APPLICATIONS

Fast Analysis of Glucosinolates in Cruciferous Vegetable Extracts Using a Luna® 3 µm HILIC Column by HPLC-UV

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Abstract
Glucosinolates are very unique molecules found in cruciferous vegetables that are currently under investigation for potential cancer inhibiting properties. Luna 3 µm HILIC columns provide chromatographers a simple and robust method for providing reproducible separations of glucosinolates from cruciferous vegetable extracts. With a fast retention time (<10 min), this method provides the opportunity to substantially increase sample throughput and simultaneously decrease costs per each analysis.

Introduction
Glucosinolates are secondary metabolites normally found in cruciferous vegetables and are precursors of isothiocyanates. Over 100 different glucosinolates have been identified in a different variety of vegetables such as broccoli, mustard seed, and Brussels sprouts. The separation and identification of glucosinolates can be performed by HPLC analysis. Due to the highly polar nature of glucosinolates, hydrophilic interaction liquid chromatography (HILIC) is a more effective technique over traditional reversed phase interactions for separation of these compounds. HILIC uses mostly organic hydrophobic mobile phase with a hydrophilic stationary phase to elute polar compounds such as glucosinolates in order of increasing hydrophilicity.

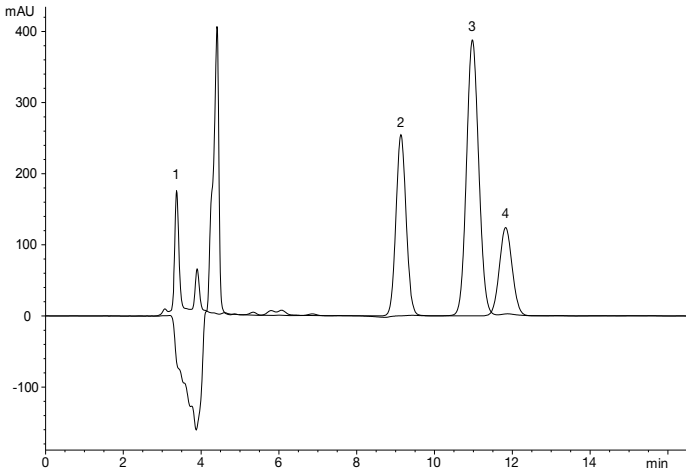
Plant Description
Cruciferous vegetables are part of the Brassicaceae family (also called Cruciferae). These vegetables are widely cultivated, with many species being raised for food production such as the cabbage family, broccoli, Brussels sprouts and similar green leaf vegetables. These vegetables are known for having flowers with petals arranged in groups of four that are situated in the form of a cross. This is the reason the family takes its alternate name Cruciferae, which means “cross-bearing” in New Latin.

Overview
Cruciferous vegetables are unique in that they are rich sources of sulfur-containing compounds known as glucosinolates. From a chemistry point of view, glucosinolates are sugar-based molecules that contain a modified form of sugar (glucose) together with sulfur and nitrogen. Glucosinolates aren’t found exclusively in cruciferous vegetables, but they are overwhelmingly absent from most other food groups. Over 100 different glucosinolates have been identified in cruciferous vegetables.

Reagents and Chemicals
HPLC analysis was performed using an Agilent® 1100 LC System (Agilent Technologies Inc., Palo Alto, CA, USA). The system was optimized in order to reduce dead volume and improve performance including increasing the UV scan rate, changing the injector needle seat, re-plumbing the system with red PEEKsil™ Tubing (SGE), and using a semi-micro flow cell. The fully porous Luna 3 µm HILIC 100 x 4.6 mm columns were from Phenomenex, Torrance, CA. The method was used to analyze cruciferous glucosinolates in vegetable extracts (broccoli, Brussels sprouts, kale, wasabi, red cabbage, mustard seeds) from Procaps Labs, Henderson, NV. The extraction protocol also used a VWR® Standard Analog Shaker and a Thermo Scientific Sorvall™ Legend XTR Centrifuge.

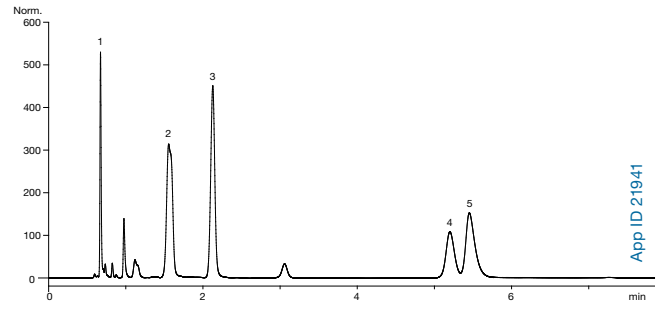
The glucosinolate reference materials: Glucotropaeolin, Sulforaphane, Singirin, Glucoraphanin, and Glucoiberin were provided by ChromaDex®.

Experimental Conditions
Figure 1.
Glucosinolate Reference Standards Mixture Using a Fully Porous 5 µm HILIC HPLC Column



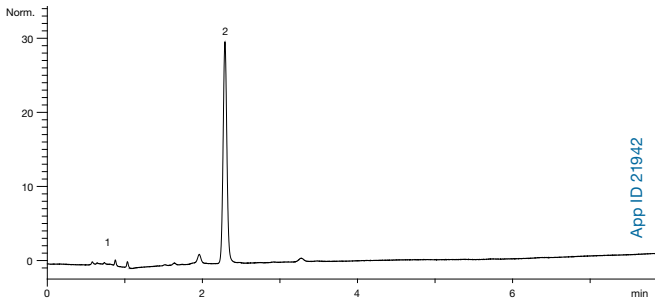
Column: Fully Porous 5 µm HILIC
Dimensions: 100 x 4.6 mm
Mobile Phase: 15 mM Ammonium Formate, (pH 4.5) in 70:30 Acetonitrile:Water
Isocratic: Hold for 20 min
Flow Rate: 0.5 mL/min
Temperature: 25 °C
Detection: UV @ 235 nm
Sample: 1. Sulforaphane
2. Sinigrin
3. Glucoraphanin
4. Glucoiberin

Figure 2.
Glucosinolate Reference Standards Mixture Using a Luna® 3 µm HILIC HPLC Column



Column: Luna 3 µm HILIC
Part No.: 00D-4449-E0
Dimensions: 100 x 4.6 mm
Mobile Phase: A : 30 mM Ammonium Formate, (pH 7.0) in 85/15 Acetonitrile/Water
B : Acetonitrile
Isocratic: A/B (70/30) hold for 8 min
Flow Rate: 2 mL/min
Temperature: 25 °C
Detection: UV @ 235 nm
Sample: 1. Sulforaphane
2. Glucotropaeolin
3. Sinigrin
4. Glucoraphanin
5. Glucoiberin

Figure 3.
Mustard Seed Extract Using a Luna 3 µm HILIC HPLC Column



Column: Luna 3 µm HILIC
Part No.: 00D-4449-E0
Dimensions: 100 x 4.6 mm
Mobile Phase: A : 30 mM Ammonium Formate, (pH 7.0) in 85/15 Acetonitrile/Water
B : Acetonitrile
Isocratic: A/B (70/30) hold for 8 min
Flow Rate: 2 mL/min
Temperature: 25 °C
Detection: UV @ 235 nm
Sample: 1. Sulforaphane
2. Sinigrin

Figure 4.
Representative Calibration Curve for Glucosinolates from 1 to 1000 µg/mL

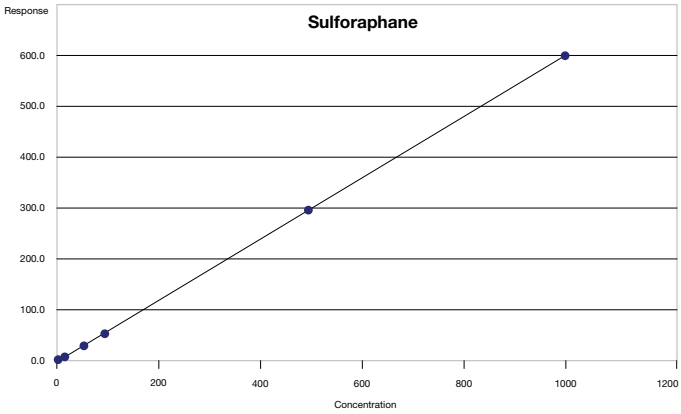


Table 1.
Calibration Curve from Luna 3 µm HILIC Column Based on ChromaDex® Reference Materials

Compound	Linearity Equation	R^2	LOD
Sulforaphane	y = 0.5954x - 2.2947	0.9998	1.0 µg/mL
Glucotropaeolin	y = 1.6421x - 7.2664	0.9997	1.0 µg/mL
Sinigrin	y = 1.6776x - 6.3077	0.9998	1.0 µg/mL
Glucoraphanin	y = 0.8239x - 4.7235	0.9997	5.0 µg/mL
Glucoiberin	y = 1.2701x - 6.3602	0.9997	5.0 µg/mL

Table 2.
Determination of Method Accuracy (%) at Low, Med, and High Concentrations

Compound	50 µg/mL	100 µg/mL	500 µg/mL
Sulforaphane	97.2	98.3	98.1
Glucotropaeolin	98.1	98.2	97.7
Sinigrin	99.3	98.1	97.3
Glucoraphanin	100.4	97.8	97.5
Glucoiberin	100.3	98.1	97.7

Accuracy based on three replicated injections of each concentrated standard.

Results and Discussion

Due to the polar nature of glucosinolates, compounds will not retain and separate as well on a HPLC using a traditional reversed phase C18 column. HILIC uses a mostly organic hydrophobic mobile phase with a hydrophilic stationary phase which elutes polar compounds such as glucosinolates in order of increasing hydrophilicity. Luna® HILIC columns retain a water-enriched layer on the surface of the silica which facilitates the transfer of polar compounds into the stationary phase for increased retention. This results in superior retention of polar compounds. For this reason, the use of HILIC columns is a more effective technique over a traditional reversed phase column to separate glucosinolates.

The original method provided by ChromaDex® incorporated a fully porous 5 µm HILIC column. The methodology had been routinely used by ChromaDex for analytical testing. The Luna 3 µm HILIC column combined with gradient condition changes provided excellent separation, reduced analysis time by over 45%, and increased sensitivity for the glucosinolates compared to the other HILIC column. Resolution and elution order were similar to the existing method (**Figures 1** and **2**). The method was also used to analyze commercially available extracts provided by Procaps Lab and to identify certain glucosinolates in vegetable extract such as mustard seeds (**Figure 3**).

Having demonstrated that the new method provided improved results, we performed experiments to determine linearity, accuracy, range, and limit of detection (LOD). To quantitate the components of interest with this methodology, a linearity curve was produced for each component based on the ChromaDex reference standard and the corresponding linear equation from this data was used for each component. Methods were shown to be linear over a range of 1 to 1,000 µg/mL (**Figure 4**).

The LOD was determined to be in the range of 1 to 5 µg/mL for all of the compounds (**Table 1**). This methodology produced a Signal to Noise ratio > 10 for the glucoiberin peak, which was the smallest peak of interest. Accuracy was determined at three different levels, 50, 100, and 500 µg/mL and was found to be less than 4% indicating the method is quite reproducible (**Table 2**).

Conclusion

The benefits of glucosinolates may lead to many neutraceutical companies adding glucosinolates to their formulations. As a result we have proposed an improved methodology for determination of glucosinolates in cruciferous vegetable extracts.

HPLC analysis times were reduced by over 45% by adapting the current method to the Luna 3 µm HILIC. We performed experiments to verify that the methodology was suitable for nutraceutical products. Final results indicate that this method is robust and ready for routine sample analysis.

References

Linus Pauling Institute: Micronutrient Research for Optimum Health. Oregon State University. *http:///lpi.oregonstate.edu/infocenter/foods/cruciferous/*

Shapiro T. A., Fahey J. W., Wade K. L., Stephenson K. K., Talalay P., Human metabolism and excretion of cancer chemoprotective glucosinolates and isothiocyanates of cruciferous vegetables. Cancer Epidemiol. Biomarkers Prev., 7: 1091-1100, 1998.

Troyer JK, Stephenson KK, Fahey JW. Analysis of glucosinolates from broccoli and other cruciferous vegetables by hydrophilic interaction liquid chromatography. J Chromatogr A. 2001 Jun 15; 919(2): 299-304.

Wade KL, Garrard IJ, Fahey JW., Improved hydrophilic interaction chromatography method for the identification and quantification of glucosinolates. J Chromatogr A. 2007 Jun 22; 1154(1-2): 469-72. Epub 2007 Apr 20.

ChromaDex Ordering Information: Phytochemical Reference Standards

Description	Quantity	Part No.
SULFORAPHANE, DL-(SH)	5 mg	ASB-00019385-005
SULFORAPHANE, DL-(SH)	10 mg	ASB-00019385-010
SULFORAPHANE, DL-(SH)	25 mg	ASB-00019385-025
GLUCOTROPAEOLIN POTASSIUM SALT(SH)	5 mg	ASB-00007300-005
GLUCOTROPAEOLIN POTASSIUM SALT(SH)	10 mg	ASB-00007300-010
GLUCOTROPAEOLIN POTASSIUM SALT(SH)	100 mg	ASB-00007300-100
SINIGRIN POTASSIUM SALT(P)	10 mg	ASB-00019270-010
SINIGRIN POTASSIUM SALT(P)	25 mg	ASB-00019270-025
GLUCORAPHANIN POTASSIUM SALT(P)	10 mg	ASB-00007298-010
GLUCORAPHANIN POTASSIUM SALT(P)	25 mg	ASB-00007298-025
GLUCORAPHANIN POTASSIUM SALT(SH)	10 mg	ASB-00007297-010
GLUCORAPHANIN POTASSIUM SALT(SH)	25 mg	ASB-00007297-025
GLUCOIBERIN POTASSIUM SALT(SH)	10 mg	ASB-00007250-010

See reverse for ChromaDex contact information

Phenomenex Ordering Information: Luna 3 µm and 5 µm HILIC Columns

3 µm Analytical Columns (mm)									SecurityGuard™ Cartridges (mm)	
Phases	50 x 2.0	100 x 2.0	150 x 2.0	50 x 3.0	100 x 3.0	150 x 3.0	100 x 4.6	150 x 4.6	4 x 2.0*	4 x 3.0*
HILIC	00B-4449-B0	00D-4449-B0	00F-4449-B0	00B-4449-Y0	00D-4449-Y0	00F-4449-Y0	00D-4449-E0	00F-4449-E0	AJ0-8328	AJ0-8329
									for ID: 2.0-3.0 mm	3.2-8.0 mm

5 µm Analytical Columns (mm)							SecurityGuard Cartridges (mm)	
Phases	100 x 2.0	100 x 3.0	150 x 3.0	100 x 4.6	150 x 4.6	250 x 4.6	4 x 2.0*	4 x 3.0*
HILIC	00D-4450-B0	00D-4450-Y0	00F-4450-Y0	00D-4450-E0	00F-4450-E0	00G-4450-E0	AJ0-8328	AJ0-8329
							for ID: 2.0-3.0 mm	3.2-8.0 mm

5 µm Axia™ Packed Preparative Columns (mm)					SecurityGuard Cartridges (mm)	
Phases	50 x 21.2	100 x 21.2	150 x 21.2	250 x 21.2	4 x 3.0*	10 x 10‡
HILIC	00B-4450-P0-AX	00D-4450-P0-AX	00F-4450-P0-AX	00G-4450-P0-AX	AJ0-8329	AJ0-8902
					for ID: 3.2-8.0 mm	9-16 mm

For additional phases and dimensions please visit www.phenomenex.com

*SecurityGuard Analytical Cartridges require holder, Part No.: KJ0-4282
‡SemiPrep SecurityGuard Cartridges require holder, Part No.: AJ0-7220

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guarantee

If Phenomenex products in the technical note do not provide at least an equivalent separation as compared to other products of the same phase and comparable dimensions, return the product with comparative data within 45 days for a FULL REFUND.

Scientifically Valid

Section 21CFR111.320 of cGMPs for Dietary Supplements requires you to "identify and use an appropriate scientifically valid method for each established specification for which testing or examination is required to determine whether the specification is met". The FDA does not elaborate on what is considered a scientifically valid method in the cGMPs. ChromaDex has defined scientifically valid as a method that meets minimum linearity, precision, sensitivity and range requirements. These requirements are outlined in an FDA laboratory document, ORA LABORATORY PROCEDURE Food and Drug Administration, ORA-LAB.5.4.5. This laboratory guidance document defines minimal performance attributes for selected methods of analysis and has been applied by ChromaDex to the selection of methods that are fit for purpose in the dietary supplements industry. According to the above definition, the method detailed in this document is considered scientifically valid as applicable to the cGMP requirements. Product specific, full method validations according to AOAC guidelines can be applied to customer samples upon request, to further document method performance in specific samples and matrices.

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