Waters™

Application Note

Mass Directed Purification of Glucoraphanin from Broccoli Seeds using Preparative Scale Hydrophilic-Interaction Chromatography (HILIC)

Andrew J. Aubin

Waters Corporation

Abstract

Broccoli seeds have been shown to be rich in glucoraphanin and are easy to obtain, making them a good choice for the isolation of glucoraphanin on a preparative scale. This application note describes the mass-directed purification of glucoraphanin from broccoli seeds using the Waters AutoPurification System with the 3100 Mass Detector and an Atlantis Hydrophilic-Interaction Chromatography (HILIC) Silica Column.

Introduction

Broccoli has long been considered a healthy food choice. Recent research and news reports have stated that eating the sprouts of broccoli may reduce the risk of stroke, high blood pressure, and cardiovascular disease.

The phytochemical compound most often associated with these health benefits is glucoraphanin (4-methylsulfinylbutyl glucosinolate, Figure 1). Glucoraphanin is the most predominant glucosinolate in broccoli and has been thought to have cancer-prevention qualities, along with the other aforementioned health benefits.

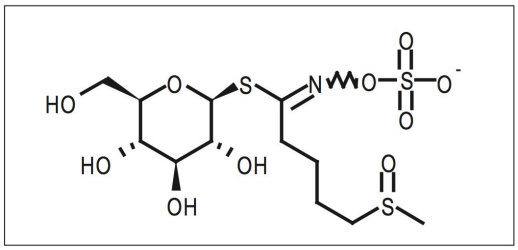

Figure 1. Glucoraphanin $C_{12}H_{22}NO_{10}S_3$.

As researchers continue to investigate the health effects of glucoraphanin, there is a need to have on hand suitable quantities of pure glucoraphanin reference material to accurately determine amounts of glucoraphanin in various vegetables, and for investigations for the use of glucoraphanin as an active compound.

Broccoli seeds have been shown to be rich in glucoraphanin (20 to 50 mg/g) and are easy to obtain, making them a good choice for the isolation of glucoraphanin on a preparative scale. This application describes the mass-directed purification of glucoraphanin from broccoli seeds using the Waters AutoPurification System with the 3100 Mass Detector (Figure 2) and an Atlantis Hydrophilic-Interaction Chromatography (HILIC) Silica Column.

Figure 2. Mass-directed AutoPurification System, with the 3100 Mass Detector.

Experimental

Glucoraphanin was extracted from broccoli seeds using a method similar to that of Rochfort et al.[1] Broccoli seeds (25 g) were boiled in deionized water (250 mL) for five minutes and then homogenized to a slurry. Following sonication for 30 minutes, 25 g of Celite 545 was added to the mix and the entire slurry passed through a glass fiber filter. The clear extracts were reduced in volume by 10 times prior to chromatography.

The isocratic separation method was first developed on an analytical scale (4.6 x 150 mm Atlantis HILIC Silica Column, 5 µm at 2 mL/min). Separation conditions were scaled up to the preparative column using the following calculations.

Scaling injection volume

$$Vol_{PREP} = Vol_{HPLC} \bullet \frac{D^2{}_{PREP}}{D^2{}_{HPLC}} \bullet \frac{L_{PREP}}{L_{HPLC}}$$

Here, Vol is the injection volume (µL), D is the inner diameter of the column (mm), and L is the column length (mm). A 5-µL injection on a 4.6 x 150 mm HPLC column corresponds to a 57-µL injection on a 19 x 100 mm preparative column.

Experiments showed that an injection volume of up to 100 µL could be used on the prep column without degradation of the chromatographic peak shape. Higher injection volumes negatively impacted peak shapes primarily due to the high solvent strength of the sample diluent.

Scaling flow rate

Based on column dimensions, the following equation is used to geometrically scale flow rate:

$$F_{PREP} = F_{HPLC} \bullet \frac{D^2{}_{PREP}}{D^2{}_{HPLC}}$$

Here, F is flow rate (mL/min) and D is the diameter of the columns (cm). A 2.0 mL/min flow rate on a 4.6 mm I.D. column equates to a 34.1 mL/min flow rate on a 19 mm I.D. column. The Waters Prep Calculator (Figure 3) was used to convert the analytical separation method to the preparatory separation method.

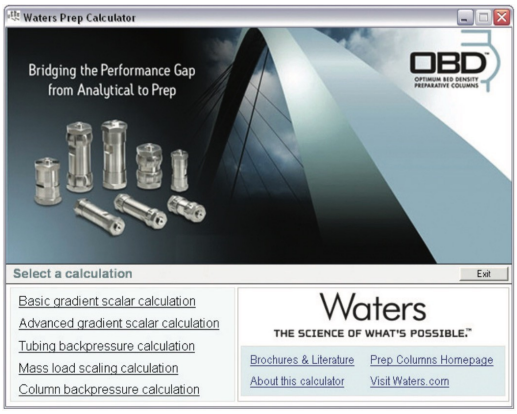

Figure 3. The Prep Calculator entry screen.

Elution of glucoraphanin from the preparatory column was monitored by mass spectrometry in electrospray negative mode and the fraction collection was triggered based on mass. The glucoraphanin fractions from each injection were combined in a single collection vessel. Collected fractions were reduced to dryness under vacuum and subsequently analyzed using a conventional HPLC system (detection by UV at 220 nm) to estimate the purity of the isolated glucoraphanin.

An exact mass measurement of the isolated material was performed using the Waters LCT Premier Mass Spectrometer. The measured *m/z* of 436.0409 was within 0.7 ppm of the theoretical value of 436.0406 for glucoraphanin.

When performing elemental composition analysis, the i-FIT algorithm was used. This algorithm compares the theoretical isotopic distributions of proposed elemental compositions against the experimentally-measured isotopic distributions. For each proposed chemical formula a numerical value is calculated that reflects the “goodness of fit” between the measured and theoretical distributions. The measured value for glucoraphanin was the top hit, with the next possibility giving an i-FIT value 32 times higher.

Analytical LC conditions

LC system:	Alliance HPLC System
Detector:	2996 PDA Detector
Column:	Atlantis HILIC, 5 µm 4.6 x 150 mm
Column temp.:	25 °C
Flow rate	2 mL/min (Isocractic 7% A, 93% B)
Mobile phase A:	50 mM Ammonium formate, pH 6.5
Mobile phase B:	Acetonitrile

Preparative LC conditions

LC system:	AutoPurification System
Pump:	1545 Binary Gradient Module
Injector/collector:	2767 Sample Manager
Detector:	3100 Mass Detector and 2996 PDA Detector
Fluidics:	System Fluidics Organizer
Column:	Atlantis HILIC Optimum Bed Density (OBD), 5 µm 19 x 100 mm
Column temp.:	Ambient
Flow rate:	34 mL/min (Isocractic 7% A, 93% B)
Mobile phase A:	50 mM Ammonium formate, pH 6.5
Mobile phase B:	Acetonitrile

MS conditions

MS system:	3100 Mass Detector
Ionization mode:	ESI Negative
Capillary voltage:	3400 V
Cone voltage:	25 V
Desolvation temp.:	500 °C
Desolvation gas:	800 L/Hr
Source temp.:	150 °C
Acquisition range:	150 to 650 *m/z*

Results and Discussion

Glucoraphanin is a relatively polar substance. Separation of glucoraphanin from other related glucosinolates and other polar extractables by reverse phase chromatography proved be difficult, with glucoraphanin eluting close to the void volume. Hydrophilic-Interaction Chromatography (HILIC) was evaluated and subsequently used for this separation.

HILIC is a separation technique that can retain and separate polar, water-soluble organic compounds. HILIC is often described as a variation of normal-phase chromatography. In normal-phase chromatography, the mobile phase is 100% organic, however using HILIC, water (usually <20%) is added to the organic mobile phase (typically an aprotic solvent like acetonitrile) making it possible to retain, separate, and elute polar compounds.

Water, a very polar solvent, competes effectively with polar analytes for the stationary phase. Polar compounds that are initially attracted to the polar packing material are eluted by modifying and increasing the polarity of the mobile phase by adding more water. Analytes are eluted in order of increasing hydrophilicity, i.e. chromatographic polarity relative to water. In the case of the broccoli seed extract, glucoraphanin is the most polar glucosinolate leaving it highly retained using HILIC.

A further advantage to using HILIC is its ability to reduce the time required to dry down samples since fractions are eluted in predominantly organic solvent. This makes evaporation and dry down significantly faster compared to reverse-phase methods where fractions are often predominantly water. Column backpressures are typically lower, due to the lower viscosity of the highly organic mobile phases, allowing for higher flow rates that in turn lead to higher sample throughput.

As was described previously, the analytical separation method was transferred to a preparatory scale. Fraction collection was triggered based on mass. In this technique (referred to as mass-directed purification), a small portion of the column effluent is spilt off and directed to the mass spectrometer. Following the split, a make-up pump is used to increase the flow rate of the splitter effluent to ensure that the data from the mass spectrometer can be processed by MassLynx Software in time to divert the column flow to the fraction collector.

Collection is triggered when the mass spectrometer detects the user-defined mass (in the case of glucoraphanin, *m/z* 436). This technique allows very precise collection control, as fraction collection is only triggered by a peak containing the mass (or masses) of interest, ultimately resulting in fewer fractions of higher quality. An example of the mass spectrometer output can be seen in Figure 4. The pink shaded area represents the collected peak fraction.

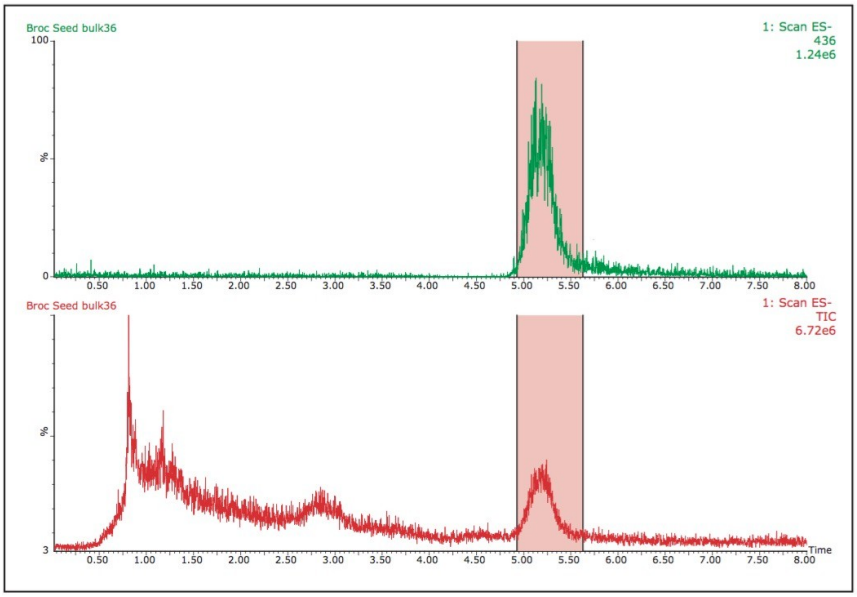

Figure 4. Mass-directed purification of broccoli seed extract. The shaded area represents the collected peak fraction.

In this purification example, a series of thirty 100-µL injections of concentrated aqueous-based broccoli extract were performed. Each injection liberated a fraction containing glucoraphanin in approximately 23 mL of mobile phase. All fractions from all injections were collected into a single container. The combined fractions were reduced to dryness with ~139 mg of dry material remaining. The identity of liberated crystal material was confirmed to be glucoraphanin using an exact mass time-of-flight mass spectrometer.

Purity of the material was evaluated using the analytical HPLC method with UV detection at 220 nm and was found to be 95% (area percent basis). The raw starting material evaluated for purity using the same technique was found to be ~ 53%. Before and after chromatograms are pictured in Figure 5.

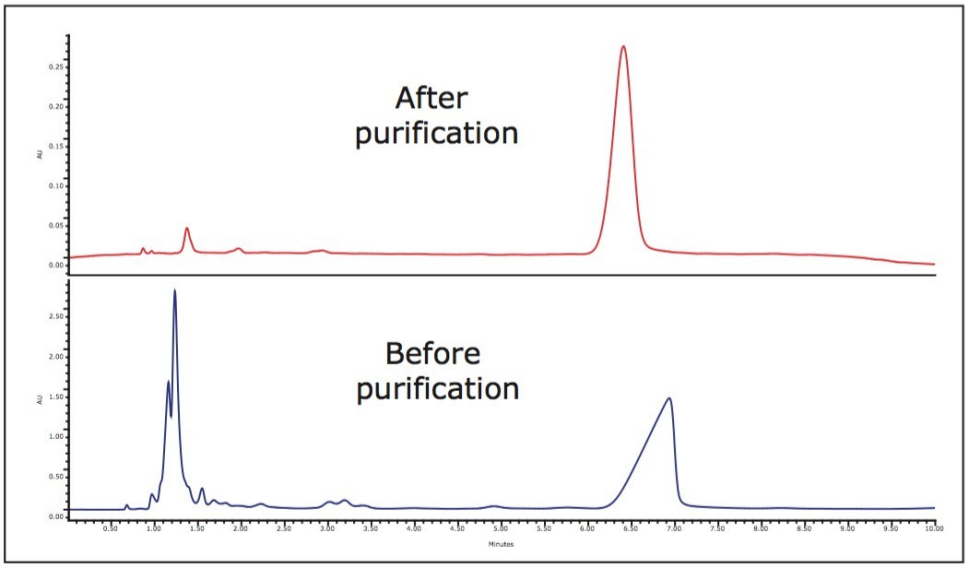

Figure 5. Broccoli extract before (bottom) and after (top) purification.

Conclusion

- Preparatory-Scale Hydrophilic-Interaction Chromatography (HILIC) provided a useful separation technique for the isolation of the polar glucoraphanin. Higher throughput is possible due to reduced fraction dry down times and the opportunity to use higher flow rates.
- An increase in purity from 53% to 95% was accomplished using mass-directed purification for the isolation of glucoraphanin from broccoli seeds.
- A total of 139 mg of glucoraphanin was isolated in this example. This amount is consistent with published values.
- Exact mass TOF-MS analysis of the purified fraction confirmed the presence of glucoraphanin.

References

1. Rochfort et al. *Journal of Chromatography A*. 2006; 1120: 205-210.

Featured Products

AutoPurification System

Alliance HPLC System

MassLynx MS Software

720002699, June 2008