allow full identification of all the major components in each one. Twenty µL of each were injected at a time.

### Conditions

| | |
|---|---|
| Columns | Agilent Hi-Plex H, 7.7 × 300 mm, 8 µm (p/n PL1170-6830)<br>Alternative crosslinked PS/DVB, 7.8 × 300 mm, 9 µm |
| Mobile phase | 0.004 M $H_2SO_4$ |
| Flow rate | 0.4 mL/min |
| Temperature | 75 °C |
| Detector | RI |

## Results and Discussion

The results of this investigation highlight differences in the selectivities of the Agilent Hi-Plex H column and the column from another manufacturer. As can be seen in both the reference standard (Figure 1) and the wine samples (Figures 2 and 3), the alternative column is less retentive than Hi-Plex H and is unable to separate malic acid and glucose to any great extent, whereas glucose, malic acid, and fructose are equally spaced (with maximum possible resolution) by a Hi-Plex H column.

Exact quantitation of the organic acids and sugars in these wine samples can be achieved by preparing four aqueous calibration standards (containing the ten compounds listed in Table 1), covering a broad concentration range. After injecting each of these into the RI, the resulting peak heights can be determined and plotted against concentration. The slopes of these lines are the response factors for each compound, and the linear correlation coefficients and y-intercepts provide a measure of analytical precision. From this information, the exact concentration of each compound in the wine samples can be determined. Based on the selectivity of these two columns, it is much easier to quantify the exact concentration of each compound when the sample is run on Hi-Plex H, as the co-elution problems of the alternative column make it very difficult to determine the peak heights of malic acid and glucose.

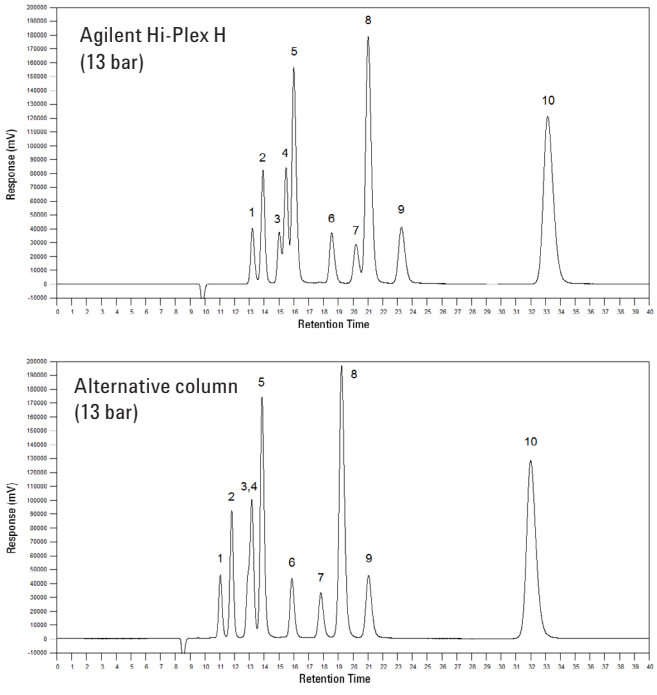


*Figure 1. Plots of standard solution on an Agilent Hi-Plex H column (above) and on an alternative column (below). Note the superior discrimination of the Hi-Plex H column, specifically with peaks 3 (glucose) and 4 (malic acid).*

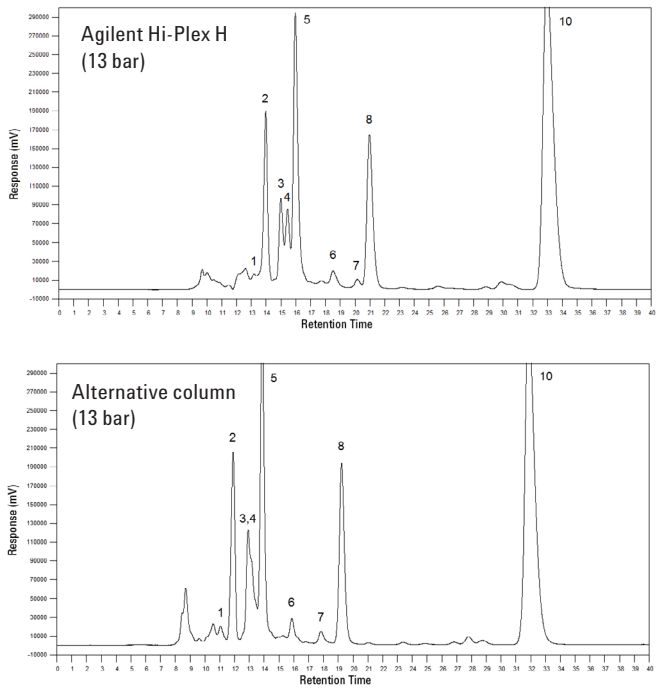


*Figure 2. A sample of Shiraz rosé wine on an Agilent Hi-Plex H column (above) and on an alternative column (below).*

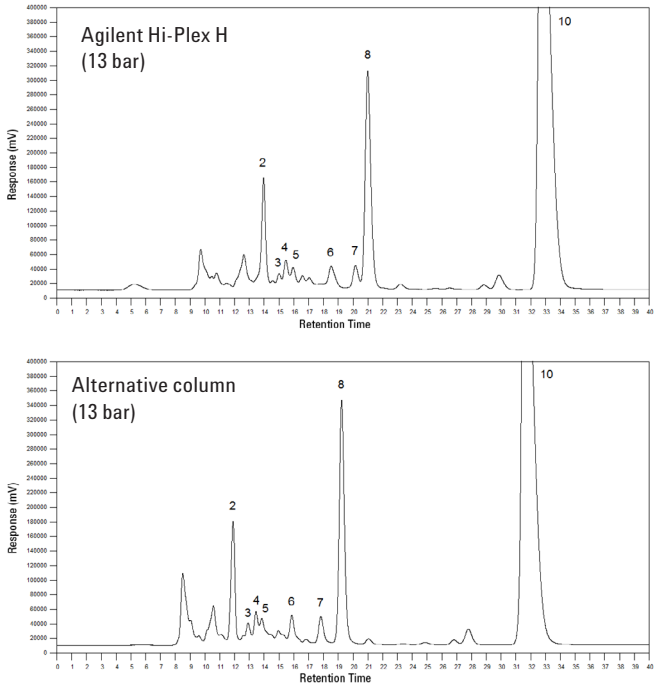


*Figure 3. A sample of Shiraz red wine on an Agilent Hi-Plex H column (above) and on an alternative column (below).*

## Conclusion

The analysis of wines demonstrates how Agilent Hi-Plex H columns provide optimum resolution of closely eluting compounds, enabling quantitation of each. These columns are ideal for the analysis of sugar alcohols and sugar molecules, using water as the mobile phase. Hi-Plex H is also the column of choice for the analysis of organic acids, using dilute mineral acid as eluent. By using the columns at higher operating temperatures, closely eluting compounds can be resolved.

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# Temperature Effects on Invert Sugar Analysis

## Application Note

Food and Pharmaceutical


## Authors

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## Introduction

Invert sugar is produced by the action of the glycoside hydrolase enzyme invertase or an acid on sucrose, which splits each sucrose disaccharide molecule into its component glucose and fructose monomer molecules, one of each. In practical terms, measured on equivalent dissolved weights, invert syrups are sweeter than sucrose solutions.

The separation of invert sugar on an Agilent Hi-Plex Ca column is affected by changes in operating temperature. According to the USP, this separation should be run at 40 °C. However, at this temperature, the peak shape of glucose is particularly poor.


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## Experimental

A standard solution containing the main components of invert sugar was made to contain 20 mg/mL of glucose and fructose and 10 mg/mL of sucrose. Ten µL of the solution were injected at temperatures ranging from 40 °C to 85 °C.

### Conditions

| | |
|---|---|
| Column | Agilent Hi-Plex Ca, 7.7 x 300 mm, 8 µm (p/n PL1170-6810) |
| Mobile phase | 100% DI $H_2O$ |
| Flow rate | 0.4 mL/min |
| Temperature | Various |
| Detector | RI |

## Results and Discussion

At 40 °C, the peak for glucose was split and maintained this shape all the way up to 60 °C. This could be a result of continuous interconversion of the glucose molecules between open-chain and cyclic form, in addition to $\alpha$- and ß-ring structures. At these temperatures, the rate of interconversion is slow enough to be detected on the RI. At higher temperatures ≥ 65 °C, where the speed of interconversion is much greater, a single glucose peak is eluted. For all of the sugars, peak width decreases and efficiency increases continuously to 85 °C. At 85 °C, the resolution between the sugar molecules would allow the flow rate to be increased to give a faster separation.

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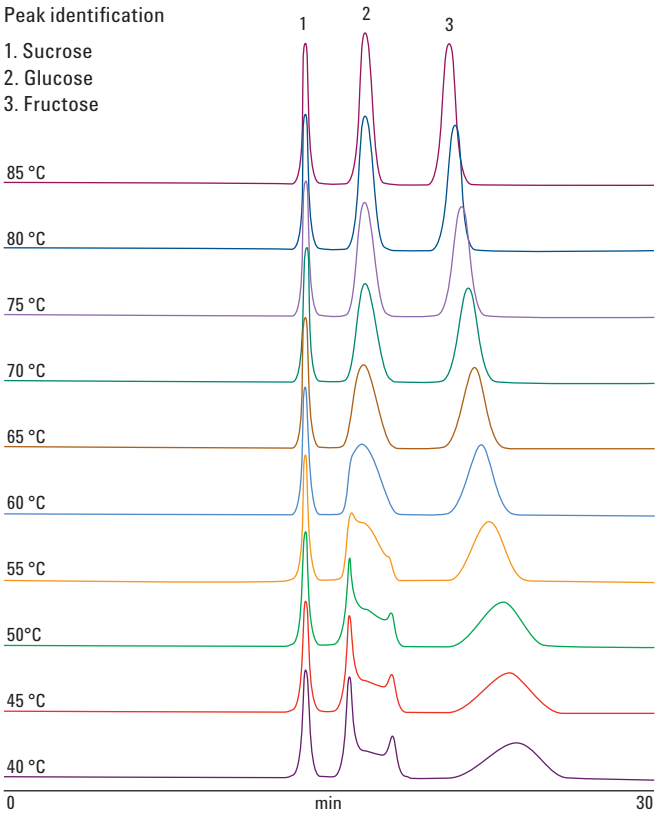


*Figure 1. Raw data chromatograms showing the main components of invert sugar on an Agilent Hi-Plex Ca column at different operating temperatures.*

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Agilent Technologies

# Analysis of Tequila Carbohydrates

Application Note

Food and Beverage


## Authors

Stephen Ball, Linda Lloyd

Agilent Technologies, Inc.


## Introduction

Tequila is a spirit made from the blue agave plant, which is known to contain raffinose, a trisaccharide commonly found in a variety of vegetables and whole grains. The Agilent Hi-Plex Ca ligand-exchange chromatography column is able to separate raffinose from the other sugars and ethanol that make up this alcoholic beverage.


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## Conditions

| | |
|---|---|
| Column | Agilent Hi-Plex Ca, 7.7 × 300 mm, 8 µm (p/n PL1170-6810) |
| Sample | Sugars in tequila |
| Sample size | 20 mg/mL |
| Mobile phase | 100% DI $H_2O$ |
| Flow rate | 0.6 mL/min |
| Injection volume | 20 µL |
| Temperature | 85 °C |
| Detector | RI |

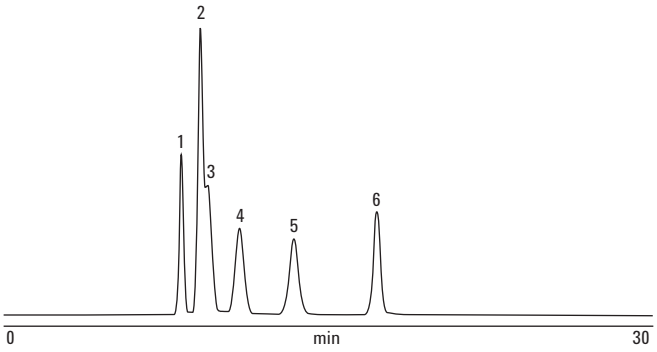


*Figure 1. Separation of sugars in tequila using an Agilent Hi-Plex Ca 8 µm column. See Table 1 for peak identification.*

*Table 1. Peak Identification for Figure 1*

| Peak | Name | Time (min) | Height (µV) | Area (%) | Width 50% (min) | As. USP | 10% Asymmetry | Res. HW | Plate counts | Plates/m |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Raffinose | 8.27 | 338184.7 | 12.365 | 0.21 | 1.01 | 1.01 | 0.00 | 8972 | 29906 |
| 2 | Sucrose, maltose | 9.13 | 608399.4 | 29.454 | 0.29 | 0.98 | 1.06 | 2.08 | 5657 | 18858 |
| 3 | Lactose | 9.45 | 273004.4 | 14.285 | 0.30 | 3.67 | 3.19 | 0.65 | 5607 | 18690 |
| 4 | Glucose | 10.92 | 184788.9 | 14.740 | 0.44 | 1.04 | 1.04 | 2.35 | 3401 | 11337 |
| 5 | Fructose | 13.43 | 161682.3 | 14.199 | 0.49 | 1.01 | 1.00 | 3.19 | 4217 | 14055 |
| 6 | Mannitol | 17.24 | 219822.6 | 14.958 | 0.35 | 0.96 | 0.96 | 5.34 | 13125 | 43750 |
| **Total** | | | **1785882.3** | **100.000** | | | | | | |

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# Analysis of Typical Components of Alcoholic Beverages

Application Note

Food and Beverage


## Authors

Stephen Ball, Linda Lloyd

Agilent Technologies, Inc.


## Introduction

Alcoholic beverages are produced by the fermentation of sugar- or starch-containing plant material. Therefore, when analyzed by HPLC, they are found to contain a mixture of mono-, di-, and trisaccharides, as well as products of the fermentation process, such as lactic acid and ethanol. The Agilent Hi-Plex H ligand-exchange chromatography column is able to separate these individual components, allowing quantification and isolation, if required.


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## Conditions

| | |
|---|---|
| Column | Agilent Hi-Plex H, 7.7 x 300 mm, 8 µm (p/n PL1170-6830) |
| Sample | Typical compounds in alcoholic beverages |
| Mobile phase | 100% 0.01 M $H_2SO_4$ |
| Flow rate | 0.4 mL/min |
| Injection volume | 20 µL |
| Temperature | 65 °C |
| Detector | RI |

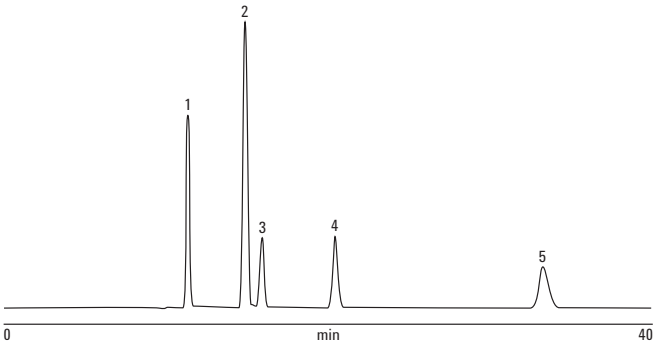


*Figure 1. Separation of typical compounds in alcoholic beverages using an Agilent Hi-Plex H 8 µm column. See Table 1 for peak identification.*

*Table 1. Peak Identification for Figure 1*

| Peak | Name | Time (min) | Height (µV) | Area (%) | Width 50% (min) | As. USP | 10% Asymmetry | Res. HW | Plate counts | Plates/m |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | Maltotriose | 11.37 | 644805.0 | 21.490 | 0.20 | 1.09 | 1.09 | 0.00 | 18156 | 60522 |
| 2 | Glucose | 14.86 | 956301.9 | 38.959 | 0.25 | 1.07 | 1.08 | 9.20 | 19732 | 65775 |
| 3 | Fructose | 15.91 | 237715.5 | 10.981 | 0.26 | 1.04 | 1.04 | 2.41 | 20430 | 68101 |
| 4 | Lactic acid | 20.42 | 243839.5 | 13.985 | 0.34 | 1.11 | 1.09 | 8.92 | 20545 | 68483 |
| 5 | Ethanol | 33.19 | 138850.4 | 14.585 | 0.62 | 1.20 | 1.18 | 15.74 | 15779 | 52595 |
| **Total** | | | **2221512.3** | **100.000** | | | | | | |

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Agilent Technologies

# USP Analysis of Sugar Alcohols on an Agilent Hi-Plex Ca Column – Mobile Phase Effects

Application Note

Pharmaceutical


## Authors

Stephen Ball, Linda Lloyd

Agilent Technologies, Inc.


## Introduction

Sugar alcohols, or polyols, are hydrogenated carbohydrates commonly used to replace sucrose in foods. They are often used with high-intensity artificial sweeteners to counter their low sweetness.

The separation of seven sugar alcohols on an Agilent Hi-Plex Ca column can be altered by introducing acetonitrile into the mobile phase.

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## Experimental

### Conditions

| | |
|---|---|
| Column | Agilent Hi-Plex Ca USP L19, 4.0 × 250 mm, 8 µm (p/n PL1570-5810) |
| Mobile phase | 100% DI $H_2O$ (initially) |
| Flow rate | 0.15 mL/min |
| Injection volume | 10 µL |
| Temperature | 90 °C |
| Detector | RI |

### Sample Preparation

The seven sugar alcohols — iso-erythritol, adonitol, arabitol, mannitol, xylitol, dulcitol, and sorbitol — are made up to a concentration of 10 mg/mL in water. See Figure 1.

When pure water is used for the mobile phase, several of the sugar alcohols in the sample either partially or completely co-elute. Modifying operating temperature or flow rate is very unlikely to give a good separation between these compounds.

Introducing acetonitrile into the mobile phase has a significant effect on the selectivity of the Agilent Hi-Plex Ca material and results in a doubling of the retention time. As a result, the mobile phase and flow rate conditions need to be modified as follows:

### Conditions

| | |
|---|---|
| Mobile phase | 30:70 acetonitrile:100% DI $H_2O$ |
| Flow rate | 0.30 mL/min |
| Temperature | 90 °C |

The same quantity of test solution is injected. See Figure 2.

## Conclusion

As can be seen by comparing the two chromatograms, using 30% acetonitrile gives extra retention for the sugar alcohols and, as a result, increases the resolution between them. All seven sugar alcohols are now either partially or completely separated. It also gives a change in the elution order.

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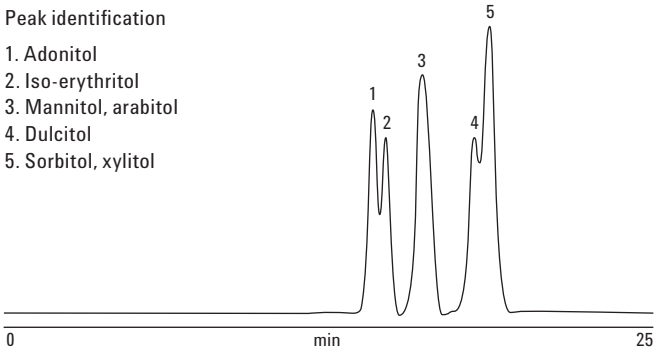


*Figure 1. Raw data chromatogram of seven sugar alcohols on an Agilent Hi-Plex Ca USP L19 column.*

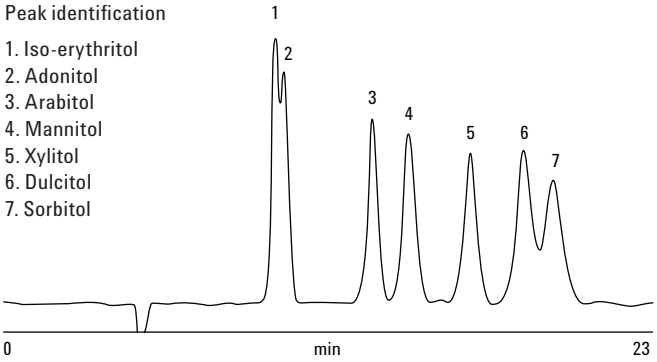


*Figure 2. Raw data chromatogram of seven sugar alcohols on an Agilent Hi-Plex Ca USP L19 column after the introduction of acetonitrile into the mobile phase.*

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Agilent Technologies

# Agilent Hi-Plex Columns for Carbohydrates, Alcohols, and Acids

## Application Note

Food and Pharmaceutical


## Authors

Stephen Ball, Linda Lloyd

Agilent Technologies, Inc.


## Introduction

Agilent Hi-Plex columns are ion-exchange ligand-exchange columns used predominantly for the separation of carbohydrates and organic acids. These columns use the preferred separation mechanism for the analysis of simple sugars, alcohols, oligosaccharides, and organic acids in foodstuffs, but can also be used for the separation of other compounds.

The range comprises a 4% crosslinked resin for the analysis of oligosaccharides and an 8% crosslinked resin, with lower exclusion limit, for mono-, di-, and trisaccharide analysis. For carbohydrate and alcohol investigations, Hi-Plex columns use isocratic conditions with water as the eluent and temperature as the main variable for control of resolution. The exception is Agilent Hi-Plex Na (Octo), which is used with sodium hydroxide eluents when pulsed amperometric detection is employed.

In these examples, we use Agilent Hi-Plex H, Agilent Hi-Plex Ca, as well as Agilent Hi-Plex Ca (Duo) for the analysis of organic acids, sugars, and sugar alcohols.


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## Organic Acids on Hi-Plex H

Where samples contain organic acids, whether or not in the presence of neutral mono- and disaccharides, Agilent Hi-Plex H is the preferred option. The column is run using water as the eluent for the analysis of sugars and organic acids or, more commonly, dilute acid for separation of organic acids. Refractive index (Figure 1 and Table 1, Figure 2 and Table 2) or UV detection (Figure 3 and Table 3) is used.

### Conditions

| | |
|---|---|
| Sample | Sugars and organic acids |
| Column | Agilent Hi-Plex H, 7.7 × 300 mm, 8 µm (p/n PL1170-6830) |
| Mobile phase | 100% 0.0085 M $H_2SO_4$ |
| Flow rate | 0.4 mL/min |
| Injection volume | 20 µL |
| Temperature | 65 °C |
| Detector | RI |

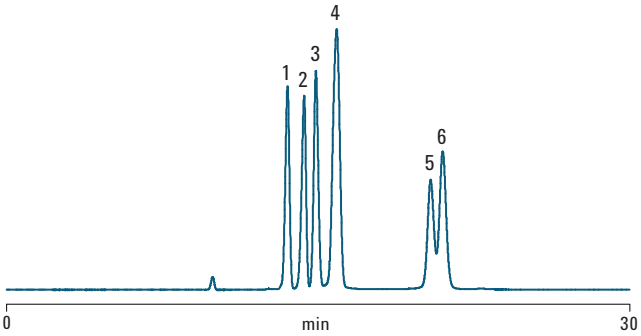


*Figure 1. Separation of monosaccharides, organic acids, and glycerol on an Agilent Hi-Plex H column with RI detection.*

### Conditions

| | |
|---|---|
| Sample | Organic acids |
| Column | Agilent Hi-Plex H, 7.7 × 300 mm, 8 µm (p/n PL1170-6830) |
| Mobile phase | 0.1 M $H_2SO_4$ |
| Flow rate | 0.6 mL/min |
| Temperature | 50 °C |
| Detector | RI |

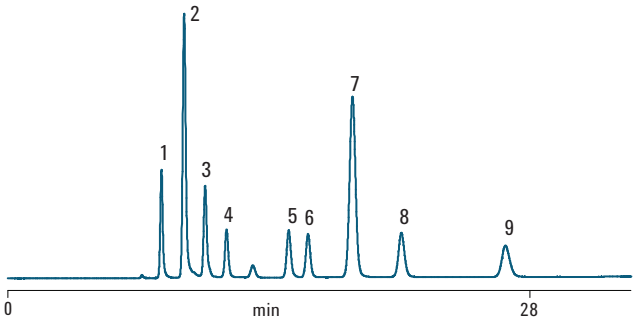


*Figure 2. Separation of nine organic acids on an Agilent Hi-Plex H column with RI detection.*

*Table 1. Peak Data from the Analysis of Monosaccharides, Organic Acids, and Glycerol on an Agilent Hi-Plex H Column Using RI Detection*

| Peak number | Analyte | As. USP | 10% Asymmetry | Plate count | Plates/m |
|---|---|---|---|---|---|
| 1 | Citric acid | 0.92 | 0.92 | 21207 | 70691 |
| 2 | Tartaric acid | 0.84 | 0.85 | 21475 | 71583 |
| 3 | Glucose | 1.04 | 1.04 | 21805 | 72684 |
| 4 | Malic acid, fructose | 0.93 | 0.93 | 10012 | 33372 |
| 5 | Lactic acid | 0.89 | 0.96 | 19685 | 65618 |
| 6 | Glycerol | 1.16 | 1.06 | 19070 | 63566 |

*Table 2. Peak Data from the Separation of Organic Acids on an Agilent Hi-Plex H Column Using RI Detection*

| Peak number | Analyte | As. USP | 10% Asymmetry | Plate count | Plates/m |
|---|---|---|---|---|---|
| 1 | Oxalic acid | 1.23 | 1.12 | 19471 | 64904 |
| 2 | *cis*-Aconitic acid | 1.29 | 1.15 | 16122 | 53741 |
| 3 | Tartaric acid | 1.30 | 1.24 | 19272 | 64240 |
| 4 | Malic acid | 1.10 | 1.07 | 20153 | 67176 |
| 5 | Lactic acid | 1.16 | 1.10 | 21469 | 71563 |
| 6 | Formic acid | 1.08 | 1.05 | 22118 | 73726 |
| 7 | Fumaric acid | 1.05 | 1.03 | 15751 | 52504 |
| 8 | Propionic acid | 1.12 | 1.09 | 20492 | 68305 |
| 9 | Butyric acid | 1.15 | 1.13 | 18181 | 60603 |

## Conditions

| | |
|---|---|
| Sample | Organic acids |
| Column | Agilent Hi-Plex H, 7.7 × 300 mm, 8 µm (p/n PL1170-6830) |
| Mobile phase | 100% 0.01 M $H_2SO_4$ |
| Flow rate | 0.6 mL/min |
| Injection volume | 20 µL |
| Temperature | 50 °C |
| Detector | UV, 210 nm |

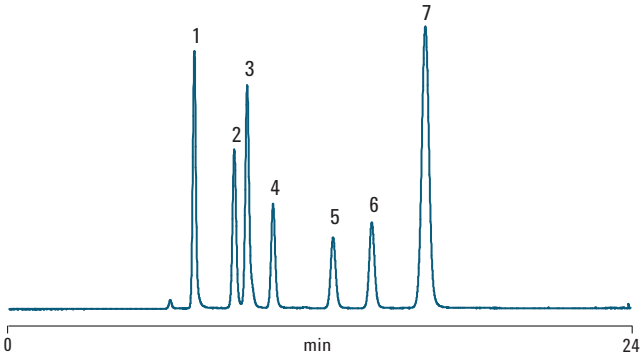


*Figure 3. Separation of seven organic acids on an Agilent Hi-Plex H column with UV detection.*

*Table 3. Peak Data from the Separation of Organic Acids on an Agilent Hi-Plex H Column Using UV Detection*

| Peak number | Analyte | As. USP | 10% Asymmetry | Plate count | Plates/m |
|---|---|---|---|---|---|
| 1 | Oxalic acid | 1.13 | 1.02 | 17164 | 57212 |
| 2 | Citric acid | 1.11 | 1.07 | 17588 | 58626 |
| 3 | Tartaric acid | 1.30 | 1.23 | 19251 | 64170 |
| 4 | Malic acid | 1.11 | 1.07 | 20170 | 67233 |
| 5 | Succinic acid | 1.08 | 1.06 | 19705 | 65684 |
| 6 | Formic acid | 1.07 | 1.05 | 21991 | 73302 |
| 7 | Fumaric acid | 1.05 | 1.03 | 15139 | 50464 |

## Sugars and Sugar Alcohols on Hi-Plex Ca

Agilent Hi-Plex Ca is recommended for the analysis of samples containing the sweetening sugars (glucose, fructose, and sucrose) and the sugar alcohols (mannitol and sorbitol) (Figure 4). The 4.0 × 250 mm column is referenced in the USP method that specifies L19 media for sugar alcohols analysis.

## Conditions

| | |
|---|---|
| Sample | Sugars and sugar alcohols |
| Column | Agilent Hi-Plex Ca, 7.7 × 300 mm, 8 µm (p/n PL1170-6810) |
| Sample size | 10 mg/mL |
| Mobile phase | 100% DI $H_2O$ |
| Flow rate | 0.6 mL/min |
| Injection volume | 10 µL |
| Temperature | 85 °C |
| Detector | RI |

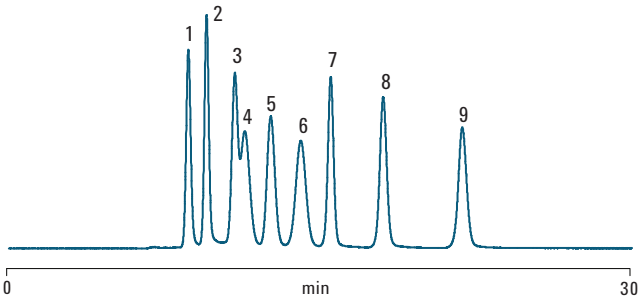


*Figure 4. Separation of a mixture of sugars and sugar alcohols on an Agilent Hi-Plex Ca column.*

*Table 4. Peak Data from the Separation of a Sugars and Sugar Alcohols Mix on an Agilent Hi-Plex Ca Column*

| Peak number | Analyte | As. USP | 10% Asymmetry | Plate count | Plates/m |
|---|---|---|---|---|---|
| 1 | Raffinose | 1.12 | 1.08 | 7138 | 23793 |
| 2 | Sucrose | 1.12 | 1.06 | 9389 | 31298 |
| 3 | Lactulose | 0.85 | 0.92 | 3858 | 12861 |
| 4 | Glucose | 1.79 | 1.59 | 2986 | 9955 |
| 5 | Galactose | 1.07 | 1.07 | 5008 | 16694 |
| 6 | Fructose | 1.01 | 1.01 | 3727 | 12423 |
| 7 | Ribitol | 1.00 | 1.00 | 14758 | 49194 |
| 8 | Mannitol | 1.04 | 1.04 | 13861 | 46204 |
| 9 | Sorbitol | 1.04 | 1.04 | 14170 | 47234 |

## Monosaccharide and Oligosaccharide Mixture on Hi-Plex Ca (Duo)

Agilent Hi-Plex Ca (Duo) is an 8% crosslinked material and therefore has a smaller pore size and less resolution for the larger oligomers. However, the Ca counter ion has improved ligand-exchange capability for monosaccharides, and so it is most suited for the analysis of samples containing both mono- and oligosaccharides (Figure 5).

## Conditions

| | |
|---|---|
| Sample | Sugars and sugar alcohols |
| Column | Agilent Hi-Plex Ca (Duo), 6.5 × 300 mm, 8 µm (p/n PL1F70-6850) |
| Sample size | 10 mg/mL |
| Mobile phase | 100% DI $H_2O$ |
| Flow rate | 0.4 mL/min |
| Injection volume | 10 µL |
| Temperature | 85 °C |
| Detector | RI |

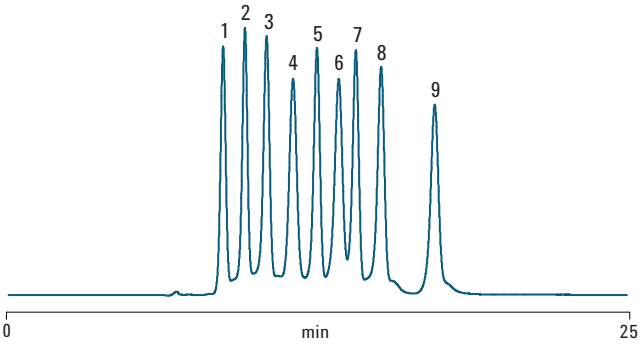


*Figure 5. Separation of a mixture of mono- and oligosaccharides on an Agilent Hi-Plex Ca (Duo) column.*

*Table 5. Peak Data from the Separation of a Sugars and Sugar Alcohols Mix on an Agilent Hi-Plex Ca (Duo) Column*

| Peak number | Analyte | As. USP | 10% Asymmetry | Plate count | Plates/m |
|---|---|---|---|---|---|
| 1 | Raffinose | 1.13 | 1.00 | 7827 | 26091 |
| 2 | Sucrose | 0.80 | 0.96 | 9363 | 31211 |
| 3 | Lactulose | 1.03 | 0.87 | 7895 | 26316 |
| 4 | Glucose | 0.99 | 0.97 | 6204 | 20680 |
| 5 | Galactose | 0.85 | 0.90 | 10869 | 36229 |
| 6 | Fructose | 0.82 | 0.88 | 7765 | 25884 |
| 7 | Ribitol | 1.02 | 0.87 | 13784 | 45948 |
| 8 | Mannitol | 0.95 | 0.87 | 13431 | 44771 |
| 9 | Sorbitol | 1.15 | 0.95 | 13807 | 46025 |

## Conclusion

Agilent Hi-Plex columns deliver improved efficiency, lower operating pressures, and longer column lifetimes from monodispersed materials. With a range of ligand counter ions for optimum selectivity and with resolution and materials matched to the USP definitions of media types L17, L19, L34, and L58, the Hi-Plex range is ideal for isocratic separations using water or dilute acid as the eluent. This simplifies system requirements for HPLC and eliminates the use of potentially hazardous organic solvents.

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Agilent Technologies

# HPLC Analysis of Sugars and Glycoproteins in Biological Systems

Application Note

Bioanalysis


## Author

Linda Lloyd

Agilent Technologies, Inc.


## Introduction

Free sugars, other than glucose and fructose, rarely occur in nature. However, other sugars such as the pentoses (arabinose, xylose, and ribose) and the hexoses (mannose and galactose) are found as integral parts of biological macromolecules. The function of the carbohydrate components of these macromolecules is diverse; they can be important either biochemically, for example targeting sequences in glycoproteins, or structurally, as in DNA and RNA. The sequences of monosaccharides are only obtained as breakdown products during fermentation.

Levels of lactate and pyruvate found in blood serum are important indicators of health. Elevated blood lactate levels are indicative of diabetes, and pyruvate of possible heavy metal poisoning. It is important to reduce sample handling to a minimum during analysis of complex biological fluids. Because samples can often be analyzed directly using Agilent Hi-Plex H columns, the potential for errors is reduced. Hi-Plex columns are well suited to the fast analysis of sugars and glycoproteins in plant and animal tissues.


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## Experimental

### Instrumentation

| | |
|---|---|
| Column | Agilent Hi-Plex H, 7.7 × 300 mm, 8 µm (p/n PL1170-6830) |
| Detector | RI |

### Materials and Reagents

| | |
|---|---|
| Mobile phase | 0.005 M $H_2SO_4$ (sugars), 0.0005 M $H_2SO_4$ (compounds of physiological significance) |

### Conditions

| | |
|---|---|
| Flow rate | 0.6 mL/min |
| Temperatures | 60 °C (sugars), 55 °C (compounds of physiological significance) |

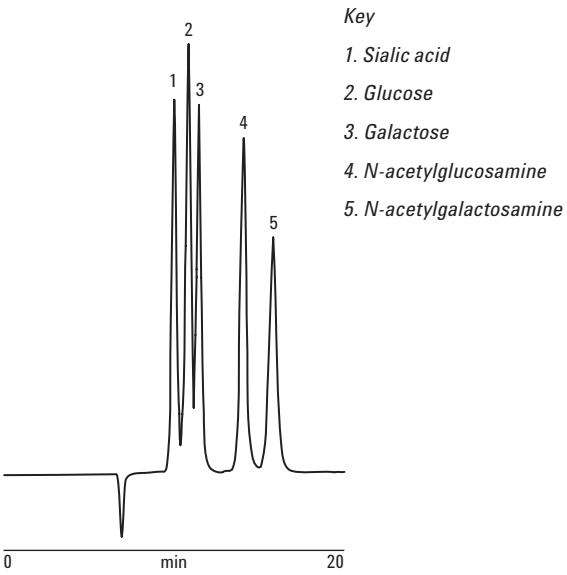


*Figure 1. Good separation of glucose and galactose from their derivatives achieved by HPLC with Agilent Hi-Plex H columns.*

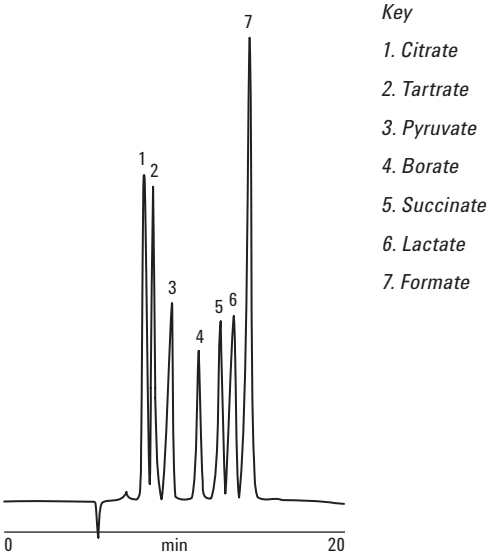


*Figure 2. The presence of lactate and pyruvate revealed by HPLC with Agilent Hi-Plex H columns.*

## Results and Discussion

Figure 1 shows how Agilent Hi-Plex H columns separate glucose- and galactose-free sugars from their derivatives N-acetylglucosamine and N-acetylgalactosamine. The latter are found in the cell membranes of higher organisms as carbohydrate residues, linking the amino acid chain to the carbohydrate component of membrane glycoproteins. Figure 2 shows good resolution of lactate and pyruvate from other salts found in animal tissues.

## Conclusion

Agilent Hi-Plex columns are packed with sulfonated resin, giving a fundamental improvement in performance to overcome the problems of low efficiencies and high back-pressures encountered with soft gels. The columns are available in hydrogen form for fast analysis of glycoproteins and sugars in biological systems. Accurate determination of composition and content is ensured.

This Agilent product is not cleared or approved by the United States FDA or other international regulatory agencies for use in clinical diagnostic procedures. No use of this product as a diagnostic medical device is implied or should be inferred.

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Agilent Technologies

# HPLC of Aloe Juice Using Evaporative Light Scattering Detection

## Application Note

Food and Pharmaceutical


## Author

Stephen Bullock

Agilent Technologies, Inc.


## Introduction

Aloe vera (syn. *Aloe barbadensis* Mill.) has been used as a medicinal plant for some 5,000 years. However, it is only in the last 20 to 30 years that information about this "miracle" plant and its possible healing power has reached a wider public. Aloe vera has been suggested as an ideal moisturizer and healing aid when applied topically on burns, sunburn, and various skin conditions. Many people also drink the juice in expectation of a balanced, healthy lifestyle or as an alternative to non-natural health supplements. In addition, the juice is consumed to aid the healing of specific illnesses or conditions.

Aloe vera juice contains over 200 active ingredients, of which the most prominent are vitamins, amino acids, minerals, phytonutrients, enzymes, and sugars (monosaccharides, disaccharides, and polysaccharides). Studies in the field of glycomics suggest that the monosaccharide content in aloe juice contributes significantly towards its anti-inflammatory activity. Normally, nonchromophoric sugar separations are performed using a refractive index (RI) detector, but RI commonly suffers from the problems of baseline instability and poor sensitivity. The Agilent 385-ELSD (evaporative light scattering detector) is a superior choice for this type of analysis.

Agilent Hi-Plex Ca columns contain a monodisperse sulfonated polystyrene incorporating 8% divinylbenzene with a calcium counter ion, and provide a separation based on a combination of both size exclusion and ligand-exchange chromatography.

To highlight the excellent resolving power of an Agilent 385-ELSD and Hi-Plex Ca system, aloe juice was analyzed, together with glucose and fructose (monosaccharides) and trehalose (disaccharide) standards. These sugars are commonly present in aloe juice.

Agilent Technologies

## Experimental

### Instrumentation

| | |
|---|---|
| Column | Agilent Hi-Plex Ca, 7.7 x 300 mm, 8 µm (p/n PL1170-6810) |
| Detector | Agilent 385-ELSD (neb = 50 °C, evap = 90 °C, gas = 1.6 SLM) |

### Materials and Reagents

| | |
|---|---|
| Mobile phase | 100% DI $H_2O$ |

### Sample Preparation

Glucose, fructose, and trehalose were dissolved in water to 1 mg/mL. Aloe juice was used as received.

### Conditions

Soft gel columns should be operated at elevated temperature to reduce operating pressure and permit the use of regular flow rates.

| | |
|---|---|
| Flow rate | 0.6 mL/min |
| Injection volume | 20 µL |
| Temperature | 80 °C |

## Results and Discussion

Figure 1 illustrates how well the three saccharide standards are resolved. Comparison of the chromatograms of the standards (lower trace) with that of the aloe juice sample (upper trace) confirms that all three saccharides are present in different quantities.

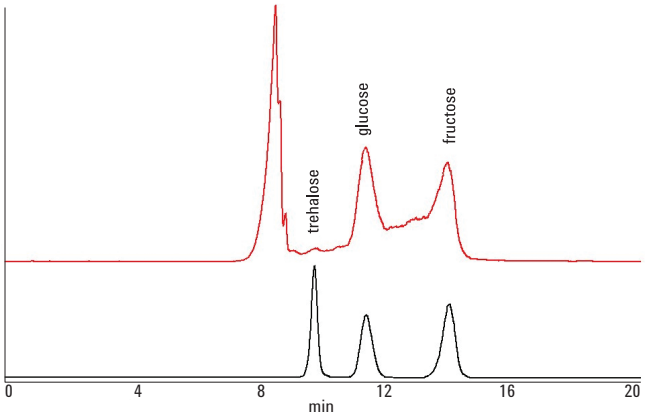


*Figure 1. Extremely stable baseline achieved by Agilent Hi-Plex Ca and Agilent 385-ELSD in profiling sugars in aloe vera juice.*

## Conclusion

An HPLC system comprising the Agilent 385-ELSD and Agilent Hi-Plex Ca column produced good separations and a very stable baseline in the analysis of aloe juice.

Hi-Plex columns are packed with sulfonated resin, giving a fundamental improvement in performance to overcome the problems of low efficiencies and high backpressures encountered with soft gels. The columns are available in calcium form for the analysis of carbohydrates in juices, to meet the growing demand for more detailed product information for labeling and control purposes.

The Agilent 385-ELSD surpasses other ELSDs for low-temperature HPLC applications with semivolatile compounds. Its innovative design represents the next generation of ELSD technology, providing optimum performance across a diverse range of HPLC applications. The unique gas control of the detector permits evaporation of high boiling solvents at very low temperatures. The instrument's novel design provides superior performance for the analysis of semivolatile compounds. Accurate determination of composition and content is assured using the Agilent 385-ELSD and Hi-Plex Ca columns.

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Agilent Technologies

# Temperature Effects on Invert Sugar Analysis

## Application Note

Food and Pharmaceutical


## Authors

Stephen Ball, Linda Lloyd

Agilent Technologies, Inc.


## Introduction

Invert sugar is produced by the action of the glycoside hydrolase enzyme invertase or an acid on sucrose, which splits each sucrose disaccharide molecule into its component glucose and fructose monomer molecules, one of each. In practical terms, measured on equivalent dissolved weights, invert syrups are sweeter than sucrose solutions.

The separation of invert sugar on an Agilent Hi-Plex Ca column is affected by changes in operating temperature. According to the USP, this separation should be run at 40 °C. However, at this temperature, the peak shape of glucose is particularly poor.


Agilent Technologies

## Experimental

A standard solution containing the main components of invert sugar was made to contain 20 mg/mL of glucose and fructose and 10 mg/mL of sucrose. Ten µL of the solution were injected at temperatures ranging from 40 °C to 85 °C.

### Conditions

| | |
|---|---|
| Column | Agilent Hi-Plex Ca, 7.7 x 300 mm, 8 µm (p/n PL1170-6810) |
| Mobile phase | 100% DI $H_2O$ |
| Flow rate | 0.4 mL/min |
| Temperature | Various |
| Detector | RI |

## Results and Discussion

At 40 °C, the peak for glucose was split and maintained this shape all the way up to 60 °C. This could be a result of continuous interconversion of the glucose molecules between open-chain and cyclic form, in addition to $\alpha$- and ß-ring structures. At these temperatures, the rate of interconversion is slow enough to be detected on the RI. At higher temperatures ≥ 65 °C, where the speed of interconversion is much greater, a single glucose peak is eluted. For all of the sugars, peak width decreases and efficiency increases continuously to 85 °C. At 85 °C, the resolution between the sugar molecules would allow the flow rate to be increased to give a faster separation.

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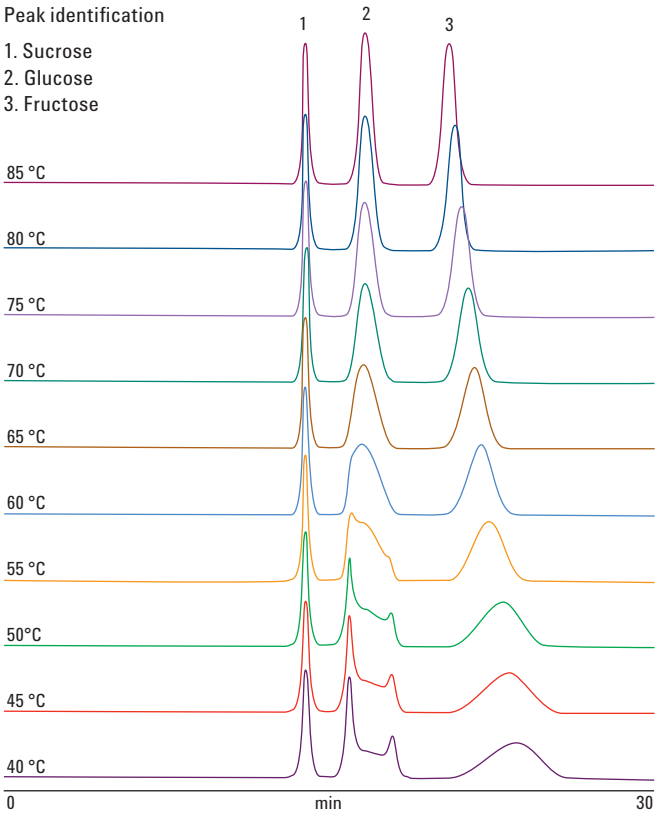


*Figure 1. Raw data chromatograms showing the main components of invert sugar on an Agilent Hi-Plex Ca column at different operating temperatures.*

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SI-01674

Agilent Technologies

# USP Analysis of Malic Acid on an Agilent Hi-Plex H Column

Application Note

Pharmaceutical


## Author

Stephen Ball

Agilent Technologies, Inc.


## Introduction

Maleic acid and fumaric acid are dicarboxylic acids consisting of an ethylene group connecting the two carboxylic acid groups. Maleic acid is the *cis* isomer of butenedioic acid, and fumaric acid is the *trans* isomer; therefore, both compounds are very similar in structure to each other and are closely related to malic acid.

The USP method for malic acid, requiring it to be separated from maleic acid and fumaric acid, specifies a 6.5 × 300 mm column that contains packing L17 (a strong cation exchange resin consisting of sulfonated crosslinked styrene/divinylbenzene copolymer in the hydrogen form). UV detection at 210 nm is required.

Agilent Technologies

## Materials and Reagents

| | |
|---|---|
| Column | Agilent Hi-Plex H, 6.5 × 300 mm, 8 µm (p/n PL1F70-6830) |
| Mobile phase | 0.01 M $H_2SO_4$ |
| Flow rate | 0.6 mL/min |
| Temperature | 37 °C |
| Detector | UV at 210 nm |

### Standard Preparation

A solution containing 1 mg/mL of malic acid, 10 µg/mL of fumaric acid, and 4 µg/mL of maleic acid was made up in mobile phase. Twenty microliters of this solution was then injected on to the column.

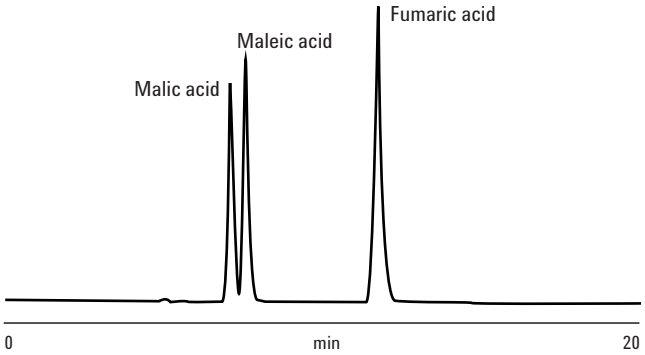


*Figure 1. USP analysis of malic acid on an Agilent Hi-Plex H column.*

## Conclusion

Maleic acid and fumaric acid, despite being structural isomers of the same compound, elute at very different retention times. Therefore, they are very well separated by the Agilent Hi-Plex H column. Malic acid, however, elutes closer to maleic acid but can be separated to at least baseline resolution. These results show that the Hi-Plex H is a good choice of column for this particular application.

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www.agilent.com/chem


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SI-02029

Agilent Technologies

# USP Analysis of Mannitol on an Agilent Hi-Plex Ca Column

Application Note

Life Sciences and Pharmaceutical


## Authors

Stephen Ball, Linda Lloyd

Agilent Technologies, Inc.


## Introduction

Mannitol is a polyol that is used as an osmotic diuretic agent and a weak renal vasodilator. It is a sorbitol stereoisomer.

Chemically, mannitol is a sugar alcohol, or a polyol, which is similar to xylitol and sorbitol.

According to the USP method, mannitol is analyzed using a liquid chromatograph equipped with a refractive index (RI) detector maintained at a constant temperature and a 4 × 250 mm column that contains packing L19 (a strong cation-exchange resin consisting of sulfonated crosslinked styrene/divinylbenzene copolymer in the calcium form). Column temperature should be maintained between 30 °C and 85 °C with a flow rate of 0.5 mL/min.

Agilent Technologies

## Experimental

### Conditions

| | |
|---|---|
| Column | Agilent Hi-Plex Ca USP L19, 4.0 × 250 mm, 8 µm (p/n PL1570-5810) |
| Mobile phase | 100% DI $H_2O$ |
| Flow rate | 0.5 mL/min |
| Temperature | 70 °C |
| Detector | RI |

### Sample Preparation

A solution of mannitol and sorbitol, used as an internal standard, was made up to contain 4.8 mg/mL of each compound. The only requirement for this assay is that the resolution between mannitol and sorbitol is not less than 2.0.

*Peak identification*

*1. Mannitol*
*2. Sorbitol*

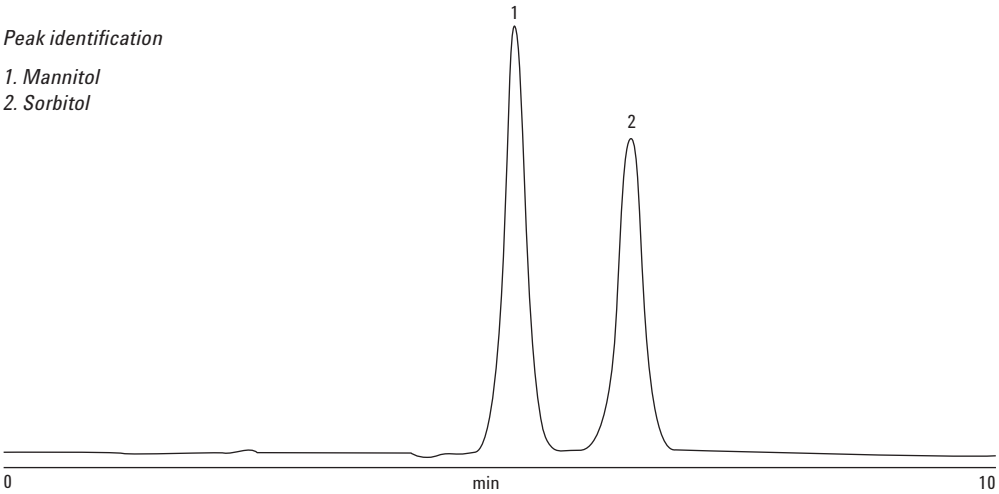


*Figure 1. Raw data chromatogram of mannitol and sorbitol on an Agilent Hi-Plex Ca USP L19 column.*

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SI-01670

Agilent Technologies

# USP Analysis of Ribavirin Using an Agilent Hi-Plex H Column

Application Note

Pharmaceutical


## Authors

Stephen Ball, Linda Lloyd

Agilent Technologies, Inc.


## Introduction

Ribavirin is an anti-viral drug, which is active against a number of DNA and RNA viruses. It is a member of the nucleoside antimetabolite drugs that interfere with duplication of viral genetic material. Though not effective against all viruses, ribavirin is remarkable as a small molecule for its wide range of activity, including important activities against influenzas, flaviviruses, and agents of many viral hemorrhagic fevers.

According to the USP method, ribavirin is analyzed using a liquid chromatograph equipped with a 207 nm detector and a 7.7 × 100 mm column that contains packing L17 (a strong cation-exchange resin consisting of sulfonated crosslinked styrene/divinylbenzene copolymer in the hydrogen form). The mobile phase must be water that has been adjusted with sulfuric acid to pH 2.5 (± 0.1), and the column should be maintained at a temperature of 65 °C and flow rate of 1.0 mL/min.


Agilent Technologies

## Experimental

### Conditions

| | |
|---|---|
| Column | Agilent Hi-Plex H USP L17, 7.7 × 100 mm, 8 µm (p/n PL1170-2823) |
| Mobile phase | 0.002 M $H_2SO_4$ (adjusted to pH 2.5) |
| Flow rate | 1.0 mL/min |
| Temperature | 65 °C |
| Detector | UV, 207 nm |

### Sample Preparation

A solution of ribavirin was made up with a concentration of 0.025 mg/mL for injection. Requirements for this assay are that the tailing factor for the ribavirin peak is not less than 0.7 and not more than 1.5 and that the relative standard deviation for replicate injections is not more than 0.5%.

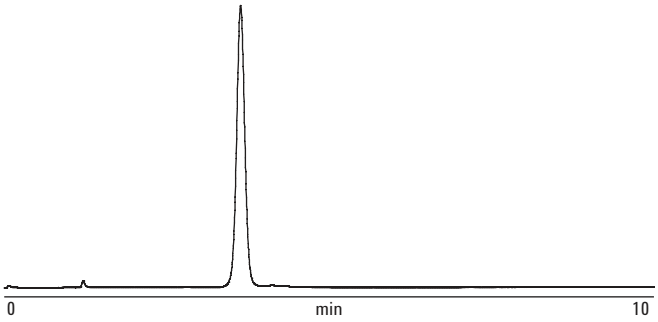


*Figure 1. Raw data chromatogram of ribavirin on an Agilent Hi-Plex H USP L17 column.*

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SI-01669

Agilent Technologies

# USP Analysis of Sorbitol on an Agilent Hi-Plex Pb Column

Application Note

Pharmaceutical


## Authors

Stephen Ball, Linda Lloyd

Agilent Technologies, Inc.


## Introduction

Sorbitol is a sugar alcohol that is metabolized slowly by the body. Also known as glucitol, sorbitol is commercially produced by the reduction of glucose, during which the aldehyde group is changed to an additional hydroxyl group.

Sorbitol is found in stone fruits, such as peaches and plums, as well as in berries. It is often used as a sugar substitute in foods such as ice cream, as well as in sugar-free gum. It is nutritive in that it contains calories, but it is not sticky like dextrose or glucose solutions. It is also used in tobacco products to give texture and maintain moisture as a humectant. Sorbitol is resistant to metabolism by oral bacteria that break down sugars and starches to release acids, which may lead to cavities or erode tooth enamel, and so it is a common ingredient in toothpaste.

Sorbitol is also found in pharmaceutical applications, where it is used as an excipient or as a primary ingredient. As an excipient, it is useful because it can be compressed into tablet forms and its sweetness provides an excellent mask to many drugs. As an active ingredient, it can be used to treat constipation or as an irrigation solution during urinary tract surgery.


Agilent Technologies

## Instrumental

According to the USP method, sorbitol is analyzed using a liquid chromatograph equipped with a refractive index (RI) detector, maintained at 50 °C, and a 7.7 × 100 mm column packed with a strong cation-exchange resin consisting of sulfonated crosslinked styrene/divinylbenzene copolymer in the lead form, classified as L34. The column temperature is maintained at 50 °C, with a flow rate of 0.7 mL/min. The system suitability test (SST) used in this analysis is a mixture of sorbitol and mannitol, which is an isomer of sorbitol.

### Conditions

| | |
|---|---|
| Column | Agilent Hi-Plex Pb USP L34, 7.7 × 100 mm, 8 µm (p/n PL1170-2820) |
| Mobile phase | 100% DI $H_2O$ |
| Flow rate | 0.7 mL/min |
| Temperature | 50 °C |
| Detector | RI |

### Sample Preparation

A solution of sorbitol and mannitol, used as an internal standard, is made up to contain 4.8 mg/mL of each compound. The requirement for this assay is that the relative retention times are about 0.6 for mannitol and 1.0 for sorbitol, with a resolution of not less than 2.0 between them.

*Peak identification*

*1. Mannitol*
*2. Sorbitol*

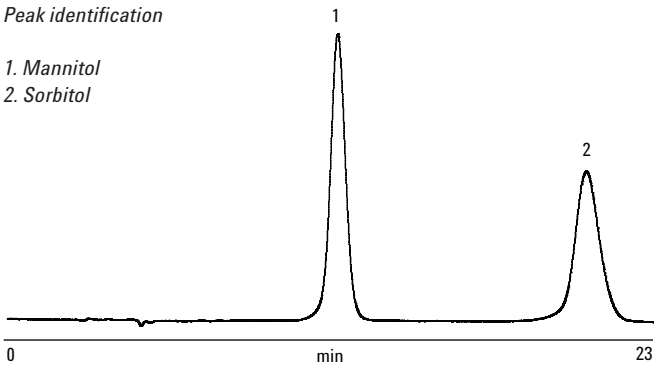


*Figure 1. Raw data chromatogram of sorbitol and mannitol on an Agilent Hi-Plex Pb USP L34 column.*

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SI-01671

Agilent Technologies

# USP Analysis of Sugar Alcohols on an Agilent Hi-Plex Ca Column – Mobile Phase Effects

Application Note

Pharmaceutical


## Authors

Stephen Ball, Linda Lloyd

Agilent Technologies, Inc.


## Introduction

Sugar alcohols, or polyols, are hydrogenated carbohydrates commonly used to replace sucrose in foods. They are often used with high-intensity artificial sweeteners to counter their low sweetness.

The separation of seven sugar alcohols on an Agilent Hi-Plex Ca column can be altered by introducing acetonitrile into the mobile phase.

Agilent Technologies

## Experimental

### Conditions

| | |
|---|---|
| Column | Agilent Hi-Plex Ca USP L19, 4.0 × 250 mm, 8 µm (p/n PL1570-5810) |
| Mobile phase | 100% DI $H_2O$ (initially) |
| Flow rate | 0.15 mL/min |
| Injection volume | 10 µL |
| Temperature | 90 °C |
| Detector | RI |

### Sample Preparation

The seven sugar alcohols — iso-erythritol, adonitol, arabitol, mannitol, xylitol, dulcitol, and sorbitol — are made up to a concentration of 10 mg/mL in water. See Figure 1.

When pure water is used for the mobile phase, several of the sugar alcohols in the sample either partially or completely co-elute. Modifying operating temperature or flow rate is very unlikely to give a good separation between these compounds.

Introducing acetonitrile into the mobile phase has a significant effect on the selectivity of the Agilent Hi-Plex Ca material and results in a doubling of the retention time. As a result, the mobile phase and flow rate conditions need to be modified as follows:

### Conditions

| | |
|---|---|
| Mobile phase | 30:70 acetonitrile:100% DI $H_2O$ |
| Flow rate | 0.30 mL/min |
| Temperature | 90 °C |

The same quantity of test solution is injected. See Figure 2.

## Conclusion

As can be seen by comparing the two chromatograms, using 30% acetonitrile gives extra retention for the sugar alcohols and, as a result, increases the resolution between them. All seven sugar alcohols are now either partially or completely separated. It also gives a change in the elution order.

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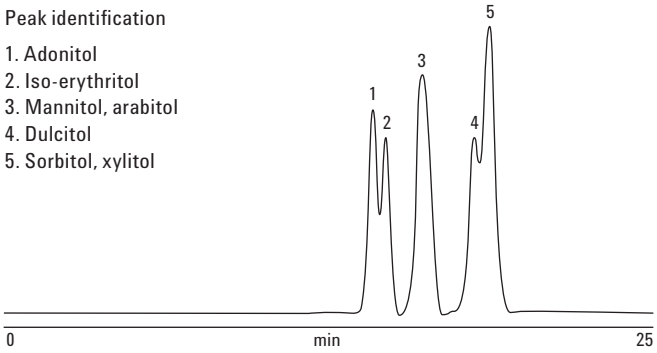


*Figure 1. Raw data chromatogram of seven sugar alcohols on an Agilent Hi-Plex Ca USP L19 column.*

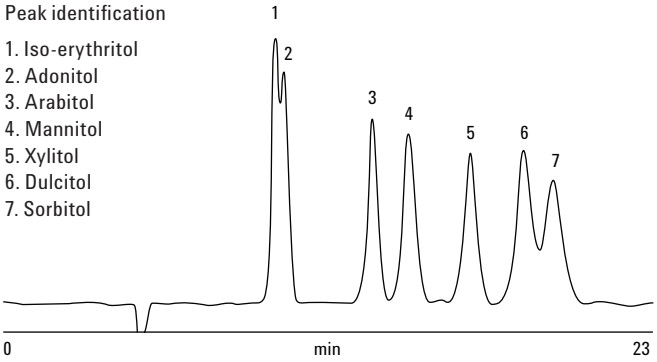


*Figure 2. Raw data chromatogram of seven sugar alcohols on an Agilent Hi-Plex Ca USP L19 column after the introduction of acetonitrile into the mobile phase.*

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Agilent Technologies

# Analysis of Bioethanol Fermentation Products Using an Agilent Hi-Plex H Column

Application Note

Biofuels


## Author

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Pirmasens

Germany


## Introduction


The fermentation of biomass to ethanol is an economically important process. For the control of the fermentation, HPLC is the method of choice because it can separate and detect carbohydrates (starting material), acids (potential inhibitors), fusel alcohols (byproducts), and ethanol (product) in one step without prior derivatization. This application note describes the analysis of a sample of a batch bioethanol fermentation product using an Agilent Hi-Plex H column.



Agilent Technologies

## Materials and Methods

The sample (a suspension of biomass and yeast in water) was pretreated by filtration through a 0.45 µm membrane.

### Conditions

| | |
|---|---|
| Column | Agilent Hi-Plex H, 7.7 × 300 mm, 8 µm (p/n PL1170-6830) |
| Mobile phase | 0.005 M $H_2SO_4$ |
| Gradient | Isocratic |
| Flow rate | 0.7 mL/min |
| Injection volume | 20 µL |
| Sample concentration | Glucose ~ 37 g/L |
| | Xylose ~ 3.5 g/L |
| | Succinic acid ~ 1 g/L |
| | Lactic acid ~ 200 mg/L |
| | Glycerol ~ 10 g/L |
| | Acetic acid ~ 700 mg/L |
| | Methanol ~ 1.5 g/L |
| | Acetaldehyde ~ 300 mg/L |
| | Ethanol ~ 87 g/L |
| Temperature | 60 °C |
| Pressure | 4.6 MPa (46 bar, 670 psi) |
| Detector | RI (55 °C) |

## Results

The chromatogram clearly indicates that the biomass sample contains large amounts of starting material, organic acids, byproducts, and final product mixed together.

## Conclusion

A sample from a batch fermentation was resolved with good separation using an Agilent Hi-Plex H column. Hi-Plex H columns are ideal for the analysis of sugar alcohols and sugar molecules using water as the mobile phase. Hi-Plex H is also the column of choice for the analysis of organic acids, using dilute acid as eluent. The use of a ligand-exchange chromatography column such as Hi-Plex H significantly reduces the need for complicated sample preparation (typically involving elution through an ion-exchange resin bed), as retention is brought about not only by ion exchange, but also by ion exclusion and partitioning on this type of column.

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*Peak identification*

1 - 4 di- and oligosaccharides
5 Glucose
6 Xylose
7 Succinic acid
8 Lactic acid
9 Glycerol
10 Acetic acid
11 Acetaldehyde
12 Methanol
13 Ethanol

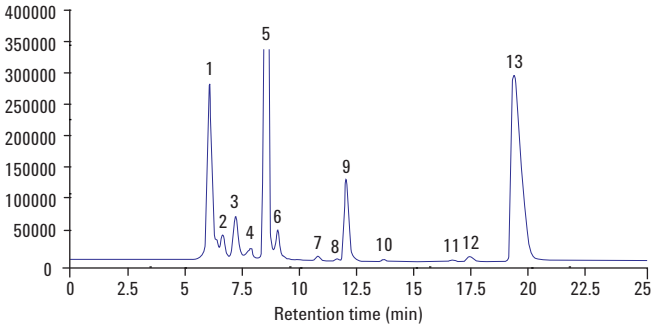


*Figure 1. HPLC chromatogram of biomass and yeast in water, using an Agilent Hi-Plex H column.*

www.agilent.com/chem


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SI-1941

Agilent Technologies

# Analysis of Byproducts in Fermentation Liquids Using an Agilent Hi-Plex H Column

Application Note

Food and Beverage


## Author

A. Ewen

Prüf- und Forschungsinstitut

Pirmasens

Germany


## Introduction

Biomass fermentation has grown in importance because diverse products such as fuel, lubricants, and chemicals can be derived. One option for this use of biomass is the fermentation of xylose from hemicelluloses, to xylitol, a sugar substitute. For the HPLC analysis of fermentation liquids, the US NREL Biomass Program method *Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples* can be applied.


Agilent Technologies

## Materials and Methods

Two fermentation samples were analyzed. The first was obtained by a hydrothermal digestion of straw (as an example of biomass) that destroys the hemicelluloses and frees the xylose. Following partial evaporation of water, the second sample was obtained after fermentation of xylose to xylitol.

### Conditions

| | |
|---|---|
| Column | Agilent Hi-Plex H, 7.7 × 300 mm, 8 µm (p/n PL1170-6830) |
| Mobile phase | 0.005 M $H_2SO_4$ |
| Gradient | Isocratic |
| Flow rate | 0.7 mL/min |
| Injection volume | 20 µL |
| Sample concentration | Xylose ~ 8 g/L |
| | Glucose ~ 1.5 g/L |
| | Xylitol ~ 13 g/L |
| | Furfural 10 ~ 500 mg/L |
| | Hydroxymethylfurfural ~ 100 mg/L |
| | Acetic acid ~ 1000 mg/L |
| | Ethanol ~ 2000 mg/L |
| | Lactic acid ~ 2500 mg/L |
| Temperature | 60 °C |
| Pressure | 4.6 MPa (46 bar, 670 psi) |
| Detector | RI (55 °C) |

## Results

After hydrothermal digestion, a large quantity of xylose is present in solution, as expected (Figure 1). Figure 2 shows that further fermentation of the sample converts a large quantity of this xylose into xylitol and gives a very large RI response for this sugar alcohol.

## Conclusion

The Agilent Hi-Plex H column is specially suited for the analysis of byproducts and degradation products (acids, alcohols, furfural, hydroxymethylfurfural), such as those produced by biomass fermentation. The column is recommended for use with samples that contain high levels of organic acids or for simultaneous analysis of these acids and sugars, using sulfuric acid as the mobile phase.

## For More Information

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*Peak identification for Figures 1 and 2*

1 Glucose
2 Xylose
3 Arabinose
4 Xylitol
5 Lactic acid
6 Glycerol
7 Acetic acid
8 Ethanol
9 Hydroxymethylfurfural (HMF)
10 Furfural

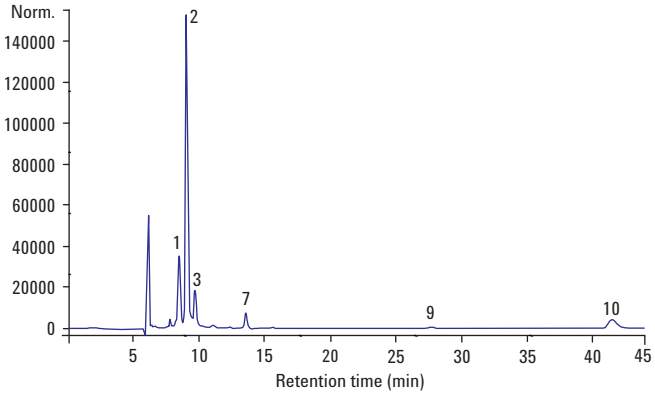


*Figure 1. Analysis of a sample of straw after hydrothermal digestion using an Agilent Hi-Plex H column.*

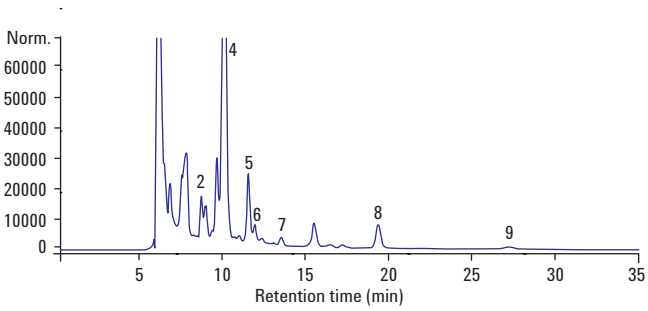


*Figure 2. Components of a straw sample after fermentation of xylose to xylitol.*

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SI-1942

Agilent Technologies

# Cellulose Hydrolysate Analysis by HPLC

Application Note

Biofuels


## Authors

Stephen Ball, Linda Lloyd,
Keeley Mapp
Agilent Technologies, Inc.


## Introduction

Cellulose is a polysaccharide consisting of a linear chain of several hundred to over ten thousand linked D-glucose units. It provides the structure of the cell wall in green plants. Cellulose can be hydrolyzed into its glucose units by treating it with concentrated acids at high temperature. Alternatively, enzymes such as the endo-acting cellulase break cellulose down into individual glucose units.

HPLC using an Agilent Hi-Plex Ca column analyzes the breakdown products of an enzymic digestion of cellulose.


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## Experimental

An isocratic HPLC system was set up with a column block heater and an RI detector.

### Conditions

| | |
|---|---|
| Column | Agilent Hi-Plex Ca, 7.7 × 300 mm, 8 µm (p/n PL1170-6810) |
| Mobile phase | 100% DI $H_2O$ |
| Flow rate | 0.6 mL/min |
| Temperature | 85 °C |

### Sample Preparation

A 10 mg/mL solution of cellulase (CAS 9012-54-8) in water was adjusted to an approximate pH of 4.5 with 0.01 M HCl. Ten milliliters of this solution were then added to 0.1 g of chromatography-grade cellulose (CAS 9004-34-6) in a 25 mL conical flask.

The contents of the flask were left in a water bath and heated to 40 °C for 24 hours, during which time 1 mL aliquots were extracted for analysis. Each sample and the liquid remaining after 24 hours were passed through a 0.45 µm syringe filter to remove any remaining cellulose from the sample (effectively preventing any further hydrolysis). All samples were stored in the freezer before analysis.

Twenty microliter injections were made of each sample to analyze for breakdown products.

## Results

The following chromatograms track the levels of the sugars resulting from the enzymic hydrolysis of cellulose over time.

Aliquots were collected at 2 hours through the process, 19 hours (after being left overnight), 21 hours, and finally 24 hours.

## Discussion

From an early stage in the process, two different sugar molecules begin to form in the solution: glucose and cellobiose.

Cellobiose is a disaccharide derived from the condensation of two glucose molecules linked in a ß (1 ⇒ 4) bond. This is a byproduct of the enzyme-catalyzed hydrolysis of cellulose.

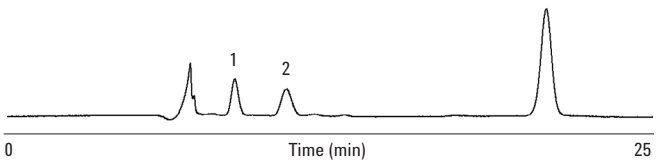
As the elapsed time increases, so does the concentration of cellobiose and glucose, indicating that increased numbers of cellulose chains are breaking down into smaller sugar units.

The additional peaks at the beginning and end of the chromatograms are likely to be additional side-products of the hydrolysis reaction or cellulase itself present in solution.

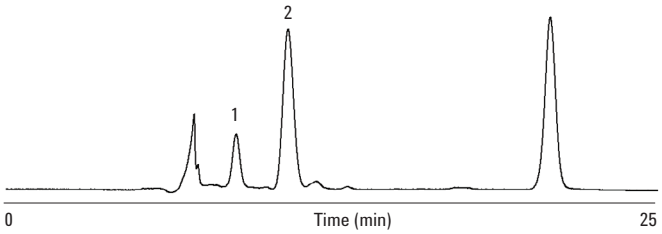
*Peak identification (for all figures)*

*1. Glucose*
*2. Cellobiose*

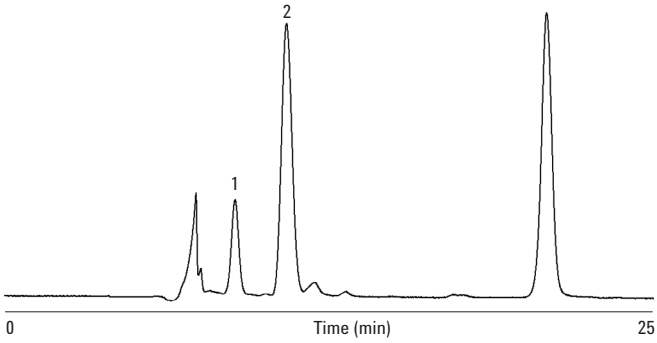
Reaction time: 2 hours



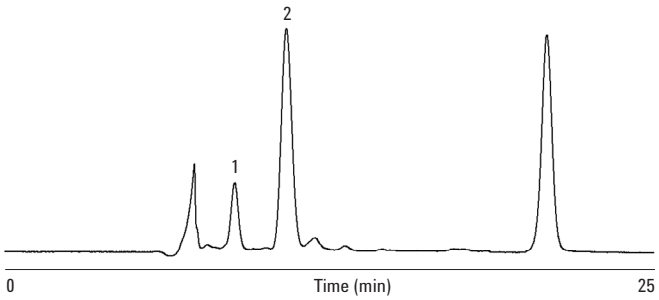

Reaction time: 19 hours




Reaction time: 21 hours




Reaction time: 24 hours



## Conclusion

The Agilent Hi-Plex Ca column can be used to quantify the levels of the sugars in solution that result from the hydrolysis of cellulose.

A potentially useful application of this HPLC procedure is in the quality control of a glucose manufacture process or in the biofuels industry, where enzymes are often used to break down cellulose and hemicelluloses.

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SI-01921

Agilent Technologies

# Analyzing Liquid Fractions of Biogas Processes by HPLC

Application Note

Biofuels


## Author

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Pirmasens

Germany


## Introduction

For process control of the ever-growing number of biogas plants, knowledge of acetic and propionic acid concentration is crucial, since high levels of propionic acid can indicate biological problems. Analysis of free fatty acids can be done by GC or by HPLC. This application note shows the analysis of a specimen of a biogas plant liquor using HPLC with an Agilent Hi-Plex H column.


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## Materials and Methods

The sample was steam distilled according to *German standard methods for the examination of water, waste water and sludge — Sludge and sediments (group S) — Part 19: Determination of the steam-volatile organic acids (S 19)* and pretreated by filtration through a 0.45 µm membrane before analysis. Since caproic acid is very seldom found in biogas plant liquors, the method can be halted after the elution of isovaleric acid.

### Conditions

| | |
|---|---|
| Column | Agilent Hi-Plex H, 7.7 × 300 mm, 8 µm (p/n PL1170-6830) |
| Mobile phase | 0.005 M $H_2SO_4$ |
| Gradient | Isocratic |
| Flow rate | 0.7 mL/min |
| Injection volume | 20 µL |
| Sample concentration | 200 mg/mL for each acid |
| Temperature | 60 °C |
| Pressure | 4.6 MPa (46 bar, 670 psi) |
| Detector | RI (55 °C) |

## Results and Discussion

Figure 1 shows a separation of a standard mixture of free fatty acids.

Figure 2 shows the main constituents of the biogas liquor, which includes some of the fatty acids in the standard mix.

## Conclusion

A sample of biogas plant liquor was successfully separated using HPLC with an Agilent Hi-Plex H column. Hi-Plex H is the column of choice for the analysis of organic acids, using dilute mineral acid as eluent. The use of a ligand-exchange chromatography column such as Hi-Plex H significantly reduces the need for complicated sample preparation (typically involving elution through an ion-exchange resin bed). This is because retention is brought about not only by ion exchange, but also by ion exclusion and partitioning on this type of column.

## For More Information

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*Peak identification (for both figures)*

1. Formic acid
2. Acetic acid
3. Propionic acid
4. Isobutyric acid
5. Butyric acid
6. Isovaleric acid
7. 3,3-Dimethyl butyric acid (Internal Standard)
8. Valeric acid
9. Isocaproic acid
10. Caproic acid

x. Ethanol

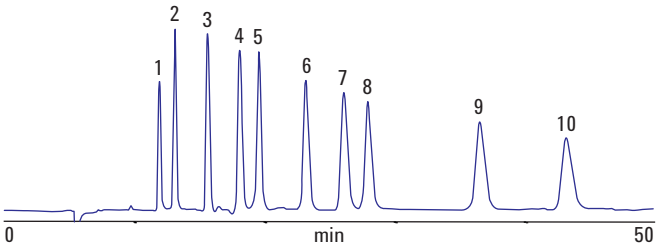


*Figure 1. Analysis of a standard mixture of free fatty acids on an Agilent Hi-Plex H column.*

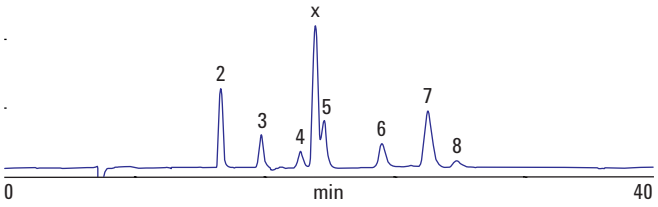


*Figure 2. Separation of a biogas plant liquor by an Agilent Hi-Plex H column.*

www.agilent.com/chem


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SI-01943

Agilent Technologies

# Organic Acids in Silage

## Application Note

Food and Environmental


## Author

A. Ewen

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Germany


## Introduction


In addition to other factors, the concentration of the three fermentation acids lactic acid, acetic acid, and butyric acid is a criterion for the quality of silages. HPLC is the choice for this analysis, since volatile and nonvolatile acids can be determined together without prior derivatization. This application note shows the analysis of several specimens of silages (grass, whole plant, and corn) using an Agilent Hi-Plex H column.



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## Materials and Methods

The extraction of the acids is done according to EN 13037 (soil improvers and growing media determination of pH) by adding 1.25 L of water to 250 mL of silage and agitating for one hour. The sample was pretreated by filtration through a 0.45 µm membrane.

## Conditions

| | | |
|---|---|---|
| Column | Agilent Hi-Plex H, 7.7 x 300 mm, 8 µm (p/n PL1170-6830) | |
| Mobile phase | 0.005 M $H_2SO_4$ | |
| Gradient | Isocratic | |
| Flow rate | 0.7 mL/min | |
| Injection volume | 20 µL | |
| Sample concentration | Glucose | 50 – 1500 mg/L |
| | Succinic acid | 50 – 125 mg/L |
| | Lactic acid | 750 – 1000 mg/L |
| | Acetic acid | 200 – 450 mg/L |
| | Ethanol | 80 – 700 mg/L |
| Temperature | 60 °C | |
| Pressure | 4.6 MPa (46 bar, 670 psi) | |
| Detector | RI (55 °C) | |

## Results and Discussion

Figure 1 shows the analysis of grass silage, which has undergone a homofermentative process leading mostly to lactic acid and a small amount of ethanol. An example for a heterofermentative process is shown in Figure 2. Here the silage of corn yielded not only lactic acid, but also acetic acid and ethanol. Figure 3 shows the analysis of whole plant silage, which has undergone an untypical process, leaving a large amount of free sugars.

## Conclusion

Samples of silage from different crops were successfully separated by HPLC with an Agilent Hi-Plex H column.

Hi-Plex H is the column of choice for the analysis of organic acids in complex matrices, using dilute mineral acid as eluent. Hi-Plex columns are packed with sulfonated resin, giving a fundamental improvement in performance. They contain monodisperse sulfonated packing to overcome the problems of low efficiencies and high backpressures encountered with soft gels.

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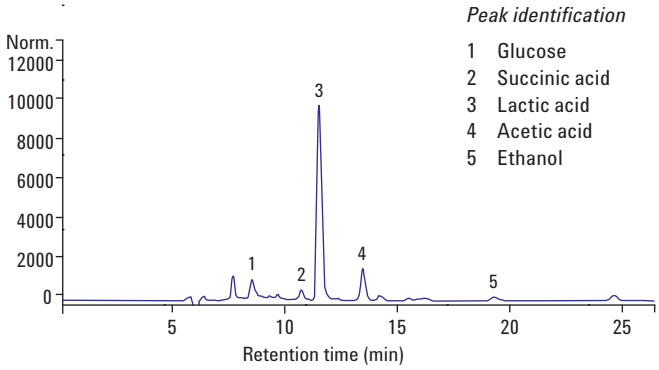


*Figure 1. Analysis of grass silage using an Agilent Hi-Plex H column.*

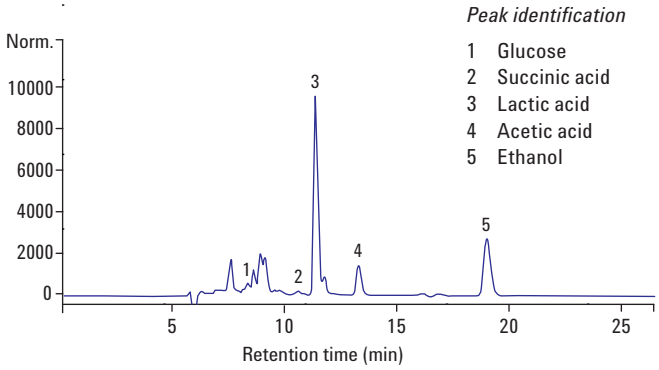


*Figure 2. Separation of corn silage using an Agilent Hi-Plex H column.*

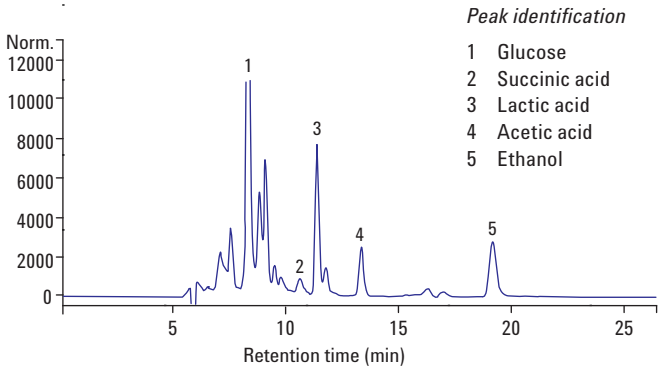


*Figure 3. Analysis of whole plant silage using an Agilent Hi-Plex H column.*

www.agilent.com/chem


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SI-1945

Agilent Technologies

# Analysis of Sugars from Biomass Fermentation

## Application Note

Biofuels


## Author

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Pirmasens

Germany


## Introduction

Biomass fermentation has grown in importance because diverse products such as fuel, lubricants, and chemicals can be derived. One option for this use of biomass is the fermentation of xylose (from hemicelluloses) to xylitol, a sugar substitute. For the HPLC analysis of fermentation liquids, the US NREL Biomass Program method *Determination of Sugars, Byproducts, and Degradation Products in Liquid Fraction Process Samples* can be applied.

This application note shows the analysis of the main hemicellulose-forming carbohydrates in a sample obtained by a hydrothermal digestion of straw. The hemicelluloses are hydrolyzed, and, in addition to other monosaccharides, free xylose can be obtained. While the Agilent Hi-Plex H column is especially suited for the analysis of byproducts and degradation products (Application Note SI-1942), the sugars are best analyzed with an Agilent Hi-Plex Pb column.


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## Materials and Methods

### Conditions

| | |
|---|---|
| Column | Agilent Hi-Plex Pb, 7.7 × 300 mm, 8 µm (p/n PL1170-6820) |
| Mobile phase | 100% DI $H_2O$ |
| Gradient | Isocratic |
| Flow rate | 0.5 mL/min |
| Injection volume | 20 µL |
| Sample concentraion | 1 g/L for each component |
| Temperature | 70 °C |
| Pressure | 2.5 MPa (25 bar, 360 psi) |
| Detector | RI (55 °C) |

## Results

Figures 1 and 2 highlight the main constituents of hemicellulose and straw after hydrothermal digestion. Clearly, this digestion process yields large quantities of xylose that elute after 29 minutes, as well as glucose and arabinose to a lesser extent.

## Conclusion

The sugar composition of straw after hydrothermal digestion is readily determined using water as the mobile phase with an Agilent Hi-Plex Pb column. Analyses with these columns avoid the use, high cost, and disposal implications of toxic acetonitrile when separations are performed on amino silica columns. In addition, Hi-Plex stays active in the presence of sugar molecules. Together with fast dissolution, this benefit results in long lifetimes compared to amino silica columns.

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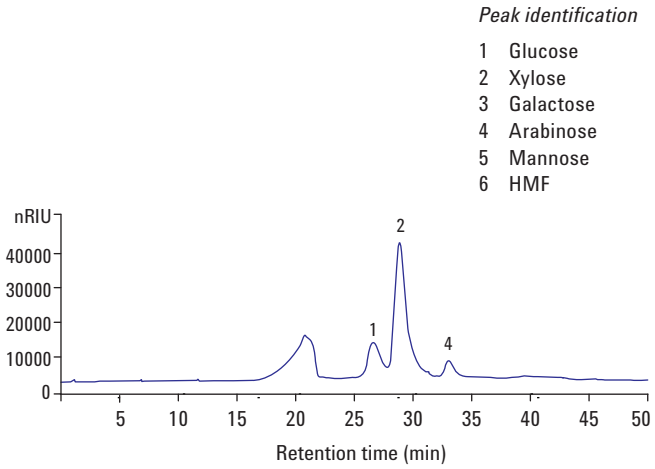


*Figure 1. Analysis of a sample of straw after hydrothermal digestion using an Agilent Hi-Plex Pb column.*

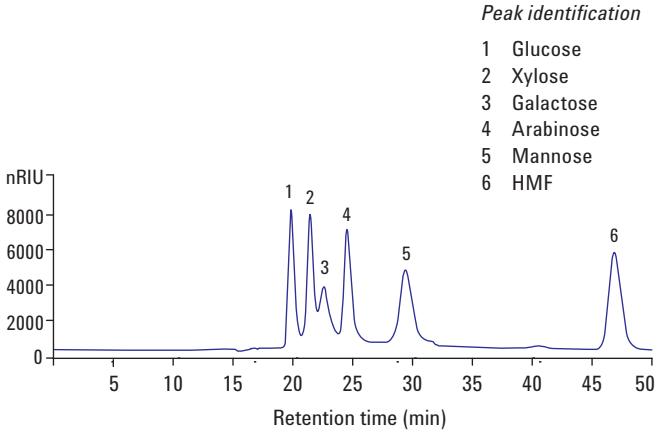


*Figure 2. Standard curve of the main hemicellulose-forming monosaccharides and hydroxymethylfurfural (HMF), an oxidation product of sugars.*

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SI-1944

Agilent Technologies

# Index

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Ethanol *continued on next page*

# Index

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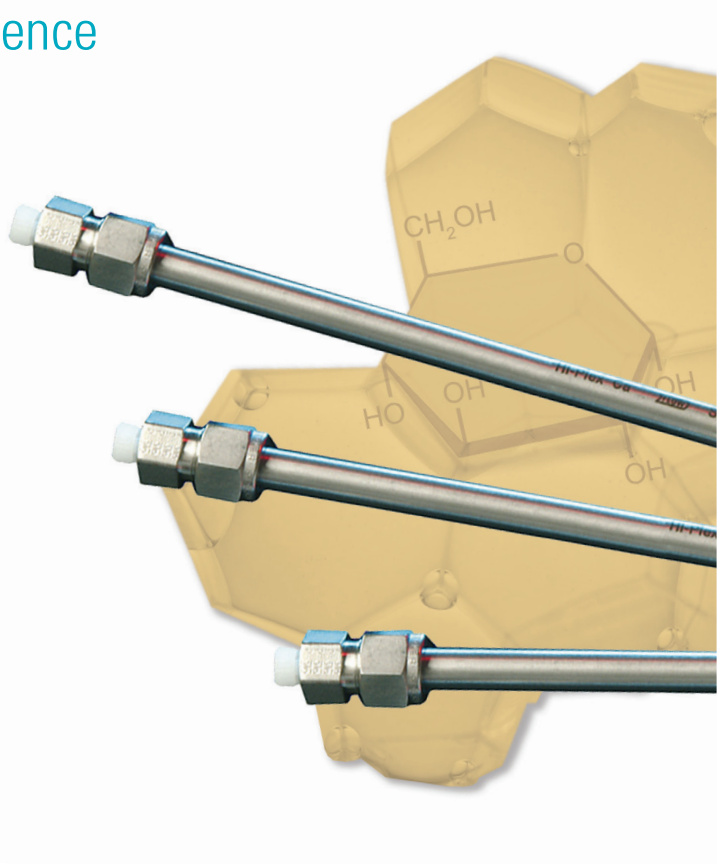


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