Analysis of sugars, small organic acids, and alcohols by HPLC-RID V.1

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DISCLAIMER

This protocol is for research purposes only.

ABSTRACT

An analytical method was developed using high performance liquid chromatography with refractive index detection (HPLC-RID) to quantify the concentration of sugars and carboxylic acids including formic acid, acetic acid, propionic acid, butyric acid, lactic acid, glucose, xylose, arabinose, and glycerol in aqueous samples. This list is non-exhaustive as this isocratic method with an acid modified mobile phase is compatible with analysis of many alcohols, small acids, and sugars. This method utilizes a Bio-Rad Aminex HPX-87H Ion Exclusion Column to provide chromatographic separation.

GUIDELINES

This protocol utilizes an high pressure liquid chromatography refractive index detector (HPLC-RID) system manufactured by Agilent Technologies as referenced in '*Materials*'. A similar HPLC-RID system can be utilized; however, some parameter nomenclature may deviate depending on the manufacturer.

Protocol status: Working
We use this protocol and it's working

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PROTOCOL integer ID: 62288

Keywords: formic acid, acetic acid, propionic acid, butyric acid, lactic acid, glucose, xylose, arabinose, glycerol, carboxylic acids, 87H, HPLC, refractive index detector, RID, monomeric sugars

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MATERIALS

Reagents:

10N Sulfuric Acid **Fisher Scientific Catalog #SA200-1**

Standards:

Carboxylic Acid Standards contain the following analytes:
Glucose
Xylose
Lactic acid
Formic acid
Acetic acid
Propionic acid
Butyric acid
Glycerol

Carboxylic Acids COA.pdf

Carboxylic Acids Standard Kit **Absolute Standards Catalog #99819**

Coferm Standards contain the following analytes:
Cellobiose
Glucose
Xylose
Arabinose
Xylitol
Lactic acid
Glycerol
Acetic acid
Ethanol

COFERM COA.pdf

Coferm Standard Kit **Absolute Standards Catalog #94588**

Acids Standards contain the following analytes:

Acetic acid
Levulinic acid
Formic acid

Acids COA.pdf

Acids Standard Kit **Absolute Standards Catalog #93743**

Materials:

Syringe filters for aqueous matrices

Equipment	
syringe filters, 13mm nylon membrane	NAME
syringe filter	TYPE
Cytiva	BRAND
4550	SKU
https://www.cytivalifesciences.com/en/us/shop/lab-filtration/syringe-filters-non-sterile/nylon-non-sterile-syringe-filters/acrodisc-syringe-filters-with-nylon-membrane-p-36371	LINK
13mm	SPECIFICATIONS

Syringe filters for organic matrices-

Equipment

syringe filters, 13mm nylon membrane

NAME

syringe filter

TYPE

Cytiva

BRAND

4550

SKU

https://www.cytivalifesciences.com/en/us/shop/lab-filtration/syringe-filters-non-sterile/nylon-non-sterile-syringe-filters/acrodisc-syringe-filters-with-nylon-membrane-p-36371

LINK

13mm

SPECIFICATIONS

Instrumentation:

Equipment

Agilent 1260 Infinity II LC System

NAME

HPLC System

TYPE

Agilent

BRAND

Agilent 1260 Infinity II LC System

SKU

https://www.agilent.com/en/product/liquid-chromatography/hplc-systems/analytical-hplc-systems/1260-infinity-ii-lc-system

LINK

G7111B Quat Pump
G7167A Multisampler
G7116A 1260 MCT
G7117C 1260 DAD HS
G7162A 1260 RID

SPECIFICATIONS

Column:

Analytical Column

Equipment	
HPX-87H Column	NAME
Column	TYPE
Bio-Rad	BRAND
125-0140	SKU
https://www.bio-rad.com/en-us/sku/1250140-aminex-hpx-87h-column?ID=1250140&WT.mc_id=170208017160&WT.srch=1&WT.knsh_id=_kenshoo_clickid_&gclid=EAIaIQobChMI5vbXvujS9wIVTxPUAR2GPgz2EAAYAiAAEgIuV_D_BwE	LINK

Guard Column

Equipment	
Micro-Guard Cation H Cartridge	NAME
Guard Column	TYPE
Bio-Rad	BRAND
1250129	SKU
https://www.bio-rad.com/en-us/sku/1250129-micro-guard-cation-h-refill-cartridges?ID=1250129	LINK

SAFETY WARNINGS

All chemicals used for this procedure are hazardous. Read the Safety Data Sheet (SDS) for all chemicals and follow all applicable chemical handling and waste disposal procedures. Manufacturer specific SDS information can be found by following the CAS numbers of compounds in 'Materials' list.

Sulfuric acid can cause serious chemical burns. See SDS for additional information:

https://beta-static.fishersci.com/content/dam/fishersci/en_US/documents/programs/education/regulatory-documents/sds/chemicals/chemicals-s/S25899.pdf

BEFORE START INSTRUCTIONS

All solvents and chemicals used are listed in the 'Materials' section. These are excluded from in-line references to maintain clarity and keep the steps concise.

Preparation of mobile phase and instrument equilibration

1 Mobile phases

1. To make 0.01 N sulfuric acid (0.005 M), dilute 1.0 mL of 10 N sulfuric acid into 1.0 L of 18.2MΩ·cm ultrapure water (UPW). Volumetric preparation of this mobile phase will yield the most reproducible chromatography. See note below.

Note

It is advised to prepare sufficient mobile phase for the entire analysis to reduce the need to add additional mobile phase during an active sequence. Adding mobile phase during an active sequence may cause retention time shifting if the new mobile phase is not identical to the original mobile phase. This method uses roughly 18.0 mL of mobile phase per injection. Calculate how much mobile phase is needed before beginning analysis to prepare enough for the entire analysis.

2 Instrument equilibration

1. Purge instrument with 0.01 N sulfuric acid solution made in the previous step. Be certain the instrument is purged through the entire flow path including the detector, before the analytical column is plumbed in. The Aminex 87H column is sensitive to solvents, for example, a higher amount of methanol will damage the column. The purge step is needed to remove all solvents/mobile phases from the analytical system. (See Bio-Rad's 'Instruction Manual' for Aminex resin-based columns for solvent compatibility and installation details).
2. Add the guard column and analytical column to the system and begin equilibrating the column at a low flow rate of 0.2 mL/min while the column reaches analysis temperature.
3. During the column equilibration, begin purging the reference cell of the refractive index detector (RID), this process will continue through the final equilibration of the column.
4. At intervals of at least 10 minutes, increase the flow by 0.2 mL/min until you reach the method flow of 0.6 mL/min.
5. Once the column is up to method flow and both the column compartment and the RID are at method temperature and stable, the RID reference cell can be closed. This typically takes around 30 minutes after method flow is reached. A longer purge of the reference cell is not detrimental.
6. After the reference cell is closed, wait for the RID signal to stabilize before starting analysis.

Preparation of standards

3 Standards

1. Allow standard level ampules (listed in 'Materials' section) to come to room temperature, vortex, and transfer contents of ampules into 2 mL amber HPLC vials.
2. To create lower concentration calibration levels than provided in the kit, dilute the lowest concentration ampule to your desired concentration.

Concentrations are displayed in µg/mL (ppm)								
level	glucose	xylose	lactic acid	glycerol	formic acid	acetic acid	propionic acid	butyric acid
1-10x	50.2	50.2	50.2	10.0	10.0	10.1	10.0	50.3
1-5x	100.4	100.4	100.4	20.1	20.1	20.1	20.1	100.5
1-2x	251.1	251.0	251.0	50.2	50.2	50.3	50.2	251.3
1	502.1	501.9	501.9	100.4	100.4	100.5	100.3	502.5
2	2502.9	1501.6	1002.4	502.1	501.9	502.0	502.0	2502.9
3	10000.9	7500.6	5000.7	2501.2	2500.5	2501.4	2500.7	10000.8
4	20001.8	15001.2	10001.3	5002.4	5001.0	5002.7	5001.5	20001.5
5	40007.7	30005.5	20004.7	10005.9	10003.1	10006.5	10004.0	40007.2
CVS	5002.3	5002.1	3003.0	1007.0	1003.4	1002.9	1002.5	5001.9

Example calibration concentration table created from commercial standards 'Carboxylic Acids Standards Kit' by Absolute Standards referenced in 'Materials'. Notice the calibration range was extended to lower concentrations by diluting "level 1" by 2x, 5x, and 10x, respectively. Calibration verification standard (CVS) level is a commercial quality control level described in section 6.2. (Click to enlarge)

Note

Reporting limits and linear ranges may vary and should be determined for each instrument individually. The standard ranges provided by the manufacturer are suggested starting amounts and may change depending on instrument response.

Preparation of samples

4 **Samples**

- Samples must be filtered through a 0.2 µm or smaller filter prior to injection on the HPLC ('Materials' section includes part numbers for filters to use depending on matrix composition)
- Samples expected to be over the linear range of the instrument should be diluted to ensure accurate analysis and avoid carryover.

HPLC-RID analysis

5 **Method Specifications**

Analysis is performed using an Agilent 1200 Series High Performance Liquid Chromatography (HPLC) system. An isocratic concentration of 0.01N sulfuric acid through an Aminex HPX-87H column (300 x 8.7 mm, 9 µm particle size) is used to achieve separation at a flow rate of 0.6 mL/min. Quantitation is determined using refractive index detection (RID). Column and RID are both held constant at 55 °C and each sample and standard is injected at a volume of 6.0 µL.

Binary pump configuration	
Flow rate	0.6 mL/min
Maximum pressure	100 bar
Mobile phase A	0.01N sulfuric acid (v/v)

Column compartment parameters	
Temperature	55 °C

Multisampler parameters	
Injection volume	6 µL
Draw speed	100 µL/min
Eject speed	100 µL/min
Wait time after draw	2 sec
Bottom sensing	enabled

Retention time of analytes is dependent on the configuration of the HPLC and will vary from instrument to instrument. Retention time markers for each analyte should be run individually to assess the total required run time of the analysis based on elution. Additional analytes not listed in this protocol may be compatible with these instrument parameters. This re-emphasizes the need to run single analyte retention time markers to prevent co-eluting peaks. For the analyte list provided here, the method run time is 27 minutes.

Note

Known co-eluting peaks on this method are:
- ethanol, butyric acid
- xylose, galactose, mannose, fructose

Note

Injection volume can be increased to 20 µL to obtain a lower reporting limit if necessary. This has the possibility to reduce the upper limit of quantitation due to signal saturation.

Analytical Quality Control

6 Multiple strategies are utilized when performing this analysis to ensure instrument stability and reproducibility.

6.1 Calibration Curves

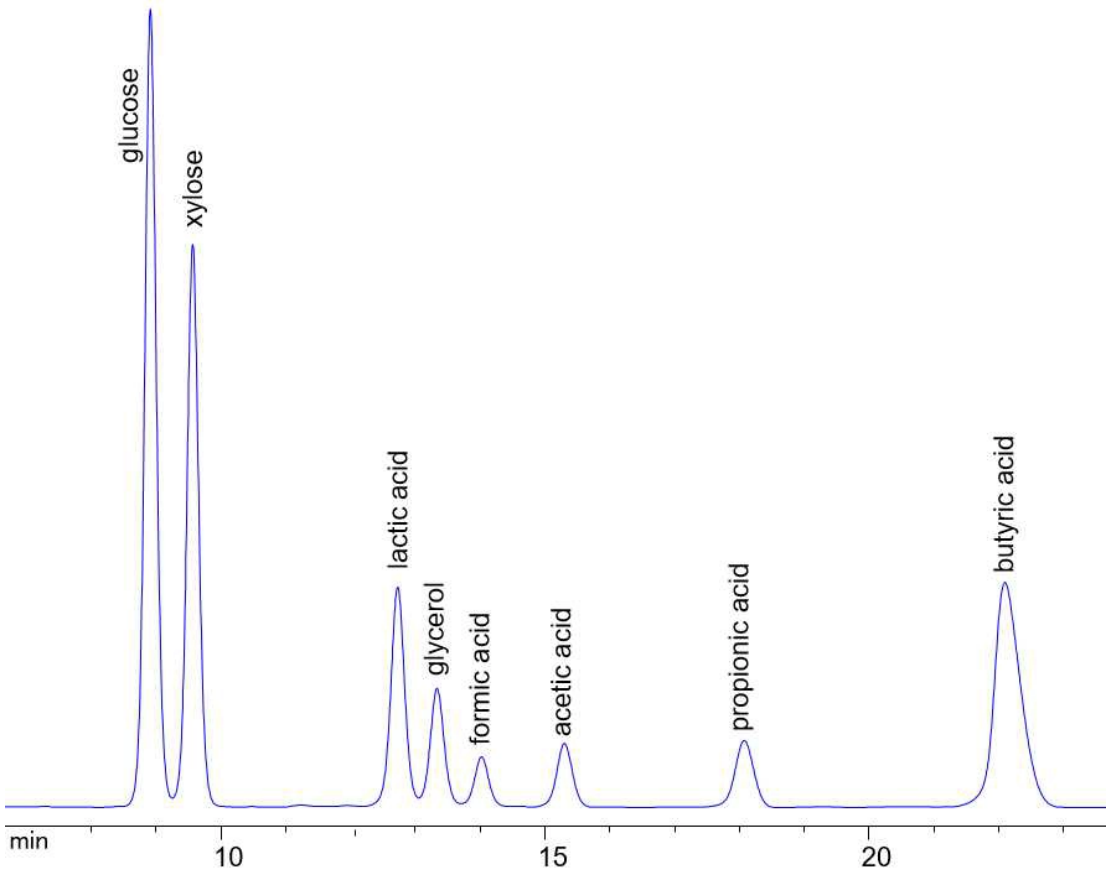
All compounds must have a correlation coefficient (r^2) of 0.995 or greater using a linear calibration fit and ignore the origin.

6.2 Calibration Verification Standards (CVS)

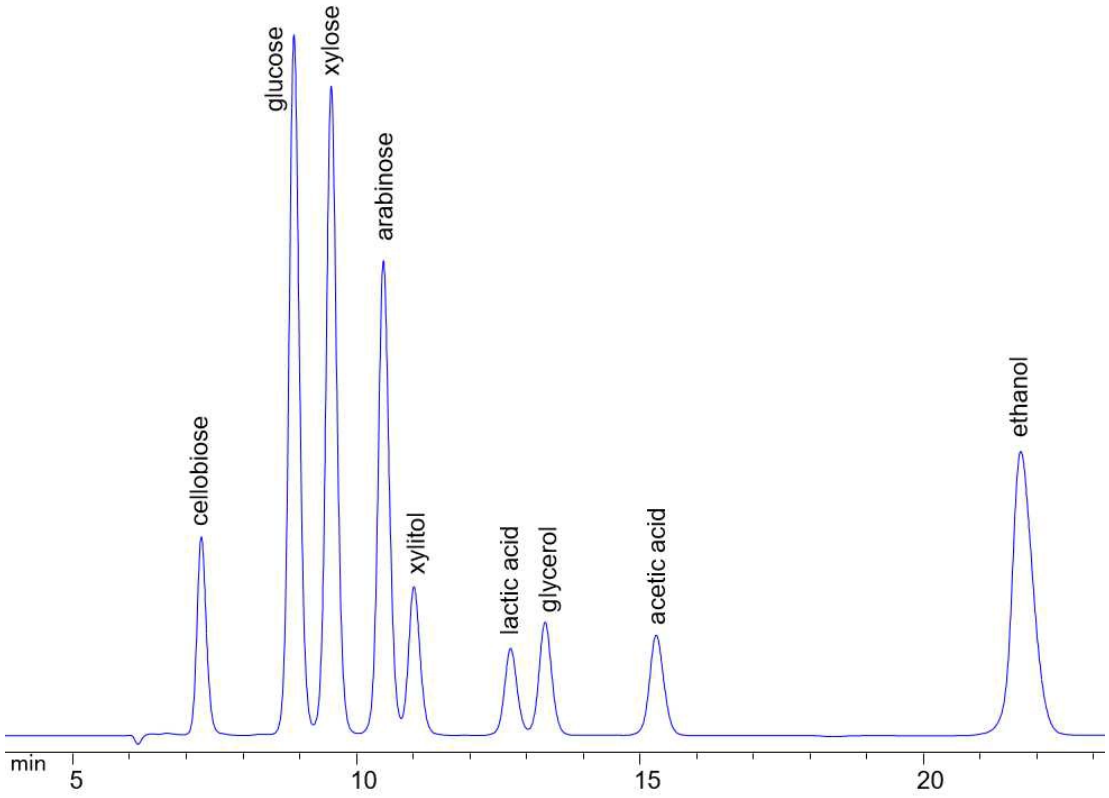
A calibration verification standard (CVS) is a level provided by the manufacturer that is re-analyzed every 20 or fewer samples to ensure instrument drift remains within the determined acceptance criteria. Acceptable CVS recoveries for this analysis are within 10% of the expected amount. Acceptance criteria may differ between instruments and should be determined experimentally.

Example Chromatography

7



Elution order for analytes included in the commercially available 'Carboxylic Acid Standard Kit' seen in 'Materials'.



Elution order for analytes included in the commercially available 'Coferm Standard Kit' seen in 'Materials'.

Note

Additional analytes are included in 'Coferm Standard Kit' preparation. These are 5-hydroxymethylfurfural which elutes at approximately 33 minutes and furfural at approximately 52 minutes. They are omitted in the chromatogram above, but should be considered if using the commercially prepared standard kit.