

Purification of Carbohydrates

by Medium Pressure Liquid Chromatography



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Abstract

The purification of carbohydrate compounds and intermediates has proven difficult because of low adsorbing and absent chromophores. Advances in Medium Pressure Liquid Chromatography (MPLC) equipment, such as the *CombiFlash* Rf system, and media allow faster and improved purification, saving time and solvent. Examples of detection and purifications will be shown. An innovative new technique, *All-Wavelength Collection*, is presented for the purification of mixtures containing weakly absorbing chromophores.

Background

Carbohydrate containing compounds generally pose a challenge for MPLC equipment because they show weak or no UV absorbance. Derivatized carbohydrate compounds may show a weak absorbance in the same wavelengths as the eluting solvent making detection and fractionation difficult. Detection of carbohydrate containing compounds is usually only difficult early in the synthesis when there are few chromophores that absorb UV light. As the synthesis proceeds closer to the targeted compound, UV detection becomes easier. Detection techniques such as evaporative light scattering detection (ELSD) and All-Wavelength Collection allow easy detection of the precursor molecules.

Improvements in MPLC media have also allowed improvements in purifying compounds including those based on carbohydrates.

Example 1: All-Wavelength Collection of glucose pentaacetate

All-Wavelength Collection in the *CombiFlash* Rf 200 system measures the average absorbance on all wavelengths detected on a photodiode array. The signal is processed to remove baseline drift caused by solvent absorbance. This creates a single voltage that allows the fraction collection program in the MPLC or Flash chromatography system to properly cut the peak.

Experimental for Example 1

A sample mixture was prepared from 180 mg (0.999 mmol) of β -D(+)-glucose (Sigma, St. Louis, MO), 5 mL anhydrous pyridine, 5 mL acetic anhydride (Sigma), and a small crystal of 4-(dimethylamino) pyridine (Aldrich, St. Louis, MO). The mixture was stoppered and heated to 50 °C. After reaching 50 °C, the mixture was removed from the water bath and stirred at room temperature. After ½ hour, all crystals were dissolved. The mixture was stirred three more hours at room temperature followed by quenching with ice water (10 mL). Methylene chloride (50 mL) was added and then the mixture was washed with 3×25 mL 1N HCl followed by 25 mL saturated sodium bicarbonate, and finally with 25 mL water. The mixture was dried over sodium sulfate. The yield was undetermined.

Three milligrams of the prepared sample mixture was dissolved in 0.5 mL methylene chloride. The mixture was eluted on a 12 g *RediSep* Rf silica column (Teledyne Isco, Lincoln, NE, PN 69-2203-312) on a *CombiFlash* Rf 200 system (Teledyne Isco, PN 68-5230-006). A gradient of 0–10% methanol in methylene chloride was run. Detection was at 210 nm and All-Wavelength Collection.

Results and discussion for Example 1

Glucose pentaacetate's UV spectrum exhibits only weak end absorption. In addition, a very small sample load of 3 mg was loaded onto the column. The small sample, weak spectrum, and baseline drift from the change in solvent absorbance makes this compound difficult to detect and fractionate with a single wavelength detector. However, the All-Wavelength Collection algorithm purified the compound (Figure 1).

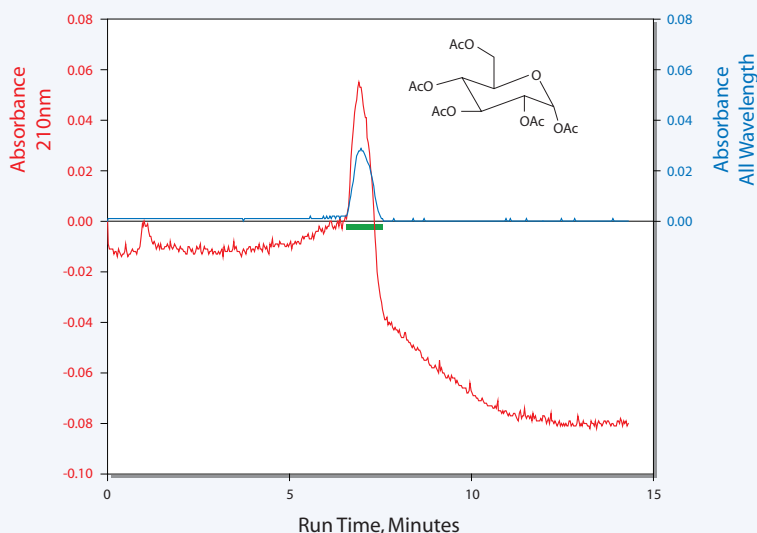


Figure 1:
Purification of glucose pentaacetate with
All-Wavelength Collection

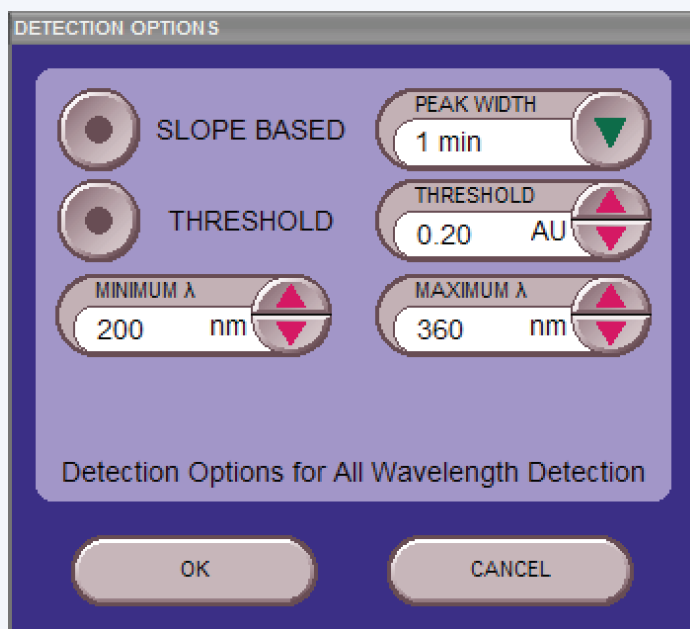


Figure 2:
All-Wavelength Collection
Detector Options dialog

Glucose pentaacetate shows only weak end adsorption which is further suppressed by the absorption of the methylene chloride. As the methylene chloride concentration is decreased, the baseline drifts downwards. This drift tends to confuse common fraction collection programs but is not an issue with All-Wavelength Collection.

All Wavelength Collection has several parameters that can be set for purifying compounds (Figure 2). The detected wavelength range is set by the Minimum λ and Maximum λ settings. The Peak width was found to be best set according to the following guidelines:

- If the wavelength range includes the solvent UV absorbance and a gradient is being run, set the peak width to twice the value for a single wavelength for the column size (wider peak widths may be required for tailing peaks).
- If the solvent doesn't absorb UV light (*i.e.* methanol–water), the wavelength range does not include the solvent, or the purification is under isocratic conditions, the All-Wavelength Collection peak width should be set to the widest value (8 minutes).

These parameters reduce artifacts while allowing optimal collection of compounds. Monitoring at a single wavelength (210 nm in Example 1) provides an overview of the collection.

In addition to suppressing solvent baselines, All-Wavelength Collection is useful if the compound absorbance is unknown (such as natural products¹) or if the detector at a single wavelength is overloaded causing multiple peaks to appear as a single peak.

Example 2: Evaporative Light Scattering Detection of 2,3-O-isopropylidene-D-ribofuranose

Evaporative Light Scattering Detection (ELSD) is useful when the compound possesses no chromophores. ELSD is not a universal detector as some low-melting compounds fail to cause a response². ELSD is also a destructive detector since a portion of the material is lost to the detector.

Experimental for Example 2

A mixture of 2.0 g D(-) ribose (Sigma) in 50 mL acetone (HPLC grade, 99.5%, Alfa Aesir) was prepared. To this solution, 0.12 mL concentrated sulfuric acid (EM Chemicals) was added with stirring at room temperature. The ribose was completely dissolved after 45 minutes; the mixture was stirred for 1.5 hours total. The mixture was neutralized to pH 8.5–9 with TEA and then evaporated to yield a yellow syrup.

A sample volume of 0.1 mL of this reaction product was injected onto a 12 g *RediSep Rf* silica column on a *CombiFlash Rf 200* system. The gradient was 0 to 100% ethyl acetate in hexane. Detection was with a SOFTA model 400 ELSD; a portion of the eluent from the system was diverted with a dispensing pump (included as part of Teledyne Isco's detector flow splitter, PN 60-5237-063) to ensure a steady flow of sample to the ELSD. The ELSD spray chamber was set to 35 °C, drift tube was 70 °C, the gain was set to "EDR".

Results and discussion for Example 2

The product was easily purified using the ELSD detector. The active flow splitter allows the purification to be easily scaled to larger reactions because the optimal sample size is delivered to the ELSD regardless of the *CombiFlash Rf 200* system's flow rate.

The flow splitter works well with other detectors as well; for example, a refractive index detector could be used with isocratic purifications.

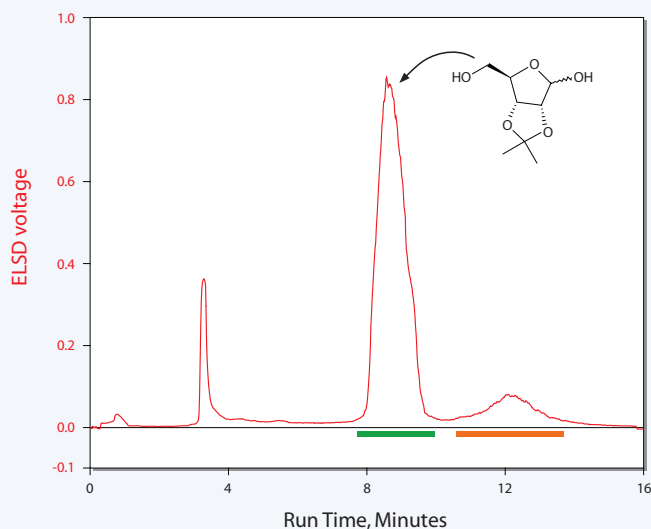


Figure 3:
ELSD detection of 2,3-O-isopropylidene-D-ribofuranose with a *CombiFlash Rf* system

Example 3: Improvements in silica gel media for MPLC

There has been a trend towards smaller particle size for MPLC applications to improve resolution. Irregular silica, while it improves resolution, is more difficult to pack reliably³. The smaller particle size also increases the back pressure from the column which can limit the column flow rate to a suboptimal value, as measured by a Van Deemter plot, which reduces resolution.

Using spherical media in a reduced particle sizes, such as RediSep Rf Gold, allow increased resolution with insignificant changes in backpressure or run times compared to larger, irregular particles.

Experimental for Example 3

A sample volume of 0.1 mL of 2,3-O-isopropylidene-D-ribofuranose reaction mixture from Example 2 was run under the same chromatographic conditions as listed in Example 2, except a 12 g high performance RediSep Rf Gold silica column (Teledyne Isco, PN 69-2203-345) was used.

Results and discussion for Example 3

The RediSep Rf Gold silica has a particle size range of 20-40 μ compared to 40-60 μ for the silica typically used for MPLC and flash chromatography.

As expected from the use of smaller particle size, the peak width of the eluted compounds are reduced with the spherical silica (Figure 4) as compared to the purification in Figure 3. In addition, the minor impurity has been resolved into three peaks. Although, in this case, the major peak is the desired compound there are situations where the minor compounds are also valuable products. The additional resolution provided by the smaller, spherical particles allow better resolution of these compounds.

The extra resolution provided by small, spherical silica can be used to reduce run time⁴ or increase the loading capacity compared to irregular silica with a similar particle size⁵.

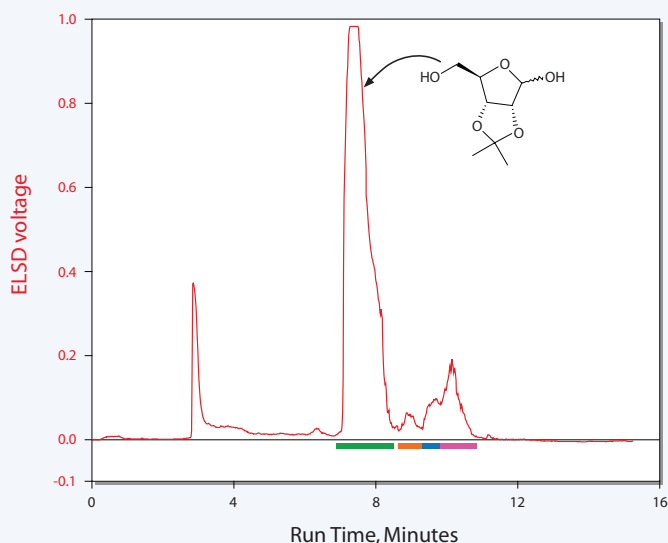


Figure 4:
Purification of 2,3-O-isopropylidene-D-ribofuranose on spherical silica with three minor impurities collected

Example 4: Improvements in C18 media for MPLC

Smaller spherical particles confer the same advantages for reverse phase columns as for silica. Using smaller particle sizes, combined with an instrument capable of 200 psi pressure allows purifications that rival prep HPLC.

Experimental for Example 4

A mixture of 100 mg of esculin monohydrate (Aldrich) and 100 mg of 4-nitrophenyl α -(D) glucopyranoside (Aldrich) was dissolved in 4.0 mL of 75:25 methanol:water. A 0.50 mL sample volume of this solution was injected onto a 12 g RediSep Rf C18 (Teledyne Isco, PN 69-2203-411). A second sample of the same volume was injected onto a high performance RediSep Rf Gold C18 column (Teledyne Isco, PN 69-2203-334). Both columns were run with the same method from 5 to 95% acetonitrile in water.

Results and discussion for Example 4

The smaller, spherical particles provided higher resolution and improved purity of closely eluting compounds.

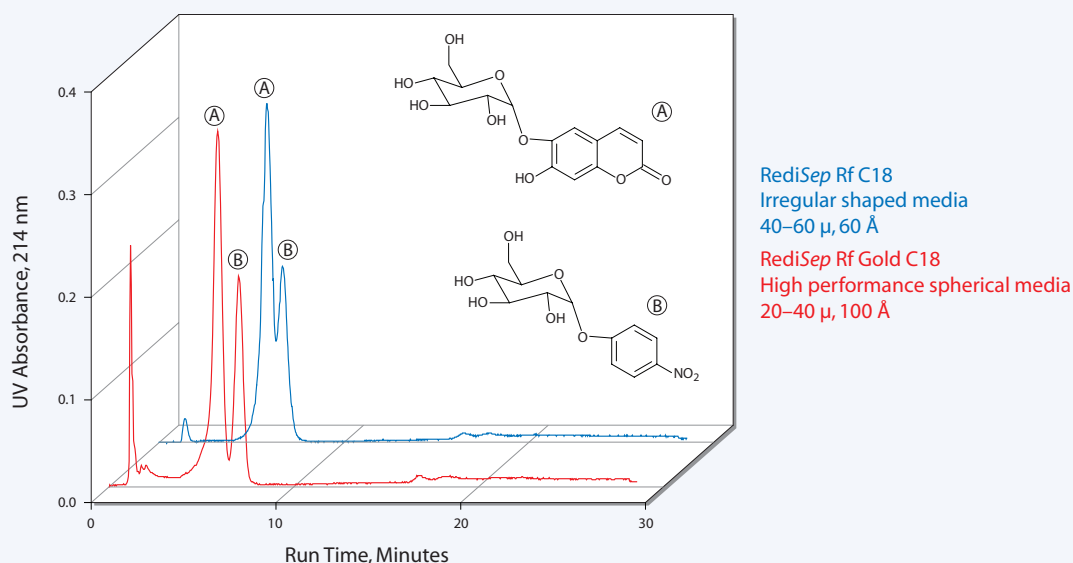


Figure 5:
Purification of esculin monohydrate and 4-nitrophenyl α -(D) glucopyranoside on RediSep Rf C18 (blue trace) and RediSep Rf Gold C18 (red trace)

Conclusion:

Compounds containing carbohydrate moieties are easily purified on Medium Pressure Liquid Chromatography equipment such as the *CombiFlash* Rf system. All-Wavelength Collection enables purification of compounds possessing weak chromophores, even when solvents also absorb UV light and cause drifting baselines. Alternative detectors such as ELSD, Refractive Index, and others can be used with MPLC equipment for compounds that do not absorb UV light. Higher performance columns enable improved purity of closely eluting compounds.

References

- 1 Silver, J.E. All-Wavelength Detection: A new detection technique for MPLC Chromatography suitable for Natural Products. Presented at the 50th meeting of The American Society of Pharmacognosy, July 2009; poster P-181.
- 2 Webster, G.K.; Jensen, J.S.; Diaz, A.R. Investigation into Detector Limitations Using Evaporative Light-Scattering Detectors For Pharmaceutical Applications. *J. Chromatographic Science*, **Oct 2004**, 42, 484
- 3 Snyder, L.R.; Kirkland, J.J. *Introduction to Modern Liquid Chromatography*, 2nd edition; John Wiley & Sons: New York, 1979; p 177
- 4 Silver, J.E.; Bellinghausen, P.; Fowler, N.; Scanlon, C. Advances in Medium Pressure Liquid Chromatography. Presented at the 237th meeting of The American Chemical Society, March 2009; poster MEDI 33.
- 5 Silver, J.E.; Bilger, E.; Crea, T.R.; Pipes, R. Spherical silica shows increased loading capacity compared to irregular silica in MPLC. Presented



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