Agilent InfinityLab

Agilent InfinityLab LC Solutions

# Methods for High-Quality, Safe, and Authentic Herbal Products

Application Compendium

Agilent

Trusted Answers

# Introduction

The American Herbal Products Association estimates that there as many as 3,000 plant species in commerce. The FDA estimates that there are about 85,000 dietary supplement products in the marketplace. The rapid rate of new product introduction far outpaces efforts to produce reliable analytical methods that verify botanical identity, quality, and strength.

Unlike single-chemical entity active pharmaceutical ingredients (APIs), botanical raw materials and their derived products are highly variable. Their chemistry and morphology depend on the genotypic and phenotypic variation, geographical origin, weather exposure, harvesting practices, and processing conditions. Unless controlled, this inherent variability in the raw materials can result in inconsistent finished products that are underpotent, overpotent, or contaminated.

The role of reliable measurements in regulatory settings has obvious public health implications. Tight control over active ingredients, nutrients, and other constituents of foods and supplements are necessary for safety and efficacy. Most validation guidelines require a demonstration of method applicability, selectivity, linearity, repeatability, accuracy, limit of detection, limit of quantitation, and stability.

Published methods for botanical analysis mostly focus on the quantitation of target compounds in raw ingredients. These methods are often tedious and not suitable for routine ingredient analysis or finished product testing because of low throughput and matrix effects. For routine testing, it is desirable to develop concise, simple methods with optimized extraction time and as few sample transfers as possible. These method attributes minimize analyte loss and improve method precision.

Agilent InfinityLab LC instruments, columns, and supplies deliver the rugged quality and robust analytical results that are needed for the routine testing of herbal products. Every component of the Agilent InfinityLab family is uniquely designed to work together and to help continuously improve workflow efficiency.

InfinityLab LC Series instruments are modular, providing flexibility to ensure the best configuration for any application. While Agilent InfinityLab Poroshell 120 LC columns are robust, flexible, and they reliably achieve the highest efficiency and resolution via superficially porous particle technology. Also, the innovative design of InfinityLab supplies enables ease-of-use and improved results for everyone in any lab. InfinityLab LC components help every scientist get the most from their applications with innovations that improve uptime, minimize rework, and simplify operation.

This application compendium contains seven validated methods for botanical raw materials and finished herbal products. All methods demonstrate the performance of Agilent InfinityLab LC Solutions to deliver the fast, accurate, and reproducible results required for routing testing—ultimately ensuring high-quality, safe, and authentic herbal products.

Learn more about InfinityLab at www.agilent.com/chem/infinitylab

# Table of Contents

# Quantifying Anthocyanins in Cranberry Raw Materials and Finished Products

## Using UHPLC/UV and an Agilent ZORBAX Rapid Resolution HT StableBond C18 column

Application Note

Food and Beverage Testing


**Authors**

Ronan Yu, Ying Liu, and Paula Brown
BCIT, Natural Health and Food Products Research Group
Burnaby, British Columbia, Canada

Udo Huber
Agilent Technologies, Inc.
Waldbronn, Germany



## Abstract

This method quantifies five anthocyanins commonly found in cranberry raw materials and finished products. The anthocyanins are cyanidin-3-O-glucoside, cyanidin-3-O-galactoside, cyanidin-3-O-arabinoside, peonidin-3-O-galactoside, and peonidin-3-O-arabinoside. The anthocyanin separation was achieved on an Agilent ZORBAX RRHT StableBond C18, 4.6 × 50 mm, 1.8 µm column in 11 minutes, including post time, on an Agilent 1290 Infinity II LC System.


## Introduction

Cranberries (genus *Vaccinium*) are cultivated from evergreen dwarf shrubs or trailing vines throughout the northern United States, Canada, and Chile for their fruit.[1] Most harvested cranberries are processed into juices, sauces, and dried cranberry products. Fresh raw cranberries are light green and turn into a deep red color when ripe due to their anthocyanin content. Anthocyanins (anthocyanidins with a sugar moiety) belong to a class of flavonoids that have been shown to have antioxidant properties *in vitro*.[2] The primary anthocyanins in cranberry are cyanidin-3-O-galactoside, cyanidin-3-O-arabinoside, peonidin-3-O-galactoside, peonidin-3-O-arabinoside, and lesser amounts of cyanidin-3-O-glucoside and petunidin-3-O-galactoside.[3]

## Experimental

The Agilent 1290 Infinity II LC System was composed of the following modules:

- Agilent 1290 Infinity II Flexible Pump (G7104A)
- Agilent 1290 Infinity II Multisampler with Integrated Thermostat (G7167B, option 101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with Agilent InfinityLab Quick Connect heat exchanger, standard flow
- Agilent 1290 Infinity II Diode Array Detector (G7117B) equipped with an Agilent InfinityLab Max-Light Cartridge Flow Cell, 10 mm

Return to Contents Section

## Software

– Agilent OpenLab software suite, version 2.3 or later

## Column

– Agilent ZORBAX RRHT StableBond C18, 4.6 × 50 mm, 1.8 µm (part number 827975-902)

## Samples

Cranberry powder and juice samples were provided by United States Pharmacopeia (USP), and anthocyanin standards were purchased from Alkemist Labs.

**Sample preparation for freeze-dried raw materials and powdered extracts:** Freeze-dried cranberries and cranberry extracts were ground into powders to ≤60 mesh size. A 250 mg (±1 mg) test sample was weighed into a 50 mL centrifuge tube. Then, 20 mL of extraction solvent was added using a graduated cylinder, vortexed for 10 seconds, and then sonicated for 15 minutes. The centrifuge tube was transferred to a wrist action shaker, where it shook at a 45° angle at 180 rpm for 30 minutes. The centrifuge tube was vortexed for 10 seconds and centrifuged at 5,000 rpm for 5 minutes. The supernatant was decanted into a 25 mL volumetric flask and brought to volume with extraction solvent. The volumetric flask was inverted 20 times to mix, and then approximately 1 mL of the solution was filtered through a 0.45 µm PTFE syringe filter into an amber HPLC vial.

**Sample preparation for juices:** The juice was thoroughly mixed by inverting the container about 20 times or until no sediment was visible. 10 mL of juice was transferred into a centrifuge tube and centrifuged at 5,000 rpm for 10 minutes. A 1 mL aliquot of the juice was diluted using a dilution factor of 1:5 (1 part juice to 4 parts of solvent) with the extraction solvent. It should be noted that the 1:5 dilution factor is a recommendation. If the juice is more concentrated, then a higher dilution might be required to quantify the anthocyanins. Then, approximately 1 mL of the juice was filtered through a syringe fitted with a 0.45 µm nylon filter into an amber HPLC vial.

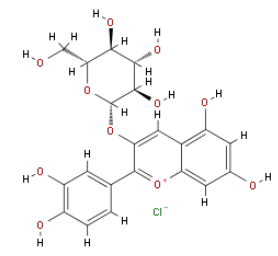
**Sample preparation for juice cocktails:** The juice cocktail was thoroughly mixed by inverting the container approximately 20 times or until no sediment was visible. 10 mL of cocktail juice was transferred into a centrifuge tube and centrifuged at 5,000 rpm for 10 minutes. A 1 mL sample of the cocktail juice was then filtered through a syringe fitted with a 0.45 µm nylon filter into an amber HPLC vial.

The reference diluent solvent was the same as the extraction solvent, which was methanol and concentrated (36.5 to 38.0%) hydrochloric acid (98:2, v/v).

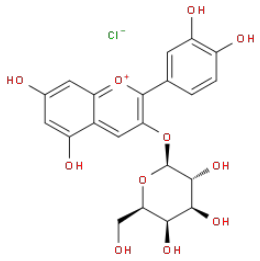
Return to Contents Section

## Compounds

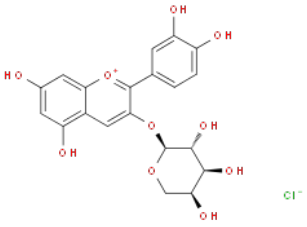
The anthocyanins of interest are shown in Figure 1 with their chemical structures.



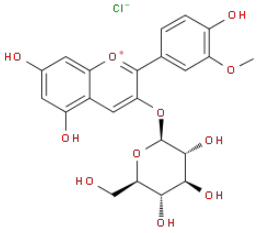
Cyanidin-3-O-glucoside chloride



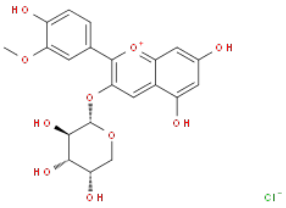
Cyanidin-3-O-galactoside chloride



Cyanidin-3-O-arabinoside chloride



Peonidin-3-O-glucoside chloride



Peonidin-3-O-arabinoside chloride

**Figure 1.** Chemical structures of the anthocyanins of interest.

A seven-point standard curve of mixed standards was used to quantify the select anthocyanins of interest. The HPLC method parameters are shown in Table 1.

**Table 1.** LC method parameters for analyzing compounds in cranberry samples.

| Parameter | Value |
|---|---|
| Column | Agilent ZORBAX RRHT StableBond C18, 4.6 × 50 mm, 1.8 µm |
| Solvent | A) water and phosphoric acid (99.5:0.5, v/v)<br>B) water, acetonitrile, acetic acid, and phosphoric acid (50.0:48.5:1.0:0.5, v/v) |
| Gradient | Time (min) %B<br>0.00 9<br>8.00 36<br>8.50 60<br>9.00 80<br>9.10 9<br>10.5 9<br>Stop time: 10.5 min<br>Post time: 0.5 min |
| Flow Rate | 2.1 mL/min |
| Temperature | 25 °C |
| Detection | 520, 8 nm; reference off |
| Injection | Injection volume: 5 µL<br>Sample temperature: 5 °C<br>Needle wash: off |



Return to Contents Section

## Results and discussion

Thanks to the excellent performance of the 1290 Infinity II LC System, all compounds were separated with a resolution of at least 0.80 (Figures 2 and 3) and have an area percent relative standard deviation (% RSD) of less than 10%. The approximate expected retention times for the following anthocyanins are: cyanidin-3-O-glucoside (3.9 to 4.0 minutes), cyanidin-3-O-galactoside (4.3 to 4.4 minutes), cyanidin-3-O-arabinoside (4.6 to 4.7 minutes), peonidin-3-O-galactoside (5.0 to 5.1 minutes), and peonidin-3-O-arabinoside (5.7 to 5.8 minutes).

The method was precise and accurate in quantifying all target compounds, as also demonstrated in a previously published single-laboratory validation study that used a longer format, 5 µm C18 column.[4] With the shorter, more efficient ZORBAX RRHT StableBond C18 column, the analysis of the five anthocyanins can be accomplished in less than 10 minutes, which translates to a shorter separation time and less solvent consumption than conventional high performance liquid chromatography methods.[4–6]

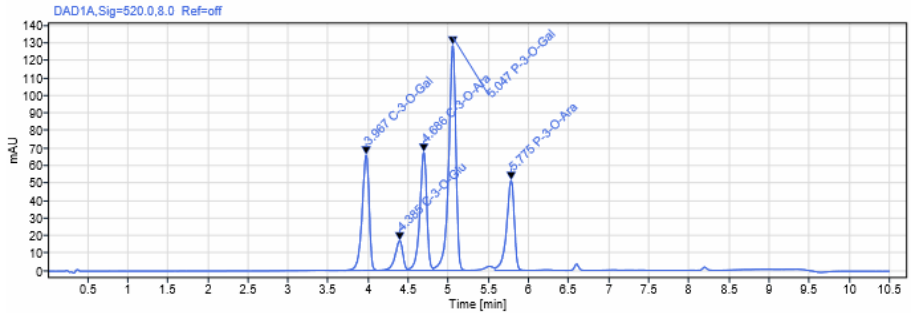



**Figure 2.** Chromatogram of the anthocyanin standards.

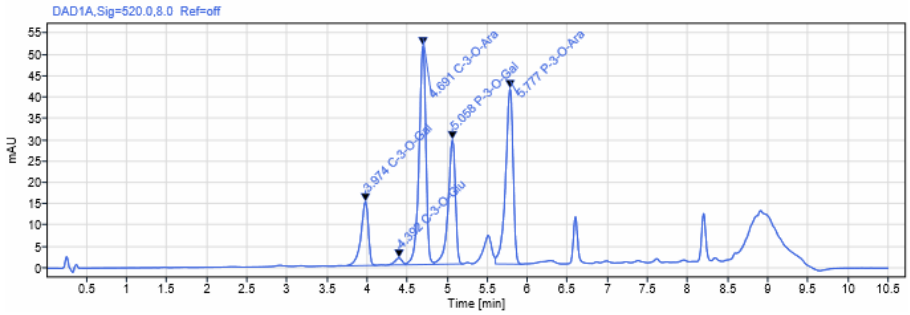



**Figure 3.** Chromatogram of a USP cranberry powdered raw material.

## Conclusion

This UHPLC method can quantify cyanidin-3-O-glucoside, cyanidin-3-O-galactoside, cyanidin-3-O-arabinoside, peonidin-3-O-galactoside, and peonidin-3-O-arabinoside in cranberry raw material and finished products in less time, with lower solvent consumption than traditional HPLC methods.

Return to Contents Section

## References


1. How Cranberries Grow: Cranberries 101 – An Introduction. Cape Cod Cranberry Growers Association **2014**.
2. De Rosso, V. *et al.* Singlet Oxygen Quenching by Anthocyanin's Flavylium Cations. *Free Radical Research* **2008**, *42(10)*, 885–91.
3. Prior, R. *et al.* Identification of Procyanidins and Anthocyanins in Blueberries and Cranberries (Vaccinium spp.) Using High-Performance Liquid Chromatography/Mass Spectrometry. *J. Agric. Food Chem.* **2001**, *49(3)*, 1270–6.
4. Brown, P.; Shipley, P. Determination of Anthocyanins in Cranberry Fruit and Cranberry Fruit Products by High-Performance Liquid Chromatography with Ultraviolet Detection: Single-Laboratory Validation. *JAOAC Int.* **2011**, *94(2)*, 459–466.
5. Chandra, A.; Rana, J.; Li, Y. Separation, Identification, Quantification, and Method Validation of Anthocyanins in Botanical Supplement Raw Materials by HPLC and HPLC-MS. *J. Agric. Food Chem*. **2001**, *49*, 3515–3521.
6. Cote, J. *et al.* Analyzing Cranberry Bioactive Compounds. *Crit. Rev. Food Sci. Nutr.* **2010**, *50(9)*, 872–88.

# Quantifying Phenolics in Echinacea Raw Materials and Finished Products

## Using UHPLC/UV and an Agilent InfinityLab Poroshell 120 SB-C18 column

Application Note

Food and Beverage Testing

**Authors**

Ronan Yu, Josie Setiawan, and Paula Brown
BCIT, Natural Health and Food Products Research Group
Burnaby, British Columbia, Canada

Udo Huber
Agilent Technologies, Inc.
Waldbronn, Germany

## Abstract

This method can quantify five phenolics commonly found in echinacea raw materials and finished products. The phenolics detected are caftaric acid, chlorogenic acid, cynarin, echinacoside, and cichoric acid. The phenolics were chromatographically separated in 14 minutes using an Agilent InfinityLab Poroshell 120 SB-C18, 3.0 × 50 mm, 2.7 µm column.

## Introduction

Echinacea is a flowering herb that is found in the Great Plains of North America.[1] Species including *Echinacea angustifolia*, *Echinacea pallida*, and *Echinacea purpurea* are widely used for medicinal and horticultural purposes.[2] Many of the harvested echinacea are processed into tablets, capsules, tinctures, and powders, which are often taken for the common cold and other upper respiratory tract infections.[3] The primary phenolic compounds include caftaric acid, chlorogenic acid, cynarin, echinacoside, and cichoric acid.

## Experimental

The Agilent 1290 Infinity II LC System was composed of the following modules:

- Agilent 1290 Infinity II Flexible Pump (G7104A)
- Agilent 1290 Infinity II Multisampler with Integrated Thermostat (G7167B, option 101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with Agilent InfinityLab Quick Connect heat exchanger, standard flow
- Agilent 1290 Infinity II Diode Array Detector (G7117B) equipped with an Agilent InfinityLab Max-Light Cartridge Flow Cell, 10 mm

Return to Contents Section

### Software

Agilent OpenLab software suite, version 2.3 or later

### Column

Agilent InfinityLab Poroshell 120 SB-C18, 3.0 × 50 mm, 2.7 µm
(part number 689975-302)

### Samples

Echinacea capsules and raw materials were obtained from commercial sources.

**Sample preparation for freeze-dried raw materials and powdered extracts:** Raw materials (root, aerials) were ground to ≤60 mesh size, and 125 mg (±1 mg) of the test sample was weighed into a 50 mL centrifuge tube. 25 mL of extraction solvent, methanol:water (60:40, v/v), was added using a volumetric pipet and vortexed for 10 seconds. The centrifuge tube was shaken on a wrist action shaker at a 45° angle for 30 minutes and then centrifuged at 5,000 rpm for 5 minutes. Approximately 1 mL of the supernatant was filtered through a 0.45 µm PTFE syringe filter into an amber HPLC vial.

**Sample preparation for soft shell dry-filled capsule samples:** 20 capsules were weighed and the total capsule weight was obtained. The capsule contents were emptied, reweighed, and the average fill weight per capsule was determined. A 125 mg (+ 1 mg) aliquot of test sample was weighed into a 50 mL centrifuge tube. 25 mL of extraction solvent, methanol:water (60:40, v/v), was added using a volumetric pipet and vortexed for 10 seconds. The centrifuge tube was shaken on a wrist action shaker at an angle for 30 minutes and then centrifuged at 5,000 rpm for 5 minutes. Approximately 1 mL of the supernatant was filtered through a 0.45 µm PTFE syringe filter into an amber HPLC vial.

**Sample preparation for tinctures (ethanol and glycerite):** The tincture was thoroughly mixed by inverting the tincture vessel several times. 1 mL of the tincture was transferred into a centrifuge tube. 24 mL of extraction solvent, methanol:water (60:40, v/v), was added using a volumetric pipet and vortexed for 10 seconds. The centrifuge tube was shaken on a wrist action shaker at an angle for 30 minutes and then centrifuged at 5,000 rpm for 5 minutes. Approximately 1 mL of the supernatant was filtered through a 0.45 µm PTFE syringe filter into an amber HPLC vial.

The diluent solvent for the reference standard was the same as the extraction solvent, which was methanol:water (60:40, v/v).



Return to Contents Section

### Compounds

The phenolics of interest are shown in Figure 1 with their chemical structures.

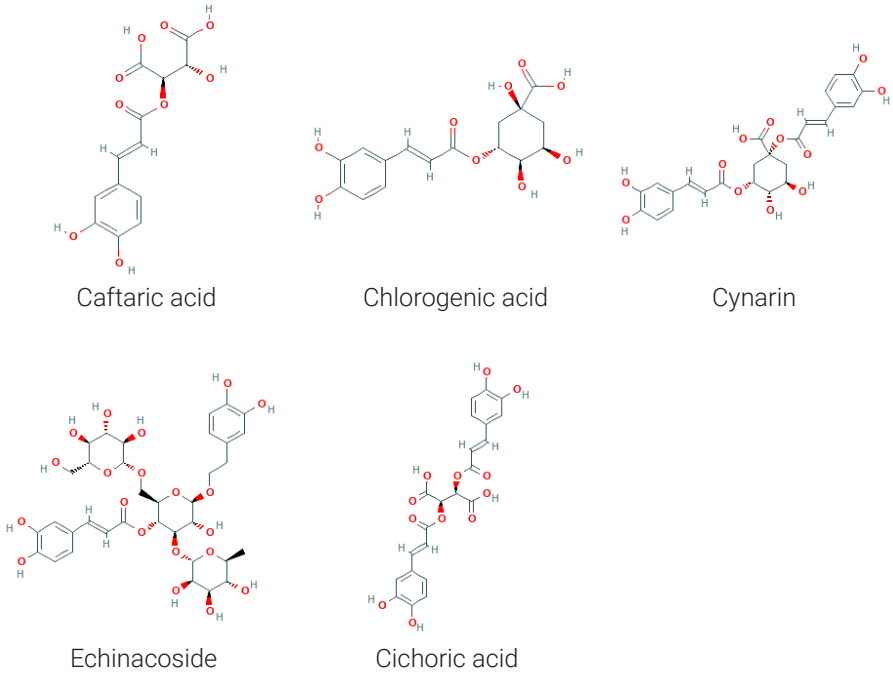



**Figure 1.** Chemical structures of the phenolics of interest.

A seven-point standard curve of mixed standards was used to quantify the select phenolics of interest. The UHPLC method details are outlined in Table 1.

**Table 1.** LC method parameters for analyzing compounds in echinacea samples.

| Parameter | Value |
|---|---|
| Column | Agilent InfinityLab Poroshell 120 SB-C18, 3.0 × 75 mm, 2.7 µm |
| Solvent | A) 0.1% o-phosphoric acid in water<br>B) acetonitrile |
| Gradient | Time (min) % B<br>0.00 2<br>10.00 17<br>11.00 95<br>14.00 95<br>Stop time: 14 min<br>Post time: 2 min |
| Flow Rate | 1.2 mL/min |
| Temperature | 25 °C |
| Detection | 330, 8 nm; reference off |
| Injection | Injection volume: 1 µL<br>Sample temperature: 5 °C<br>Needle wash: off |

## Results and discussion

This precision study was performed to show the reproducibility of the method using an InfinityLab Poroshell 120 SB-C18, 3.0 × 75 mm, 2.7 µm column. This work only required a precision study using this column, as the method used was previously validated in a matrix extension single-laboratory validation (SLV).[3] The analysis of the echinacea major phenolic compounds was accomplished

Return to Contents Section

with acceptable results with Horwitz ratio (HorRat) values of 0.31 to 1.98, and the percent relative standard deviation (%RSD) of each analyte was 1.63 to 9.88%. All compounds were separated with resolution of at least 1.5 (Figure 2). Chromatograms of the phenolic standards and three different matrices are shown in Figures 2 through 5.

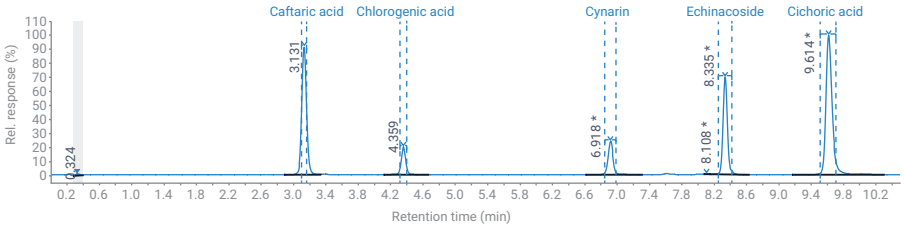



**Figure 2.** Chromatogram of the phenolic standards.

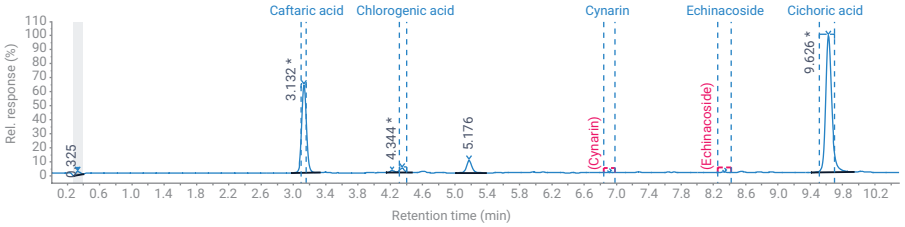



**Figure 3.** Chromatogram of an *Echinacea purpurea* aerial extract sample (diluted 2×).

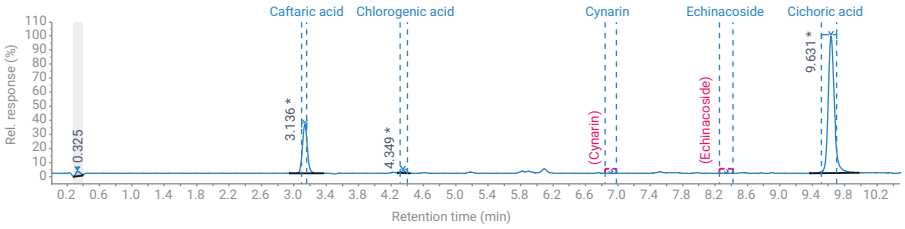



**Figure 4.** Chromatogram of an *Echinacea purpurea* tincture sample (diluted 25×).

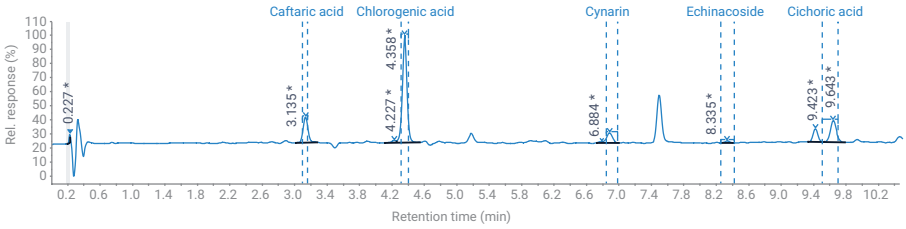



**Figure 5.** Chromatogram of an *Echinacea angustifolia* root sample.

## Conclusion

This UHPLC method can quantify caftaric acid, chlorogenic acid, cynarin, echinacoside, and cichoric acid in echinacea raw materials and finished products with reproducible results using the Agilent InfinityLab Poroshell 120 SB-C18 column.



Return to Contents Section

## References


1. Brown P.; Mudge, E.; Paley, L. Determination of Phenolic Constituents in Echinacea Raw Materials and Dietary Supplements by HPLC-UV: Collaborative Study. *JAOAC Int.* **2016**, *99(5)*, 1197–1203.
2. Binns, S.; Livesey, J.; Arnason, J.; Baum, B. Phytochemical variation in echinacea from roots and flowerheads of wild and cultivated populations. *J. Agric. Food Chem.* **2002**, *50(13)*, 3673–3687.
3. Brown, P.; Chan, M.; Paley, L. Determination of Major Phenolic Compounds in Echinacea spp. Raw Materials and Finished Products by High-Performance Liquid Chromatography with Ultraviolet Detection: Single-Laboratory Validation Matrix Extension. *JAOAC Int.* **2011**, *94(5)*, 1400–1412.


## Appendix

**Table 2.** Precision results summary of echinacea test samples.

| Matrix | Analyte | HorRat | Mean (mg/g) |
|---|---|---|---|
| *Echinacea angustifolia* Capsule | Caftaric acid | NA | <LOQ |
| | Chlorogenic acid | NA | <LOQ |
| | Cynarin | 1.27 | 0.12 |
| | Echinacoside | 0.86 | 0.52 |
| | Cichoric acid | NA | <LOD |
| Echinacea with Garlic and Ginger Capsule | Caftaric acid | 1.35 | 1.49 |
| | Chlorogenic acid | 1.74 | 0.06 |
| | Cynarin | NA | <LOD |
| | Echinacoside | NA | <LOD |
| | Cichoric acid | 1.18 | 1.89 |
| *Echinacea pallida* Root | Caftaric acid | 1.94 | 0.12 |
| | Chlorogenic acid | NA | <LOD |
| | Cynarin | 1.16 | 0.05 |
| | Echinacoside | 1.45 | 1.66 |
| | Cichoric acid | 1.95 | 0.45 |
| *Echinacea angustifolia* Root | Caftaric acid | 1.93 | 0.13 |
| | Chlorogenic acid | 1.98 | 1.26 |
| | Cynarin | 1.91 | 0.11 |
| | Echinacoside | NA | <LOD |
| | Cichoric acid | 2.00 | 0.38 |
| *Echinacea purpurea* Root | Caftaric acid | 0.55 | 0.09 |
| | Chlorogenic acid | 1.97 | 0.06 |
| | Cynarin | NA | <LOD |
| | Echinacoside | NA | <LOD |
| | Cichoric acid | 0.88 | 20.67 |
| *Echinacea purpurea* Tincture | Caftaric acid | 0.47 | 467.18* |
| | Chlorogenic acid | NA | <LOD |
| | Cynarin | NA | <LOD |
| | Echinacoside | NA | <LOD |
| | Cichoric acid | 0.64 | 1,671.74* |
| *Echinacea purpurea* Aerial Extract | Caftaric acid | 0.64 | 16.38 |
| | Chlorogenic acid | 1.06 | 1.44 |
| | Cynarin | NA | <LOD |
| | Echinacoside | NA | <LOD |
| | Cichoric acid | 0.89 | 32.16 |

* mg/L

Return to Contents Section

# Quantifying Selected Anthocyanins in European Elderberry (*Sambucus nigra* L.) and American Elderberry (*Sambucus canadensis* L.) Raw Materials and Finished Products

## A validated method using UHPLC/UV and an Agilent InfinityLab Poroshell 120 SB-C18 column

Application Note

Food and Beverage Testing

**Authors**

Ying Liu, Hazrah Moothoo, and Ronan Yu
BCIT, Natural Health and Food Products Research Group
Burnaby, British Columbia, Canada

Udo Huber
Agilent Technologies, Inc.
Waldbronn, Germany

## Abstract

This UHPLC method can detect and quantify select anthocyanin compounds that are present in European and American elderberries. In order of elution, the anthocyanins are cyanidin-3-O-sambubioside-5-O-glucoside chloride, cyanidin-3,5-di-O-glucoside chloride, cyanidin-3-O-(2-O-β-D-xylopyranosyl)-β-D-glucopyranoside chloride, cyanidin-3-O-glucoside chloride, and cyanidin-3-O-β-[6"-O-E-p-coumaroyl-sambubioside]-5-O-β-glucopyranoside. Cyanidin-3-O-β-[6"-O-E-p-coumaroyl-sambubioside]-5-O-β-glucopyranoside is present in European elderberries but not in the American counterpart. The anthocyanins were chromatographically separated in 20 minutes using an Agilent InfinityLab Poroshell 120 SB-C18, 4.6 × 150 mm, 2.7 µm column. This study demonstrated that the performance characteristics of this method—precision, limit of quantitation, limit of detection, and accuracy—are valid and suitable for analyzing these select anthocyanins in powdered root materials, extracts, syrups, and tinctures.



Return to Contents Section

## Introduction

The genus *Sambucus* comes from the Adoxaceae family of flowering plants that are commonly called elder or elderberry. Elderberries are purple-black edible raw fruits that are rich in anthocyanins and phenolic compounds.[1,2] Studies on diets that include elderberry exhibited a wide range of antioxidant and therapeutics benefits, which include a reduction in coronary heart disease and stroke and improvement in visual acuity and cognitive behavior.[2] In addition, recent studies on extracts of both European elderberry (*Sambucus nigra* L.) and American elderberry (*Sambucus canadensis* L.) demonstrated significant chemopreventive potential in controlling enzymes commonly associated with various forms of cancer.[2,3]

There are several published analytical methods in determining the anthocyanin content in both species of elderberry, but none has been validated.[4–7] It is essential to establish a reliable analytical method to quantify the levels or concentration of anthocyanins found in elderberries to support further studies on elderberry health benefits. Therefore, the objective of this study is to validate an analytical method that quantifies the select anthocyanins in European and American elderberries and also demonstrates robustness and reproducibility in different raw materials and commercial products.

## Experimental

The Agilent 1290 Infinity II LC System was composed of the following modules:

- Agilent 1290 Infinity II High-Speed Pump (G7120A)
- Agilent 1290 Infinity II Multisampler with Integrated Thermostat (G7167B, option 101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with Agilent InfinityLab Quick Connect heat exchanger, standard flow
- Agilent 1290 Infinity II Diode Array Detector (G7117B) equipped with an Agilent InfinityLab Max-Light Cartridge Flow Cell, 10 mm

### Software

- Agilent OpenLab software suite, version 2.3 or later

### Column

- Agilent InfinityLab Poroshell 120 SB-C18, 4.6 × 150 mm, 2.7 µm (part number 683975-902)

### Samples

Eight test materials of different elderberries were used in this validation study. Powdered root materials were provided by the American Herbal Pharmacopeia (CA, USA). European berries were purchased from Artemis International Nutraceuticals (IN, USA). European elderberry syrup was purchased from Sambucol Pharmacare Inc. (ON, CAN). European elderberry liquid extract with echinacea and licorice was purchased from Flora (BC, CAN). American elderberries were obtained from Missouri Botanical Garden (MOBOT) and Suro Organic Medicinal Products (QC, CAN). American elderberry liquid extract was purchased from Suro Organic Medicinal Products (QC, CAN), and American elderberry syrup was purchased from Immunia Fruitomed (QC, CAN).

The cyanidin-3-O-sambubioside-5-O-glucoside chloride, cyanidin-3,5-di-O-glucoside chloride, cyanidin-3-O-(2-O-β-D-xylopyranosyl)-β-D-glucopyranoside chloride, and cyanidin-3-O-glucoside chloride standards were purchased from

Return to Contents Section

Extrasynthase (CA, USA). Cyanidin-3-O-β-[6"-O-E-p-coumaroyl-sambubioside]-5-O-β-glucopyranoside was purchased from Polyphenols (Rogaland, NOR).

**Preparation of standards:** The standards were made in 0.1% hydrochloric acid in a 1:1 methanol and ultrapure water solution. The powdered standard was transferred into a weighing boat or paper. The container was rinsed with 500 µL of ultrapure water and then 500 µL of methanol into a 5 mL volumetric flask. The standard was transferred from the weighing boat into the volumetric flask, and any residue was rinsed off with 500 µL of ultrapure water and then 500 µL of methanol into the volumetric flask. 5 µL of concentrated HCl was added to the volumetric flask. The sample was then brought to volume with a 1:1 methanol and ultrapure water solution and capped. The volumetric flask was inverted at least 10 times to ensure adequate mixing in solution.

**Sample preparation for raw materials and powdered extracts:** All root samples and extracts were ground to approximately 50 mesh and homogenized. 175 mg of the sample was weighed into a 50 mL conical tube. 25 mL of extraction solvent that consists of methanol and 2% HCl acidified water (75:25, v/v) was added. The sample was vortexed for 10 seconds and shaken for 2 hours using a wrist-action shaker. Samples were then sonicated for 1 hour. Next, samples were centrifuged for 5 minutes at 4,500 revolutions per minute (rpm). The supernatant was then filtered using 0.22 µm polytetrafluoroethylene (PTFE) into amber sample vials.

**Sample preparation for liquids and syrups:** The sample containers were inverted for 10 seconds. The appropriate volume was transferred to create a 1:1 dilution with the extraction solvent (same extraction solvent as for raw materials). More concentrated samples required a 1:5 dilution. The mixture was vortexed for 15 seconds, then centrifuged for 5 minutes at 4,500 rpm. Finally, the sample was filtered using 0.22 µm polytetrafluoroethylene (PTFE) into amber sample vials.

For the precision study, samples were prepared in quadruplicate, and a seven-point standard curve of mixed standards was used to quantify the anthocyanins over the course of three different days. The UHPLC method that was used is outline in Table 1. The results were evaluated using the Horwitz ratio (HorRat) to indicate method performance.

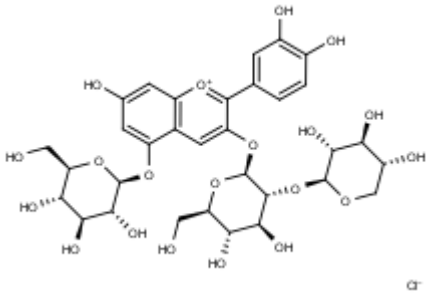
**Table 1.** LC method parameters for analyzing compounds in elderberry samples.

| Parameter | Value |
|---|---|
| Column | Agilent InfinityLab Poroshell 120 SB-C18, 4.6 × 150 mm, 2.7 µm |
| Solvent | A) 0.5% phosphoric acid in water<br>B) tetrahydrofuran and 0.5% phosphoric acid in water (60:40, v/v) |
| Gradient | Time (min) %B<br>0.00 8<br>10.00 16<br>18.00 64<br>20.00 64<br>Stop time: 20.00 min<br>Post time: 4.00 min |
| Flow Rate | 1.0 mL/min |
| Temperature | 30 °C |
| Detection | 520, 4 nm; reference off |
| Injection | Injection volume: 3 µL<br>Sample temperature: 5 °C<br>Needle wash: off |

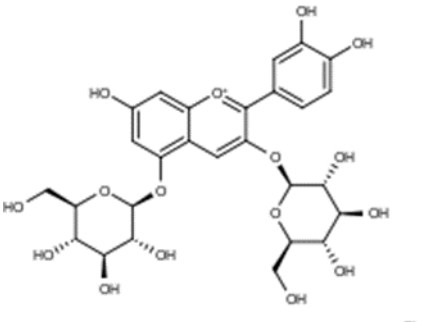
Return to Contents Section

## Compounds

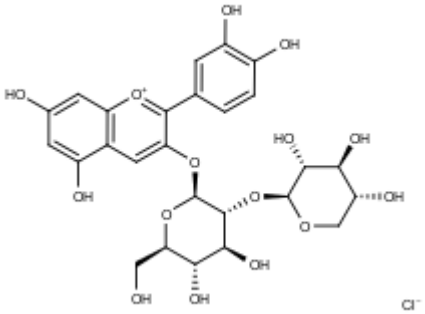
The anthocyanins of interest are shown in Figure 1 with their chemical structures.



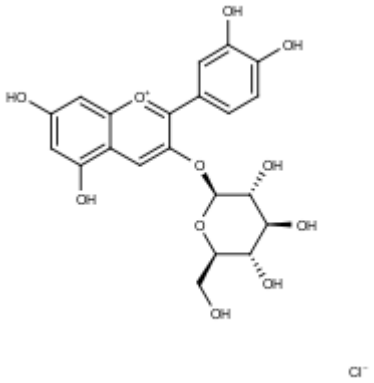
Cyanidin-3-O-sambubioside-5-O-glucoside



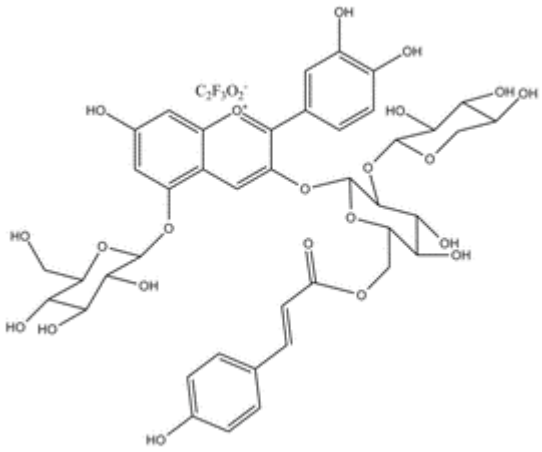
Cyanidin-3,5-di-O-glucoside chloride



Cyanidin-3-O-sambubioside chloride



Cyanidin-3-O-glucoside chloride



Cyanidin-3-O-β-[6”-O-E-p-coumaroyl-sambubioside]-5-O-β-glucopyranoside

**Figure 1.** Chemical structures of elderberry anthocyanins of interest.

Return to Contents Section

The limit of detection (LOD) and limit of quantification (LOQ) were determined using the Environmental Protection Agency Method Detection Level (EPA MDL) method. The method uses data from at least seven replicates of a sample containing a low level of the analyte. A second set of replicates at another low analyte level was prepared to ensure that variances at low levels are the same. The calculation of the EPA MDL is as follows:

$$MDL = s \times t_{(0.01, n-1)}$$

Where s is the sample standard deviation of the concentration of the replicates and $t_{(0.01, n-1)}$ is the t statistic with α = 0.01 and n – 1 degrees of freedom at a confidence interval of 99%. The LOQ is the analyte concentration calculated as 10 times the standard deviation.

A spike-and-recovery experiment at three different concentration levels was used to evaluate the accuracy of the method. For the first level, powdered gooseberry was used as blank matrix and spiked using a calibration standard that represented 60% of anthocyanin levels found in American elderberry liquid extract. For the second level, powdered gooseberry was used as a blank matrix and spiked using a calibration standard that represented 100% of anthocyanin levels found in American elderberry liquid extract. Due to the high-level concentration of anthocyanins required to represent 120% anthocyanin levels found in American elderberry liquid extract, a characterized American elderberry ground berry was used as substitute as a pseudoblank matrix (an elderberry sample with known amounts of anthocyanin that represents 100%) and was spiked with a calibration standard.

All spike-and-recovery samples were prepared in quadruplicate, and the results were evaluated based on the acceptable recovery limits under the AOAC Guidelines for Single-Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals.[8]

## Results and discussion

All five anthocyanin compounds were baseline separated. Calibration curves for each anthocyanin were linear over the following ranges (µg/mL): 0.125 to 200 for cyanidin-3-O-sambubioside-5-O-glucoside chloride, cyanidin-3,5-di-O-glucoside chloride, cyanidin-3-O-(2-O-β-D-xylopyranosyl)-β-D-glucopyranoside chloride, cyanidin-3-O-glucoside chloride, and 0.625 to 1,000 for cyanidin-3-O-β-(6"-O-E-p-coumaroyl-sambubioside)-5-O-β-glucopyranoside. All anthocyanins have a coefficient of determination of at least 99%.

The study showed that the method was precise with respect to the HorRat values for each anthocyanin (HorRat 0.04 to 0.97). See Appendix Table 2 for the HorRat results of each anthocyanin in the different test samples. The spike-and-recovery study at three different levels demonstrated that the method has acceptable average recovery values from 90.30 to 113.13% (Appendix Table 3). The following are the LOD and LOQ for the anthocyanins (µg/mL): cyanidin-3-O-sambubioside-5-O-glucoside chloride (0.17, 0.51), cyanidin-3,5-di-O-glucoside chloride (0.15, 0.45), cyanidin-3-O-(2-O-β-D-xylopyranosyl)-β-D-glucopyranoside chloride (0.16, 0.47), cyanidin-3-O-glucoside chloride (0.15, 0.43), and cyanidin-3-O-β-(6"-O-E-p-coumaroyl-sambubioside)-5-O-β-glucopyranoside (0.17, 0.52). See Appendix Table 4 for the complete table of results. Sample chromatograms are shown in Figures 2 through 10.

Return to Contents Section

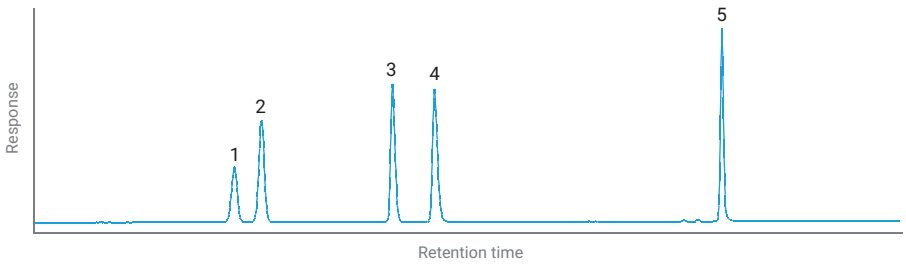



**Figure 2.** Chromatogram of a mixed standard containing the five anthocyanins. In elution order, the peaks are 1) cyanidin-3-O-sambubioside-5-O-glucoside chloride, 2) cyanidin-3,5-di-O-glucoside chloride, 3) cyanidin-3-O-(2-O-β-D-xylopyranosyl)-β-D-glucopyranoside chloride, 4) cyanidin-3-O-glucoside chloride, and 5) cyanidin-3-O-β-(6"-O-E-p-coumaroyl-sambubioside)-5-O-β-glucopyranoside.

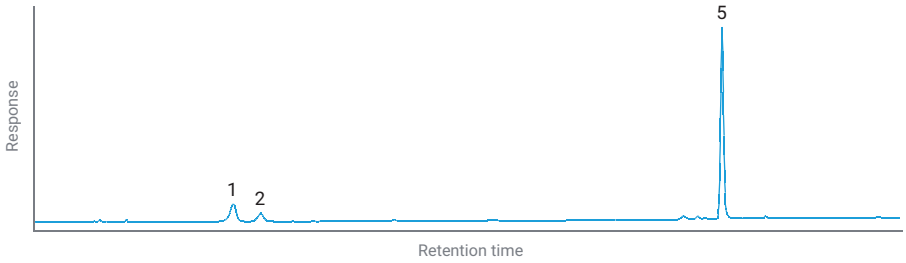



**Figure 3.** Chromatogram of Suro ground berries (*S. canadensis* L.).

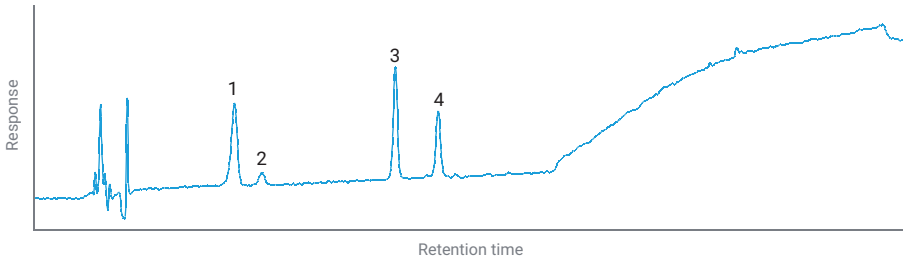



**Figure 4.** Chromatogram of Artermis ground berries (*S. nigra* L.).

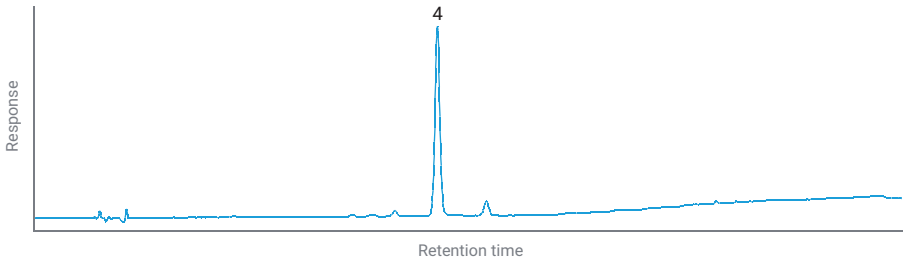



**Figure 5.** Chromatogram of Organika berry extract capsules (*S. nigra* L.).

Return to Contents Section

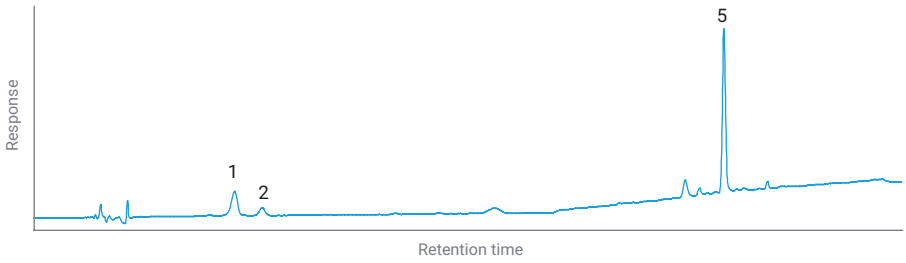



**Figure 6.** Chromatogram of MOBOT ground berries (*S. canadensis* L.).

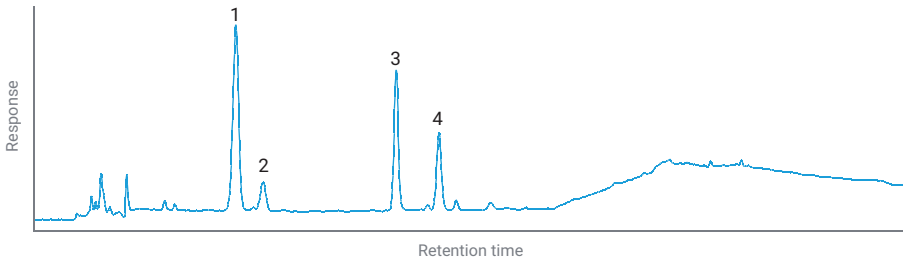



**Figure 7.** Chromatogram of Sambucol syrup (*S. nigra* L.).

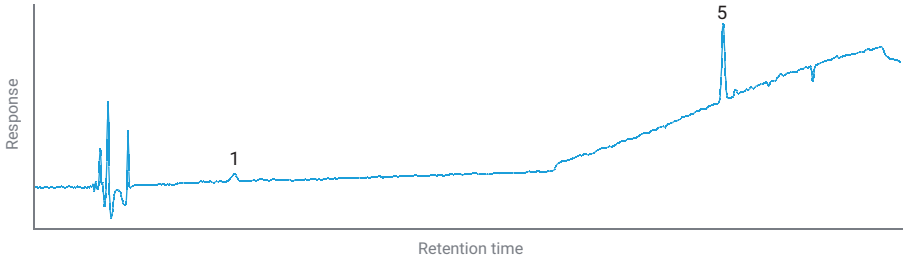



**Figure 8.** Chromatogram of Suro liquid extract (*S. canadensis* L.).

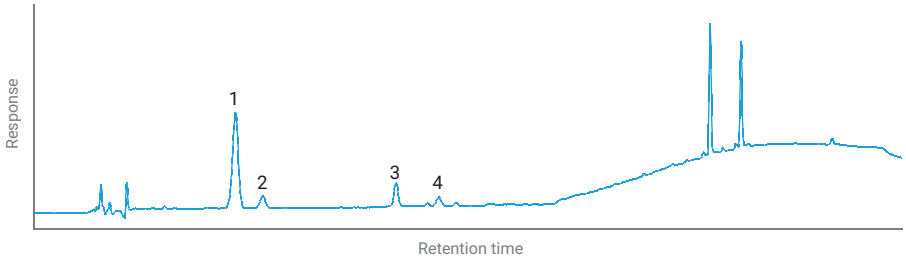



**Figure 9.** Chromatogram of Flora mixed liquid extract (*S. nigra* L.).

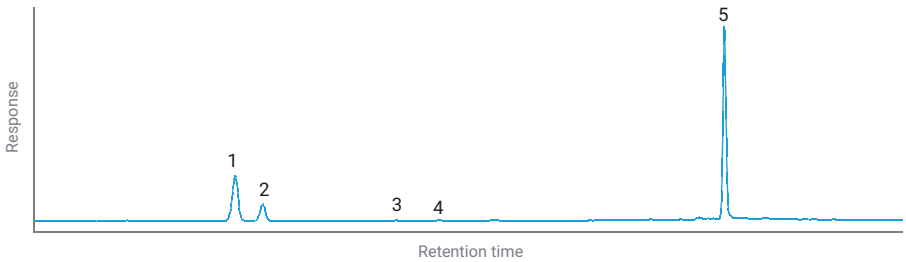



**Figure 10.** Chromatogram of Immunia mixed syrup (*S. canadensis* L.).



Return to Contents Section

## Conclusion

This study demonstrated that the method was precise and accurate in determining cyanidin-3-O-sambubioside-5-O-glucoside chloride, cyanidin-3,5-di-O-glucoside chloride, cyanidin-3-O-(2-O-β-D-xylopyranosyl)-β-D-glucopyranoside chloride, cyanidin-3-O-glucoside chloride, and cyanidin-3-O-β-(6"-O-E-p-coumaroyl-sambubioside)-5-O-β-glucopyranoside in European or American elderberry powdered root materials, extracts, syrups, and tinctures.

## References


1. Lee, J.; Finn, C. Anthocyanins and other polyphenolics in American elderberry (Sambucus canadensis) and European elderberry (S.nigra) cultivars. *J. Sci. Food Agric.* **2007**, *87*, 2665–75.
2. Ozgen, M. *et al.* Total phenolic, anthocyanin contents and antioxidant capacity of selected elderberry (Sambucus canadensis L.) accessions. *Pharmacognosy Magazine* **2010**, *6(23)*, 198–203.
3. Thole, J. *et al.* A comparative evaluation of the anticancer properties of European and American elderberry fruits. *J. Medicinal Foods* **2006**, *9*, 498–504.
4. Viapiana, A.; Wesolowski, M. HPLC Fingerprint Combined with Quantitation of Phenolic Compounds and Chemometrics as an Efficient Strategy for Quality Consistency Evaluation of Sambucus nigra Berries. *Nat. Prod. Commun.* **2016**, *11(10)*, 1449–1454.
5. Pliszka, B. Polyphenolic content, antiradical activity, stability and microbiological quality of elderberry (Sambucus nigra L.) extracts. *Acta Sci. pol. Technol. Aliment.* **2017**, *16(4)*, 393–401.
6. Vlachojannis, C.; Zimmermann, B.; Chrubasik-Hausmann, S. Quantification of anthocyanins in elderberry and chokeberry dietary supplements. *Phytother. Res.* **2015**, *29(4)*, 561–565.
7. Mikulic-Petkovsek, M. *et al.* Investigation of anthocyanin profile of four elderberry species and interspecific hybrids. *J. Agric. Food Chem.* **2014**, *62(24)*, 5573–5580.
8. AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals. *AOAC International*, **2019**.


Return to Contents Section

# Appendix

**Table 2.** Anthocyanins with their corresponding HorRat values, mean values in mg/g, and percent coefficient of variation (% CV).

| Matrix | Analyte | HorRat | mg/g($\bar{X}$) | %CV |
|---|---|---|---|---|
| Suro Ground Berries (*S. canadensis* L.) | Cyanidin-3-O-sambubioside-5-O-glucoside | 0.79 | 3.68 | 3.66 |
| | Cyanidin-3,5-di-O-glucoside chloride | 0.51 | 1.28 | 2.77 |
| | Cyanidin-3-O-sambubioside chloride | <LOQ | <LOQ | <LOQ |
| | Cyanidin-3-O-glucoside chloride | <LOQ | <LOQ | <LOQ |
| | Cyanidin 3-O-β-[6"-O-E-p-coumaroyl-sambubioside]-5-O-β-glucopyranoside | 0.86 | 53.45 | 3.42 |
| MOBOT Ground Berries (*S. canadensis* L.) | Cyanidin-3-O-sambubioside-5-O-glucoside | 0.62 | 0.53 | 3.86 |
| | Cyanidin-3,5-di-O-glucoside chloride | 0.14 | 0.52 | 0.91 |
| | Cyanidin-3-O-sambubioside chloride | <MDL | <MDL | <MDL |
| | Cyanidin-3-O-glucoside chloride | <MDL | <MDL | <MDL |
| | Cyanidin 3-O-β-[6"-O-E-p-coumaroyl-sambubioside]-5-O-β-glucopyranoside | 0.49 | 4.57 | 2.82 |
| Artemis Ground Berries (*S. nigra* L.) | Cyanidin-3-O-sambubioside-5-O-glucoside | 0.35 | 0.39 | 2.26 |
| | Cyanidin-3,5-di-O-glucoside chloride | 0.05 | 0.46 | 0.34 |
| | Cyanidin-3-O-sambubioside chloride | 0.80 | 0.13 | 6.11 |
| | Cyanidin-3-O-glucoside chloride | 0.97 | 0.12 | 7.54 |
| | Cyanidin 3-O-β-[6"-O-E-p-coumaroyl-sambubioside]-5-O-β-glucopyranoside | <MDL | <MDL | <MDL |
| Organika Berry Extract Capsules (*S. nigra* L.) | Cyanidin-3-O-sambubioside-5-O-glucoside | <LOQ | <LOQ | <LOQ |
| | Cyanidin-3,5-di-O-glucoside chloride | <LOQ | <LOQ | <LOQ |
| | Cyanidin-3-O-sambubioside chloride | <LOQ | <LOQ | <LOQ |
| | Cyanidin-3-O-glucoside chloride | 0.10 | 5.71 | 10.29 |
| | Cyanidin 3-O-β-[6"-O-E-p-coumaroyl-sambubioside]-5-O-β-glucopyranoside | <MDL | <MDL | <MDL |
| Sambucol Syrup (*S. nigra* L.) | Cyanidin-3-O-sambubioside-5-O-glucoside | 0.29 | 61.20 | 2.52 |
| | Cyanidin-3,5-di-O-glucoside chloride | 0.14 | 8.14 | 1.66 |
| | Cyanidin-3-O-sambubioside chloride | 0.28 | 17.78 | 2.87 |
| | Cyanidin-3-O-glucoside chloride | 0.31 | 9.77 | 3.46 |
| | Cyanidin 3-O-β-[6"-O-E-p-coumaroyl-sambubioside]-5-O-β-glucopyranoside | <MDL | <MDL | <MDL |
| Immunia Mixed Syrup (*S. canadensis* L.) | Cyanidin-3-O-sambubioside-5-O-glucoside | 0.24 | 784.77 | 1.43 |
| | Cyanidin-3,5-di-O-glucoside chloride | 0.20 | 161.41 | 1.51 |
| | Cyanidin-3-O-sambubioside chloride | 0.54 | 3.53 | 7.20 |
| | Cyanidin-3-O-glucoside chloride | 0.39 | 5.35 | 4.83 |
| | Cyanidin 3-O-β-[6"-O-E-p-coumaroyl-sambubioside]-5-O-β-glucopyranoside | 0.25 | 943.22 | 1.44 |
| Suro Liquid Extract (*S. canadensis* L.) | Cyanidin-3-O-sambubioside-5-O-glucoside | 0.10 | 1.94 | 1.52 |
| | Cyanidin-3,5-di-O-glucoside chloride | <MDL | <MDL | <MDL |
| | Cyanidin-3-O-sambubioside chloride | <MDL | <MDL | <MDL |
| | Cyanidin-3-O-glucoside chloride | <MDL | <MDL | <MDL |
| | Cyanidin 3-O-β-[6"-O-E-p-coumaroyl-sambubioside]-5-O-β-glucopyranoside | 0.48 | 0.65 | 8.17 |
| Flora Mixed Liquid Extract (*S. nigra* L.) | Cyanidin-3-O-sambubioside-5-O-glucoside | 0.13 | 18.03 | 1.34 |
| | Cyanidin-3,5-di-O-glucoside chloride | 0.04 | 3.52 | 0.55 |
| | Cyanidin-3-O-sambubioside chloride | 0.07 | 2.32 | 0.95 |
| | Cyanidin-3-O-glucoside chloride | 0.07 | 0.23 | 1.35 |
| | Cyanidin 3-O-β-[6"-O-E-p-coumaroyl-sambubioside]-5-O-β-glucopyranoside | <MDL | <MDL | <MDL |

Return to Contents Section

**Table 3.** Spike-and-recovery (mean percent), MDL, and LOQ results.

| Anthocyanins | MDL (µg/mL) | LOQ (µg/mL) | Level 1 ($\bar{X}$) % Recovery | Level 2 ($\bar{X}$) % Recovery | Level 3 ($\bar{X}$) % Recovery |
|---|---|---|---|---|---|
| Cyanidin-3-O-sambubioside-5-O-glucoside | 0.17 | 0.51 | 106.26 | 96.72 | 100.42 |
| Cyanidin-3,5-di-O-glucoside chloride | 0.15 | 0.45 | 110.34 | 97.22 | 113.13 |
| Cyanidin-3-O-sambubioside chloride | 0.16 | 0.47 | 97.23 | 95.08 | 96.50 |
| Cyanidin-3-O-glucoside chloride | 0.15 | 0.43 | 98.48 | 98.64 | 96.84 |
| Cyanidin 3-O-β-[6"-O-E-p-coumaroyl-sambubioside]-5-O-β-glucopyranoside | 0.17 | 0.52 | 90.30 | 93.65 | 106.89 |

Return to Contents Section

# Quantifying Ginsenosides in *Panax quinquefolius*, *Panax ginseng*, and *Panax notoginseng* Raw Materials and Finished Products

## A validated method using UHPLC/UV and an Agilent InfinityLab Poroshell 120 Phenyl-Hexyl column

Application Note

Food and Beverage Testing

**Authors**

Ronan Yu, Ying Liu, and Paula Brown
BCIT, Natural Health and Food Products Research Group
Burnaby, British Columbia, Canada

Udo Huber
Agilent Technologies, Inc.
Waldbronn, Germany

## Abstract

This UHPLC method can detect and quantify ginsenoside compounds that are present in *Panax quinquefolius*, *Panax ginseng*, and *Panax notoginseng*. The eight ginsenosides (notoginsenoside R1, Rg1, Re, Rf, Rb1, Rc, Rb2, and Rd—in order of elution) were chromatographically separated in 19 minutes using an Agilent InfinityLab Poroshell 120 Phenyl-Hexyl, 3.0 × 150 mm, 2.7 µm column. The study demonstrated that the performance characteristics of this method, including precision, limit of quantitation, limit of detection, and accuracy are valid and suitable for analyzing ginsenosides in powdered root materials, tablets, and hard-shelled capsules.

## Introduction

The genus *Panax* consists of *P. ginseng*, *P. notoginseng*, and *P. quinquefolius* and grows naturally in the wild and can be cultivated in farms. Ginseng has been used in traditional medicine systems of eastern Asia for thousands of years as a tonic and stimulant.[1] Today, ginseng is categorized as an adaptogen, a substance that improves the ability to adjust to environmental and internal distress, and is characterized by the presence of bioactive ginsenosides.[1–3] The Office of Dietary Supplements (ODS) has been addressing the importance and need for validated and publicly available methods for botanical products including ginseng.[4] AOAC International published the Standard Method Performance Requirements (SMPRs) for ginseng in 2017.[5] The method in this application note is an improvement on separation, run time, and applicable matrices over our previous First Action AOAC Official Methods of Analysis on ginsenosides quantification.[6,7]

Return to Contents Section

## Experimental

The Agilent 1290 Infinity II LC System was composed of the following modules:

- Agilent 1290 Infinity II Flexible Pump (G7104A)
- Agilent 1290 Infinity II Multisampler with Integrated Thermostat (G7167B, option 101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with Agilent InfinityLab Quick Connect heat exchanger, standard flow
- Agilent 1290 Infinity II Diode Array Detector (G7117B) equipped with an Agilent InfinityLab Max-Light Cartridge Flow Cell, 10 mm

### Software

Agilent OpenLab software suite, version 2.3 or later

### Column

Agilent InfinityLab Poroshell 120 Phenyl-Hexyl, 3.0 × 150 mm, 2.7 µm (part number 693975-312)

### Samples

Eight test materials of different ginseng species were used within this validation study. Raw root materials (*P. quinquefolius*, *P. ginseng*, and *P. notoginseng*) were provided by the American Herbal Pharmacopeia (CA, USA). Powdered root extracts (*P. quinquefolius*, *P. ginseng*, and *P. notoginseng*) were provided by United States Pharmacopeia (MD, USA). The *P. ginseng* tablet and the *P. ginseng* hard-shelled capsule (a blend of concentrated Maca and Korean ginseng) were purchased from commercial suppliers. The ginsenoside standards were purchased from Alkemist Labs (CA, USA).

**Sample preparation for raw materials and powdered extracts:** All root samples and extracts were ground to approximately 50 mesh and homogenized. For root raw materials, 400 mg was weighed into a 50 mL conical tube. For root extracts, 150 mg was transferred into a 50 mL conical tube. 30 mL of 70% methanol in water was added into each conical tube by a volumetric pipette. The samples were vortexed for a minimum of 10 seconds, centrifuged at 4,500 rpm, and then sonicated for 25 minutes at room temperature. The conical tube was vortexed again for a minimum of 5 seconds before centrifuging further for 3 minutes. A 1 mL amount of the supernatant was pipetted into a 2 mL microcentrifuge tube and 100 µL of 5% KOH solution was added. The microcentrifuge tube was vortexed for 5 seconds before left for 2 hours at room temperature and away from direct light. After 2 hours, the solution was neutralized by adding 100 µL of a 14% $KH_2PO_4$ solution. The tubes were vortexed for 5 seconds and the solution was filtered using a syringe fitted with a 0.22 µm PTFE filter. The filtered solution was then transferred into a chromatography vial.

**Sample preparation for tablets and hard-shelled capsules:** A minimum of 20 tablets or capsules were ground to make a homogeneous fine powder using a grinder/mill or mortar and pestle. The powders were mixed with a spatula and 500 mg was weighed out into a 50 mL conical tube. The extraction procedure follows the same instructions as the raw materials and powdered extract.

Return to Contents Section

## Compounds

The ginsenosides of interest are shown in Figure 1 with their chemical structures.

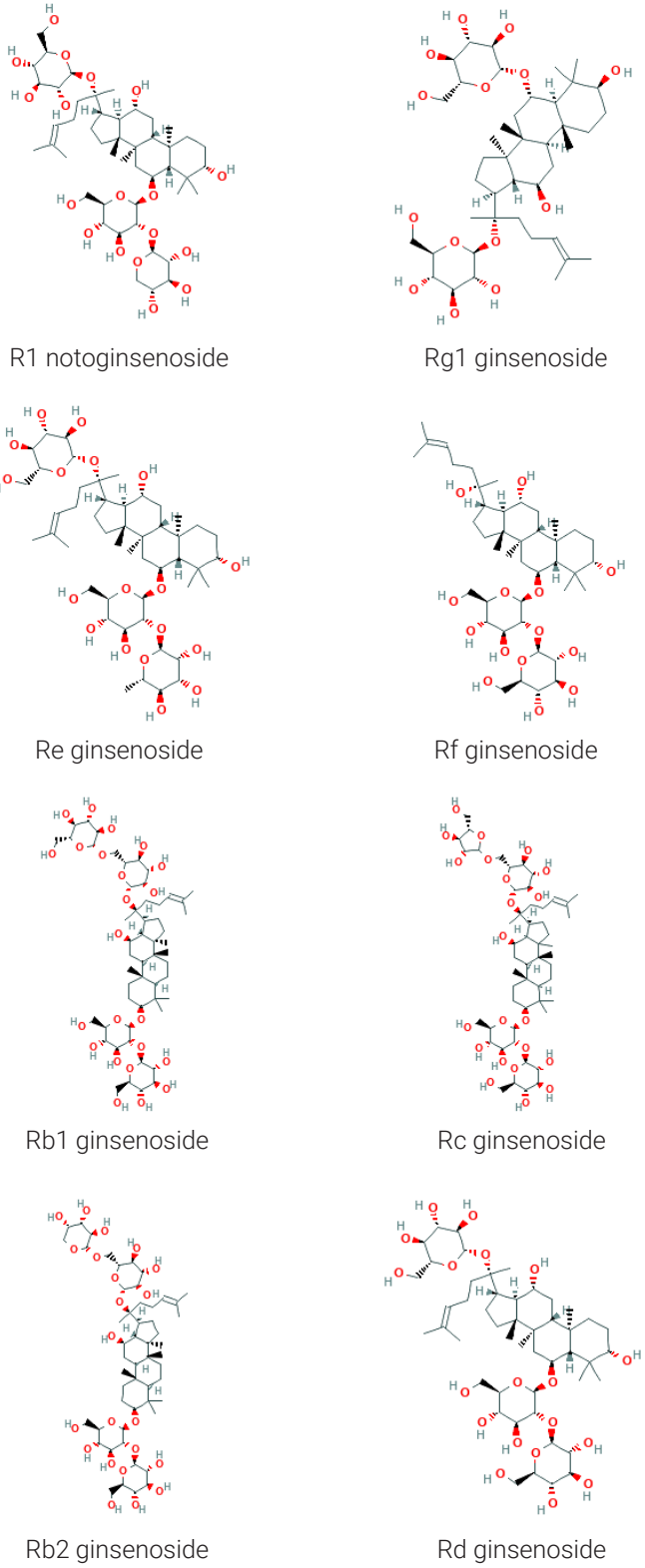



**Figure 1.** Chemical structures of the select ginsenosides.

Return to Contents Section

For the precision study, samples were prepared in quadruplicate, and an eight-point standard curve of mixed standards was used to quantify the eight ginsenosides of interest over the course of three different days. The UHPLC method used is detailed in Table 1. Standards were diluted in 100% methanol. The results were evaluated using the Horwitz Ratio (HorRat) to indicate method performance.

**Table 1.** LC method parameters for analyzing compounds in ginseng samples.

| Parameter | Value |
|---|---|
| Column | Agilent InfinityLab Poroshell 120 Phenyl-Hexyl, 3.0 × 150 mm, 2.7 µm |
| Solvent | A) water<br>B) acetonitrile |
| Gradient | Time (min) %B<br>0.00 20<br>5.00 21<br>6.00 21<br>8.00 30<br>10.00 31<br>14.00 35<br>16.00 38<br>17.00 80<br>19.00 80<br>Stop time: 19.00 min<br>Post time: 3 min |
| Flow Rate | 0.85 mL/min |
| Temperature | 30 °C |
| Detection | 203, 4 nm; reference off |
| Injection | Injection volume: 5 µL<br>Sample temperature: 5 °C<br>Needle wash: off |

The limit of detection (LOD) and limit of quantification (LOQ) were determined using the International Union of Pure and Applied Chemistry (IUPAC) method by injecting and analyzing a sample blank (70% methanol in water) seven times. The LOD is expressed as the analyte concentration corresponding to the sample blank value plus three standard deviations, and LOQ is the analyte concentration corresponding to the sample blank value plus 10 standard deviations.

A spike-and-recovery experiment at three different concentration levels was used to evaluate the accuracy of the method. For the first level, maltodextrin was used as blank matrix and spiked with a low concentration of ginsenosides (250 µL of 0.25 µg/mL). For the second level, GINX-1 North American Ginseng Root Extract Certified Reference Material (CRM) for Ginsenoside and Metals was purchased from the National Research Council of Canada (NRC) and was spiked with notoginsenoside R1 and Rf (100 µL of 2.5 µg/mL each). For the third level, the CRMs were spiked with notoginsenoside R1, Rg1, Re, Rf, Rb1, Rc, Rb2, and Rd (150 µL of 2.5 µg/mL each). All spike-and-recovery samples were prepared in triplicate, and the results were evaluated based on the acceptable recovery limits under the AOAC Guidelines for Single-Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals.[8]

## Results and discussion

All compounds were separated into individual peaks including Rg1 and Re ginsenosides. Sample chromatograms are shown in Figures 2 through 5. Calibration curves for each ginsenoside were linear over the following ranges (µg/mL): 2 to 300 for notoginsenoside R1, 4 to 500 for Rg1, 3 to 350 for Re, 2 to 200 for Rf, 4 to 500 for Rb1, 2 to 300 for Rc, 1 to 150 for Rb2, and 2 to 200 for Rd. All curves had a coefficient of determination of at least 99.9%.

Return to Contents Section

The study showed that the method is precise with respect to the HorRat values for each ginsenoside (HorRat 0.37 to 1.92). See Appendix Table 2 for the HorRat results of each ginsenoside in the different test samples. The spike-and-recovery study at three different levels demonstrated that the method has acceptable recovery values from 81.67 to 111.99% (Appendix Table 3). The LOD and LOQ for the following ginsenosides are (µg/mL): notoginsenoside R1 (0.32, 0.52), Rg1 (0.52, 1.34), Re (0.47, 0.70), Rf (0.34, 0.72), Rb1 (0.80, 1.39), Rc (0.57, 0.95), Rb2 (0.18, 0.53), and Rd (0.86, 1.37).

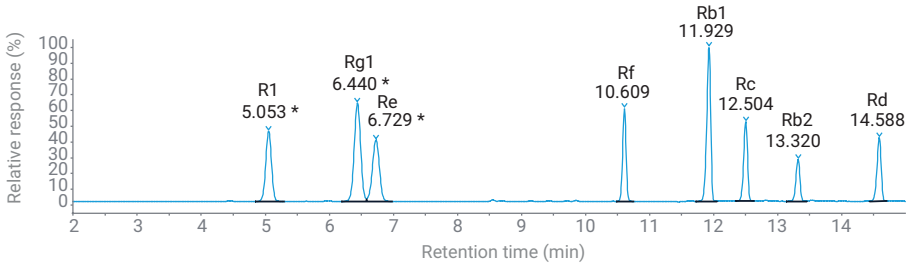



**Figure 2.** Chromatogram of standards; the eight ginsenosides, in elution order, are notoginsenoside R1, Rg1, Re, Rf, Rb1, Rc, Rb2, and Rd.

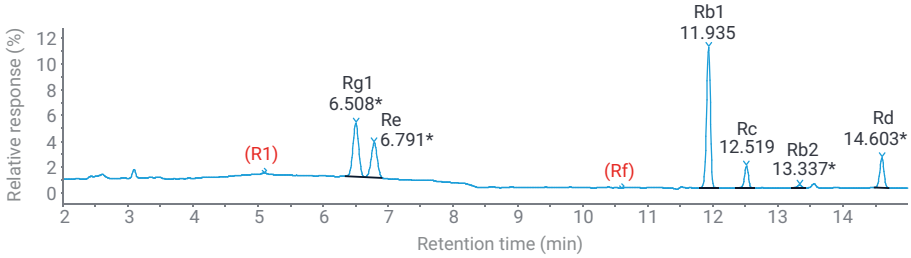



**Figure 3.** Chromatogram of a *P. quinquefolius* powdered root material.

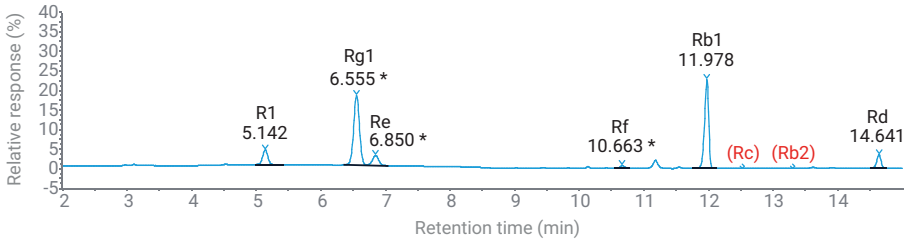



**Figure 4.** Chromatogram of a *P. notoginseng* extract.

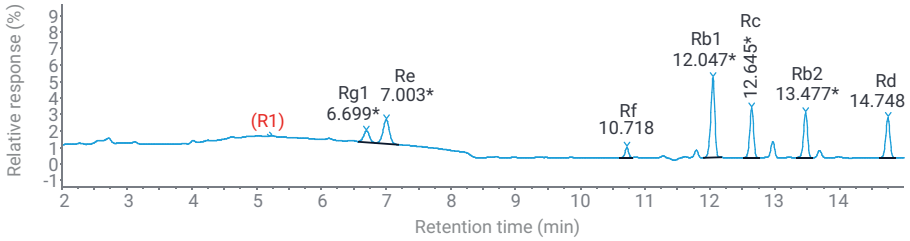



**Figure 5.** Chromatogram of a *P. ginseng* tablet.



Return to Contents Section

## Conclusion

The study demonstrated that this method is precise and accurate in determining notoginsenoside R1, Rg1, Re, Rf, Rb1, Rc, Rb2, and Rd in ginseng powdered root materials, extracts, tablets, and hard-shelled capsules. The Agilent InfinityLab Poroshell 120 Phenyl-Hexyl column delivered better resolution than a traditional high-performance liquid chromatography column, as well as consumed less solvent during analysis due to a shorter run time.[6,7]

## References


1. Witchl, M. Herbal Drugs and Phytopharmaceuticals: A Handbook for Practice on a Scientific Basis, 3rd ed. Medpharm GmbH Scientific Publishers, Stuttgart, Germany, **2004**, 255–258.
2. Wagner, H.; Norr, H.; Winterhoff, H. Plant Adaptogens. *Phytomedicine* **1994**, *1*, 63–76.
3. Meerson, F. Adaptation, Stress and Prophylaxis. Springer-Verlag Berlin Heidelberg New York, **1984**.
4. Betz, J. *et al.* The NIH analytical methods and reference materials program for dietary supplements. *Anal. Bioanal. Chem.* **2007**, *389*, 19–25.
5. Standard Method Performance Requirements (SMPRs) for Determination of Select Ginsenosides in Dietary Supplements and Dietary Ingredients. *AOAC SMPR 2017.014*, **2017**.
6. Brown, P. Determination of ginsenoside content in Asian and North American ginseng raw materials and finished products by high-performance liquid chromatography: single-laboratory validation. *J. AOAC Int*. **2011**, *94(5)*, 1391–1399.
7. Brown, P.; Yu Ronan. Determination of Ginsenoside Content in *Panax ginseng* C.A. Meyer and *Panax quinquefolius* L. Root Raw Materials and Finished Products by High-Performance Liquid Chromatography with Ultraviolet Absorbance Detection: Interlaboratory Study. *J. AOAC Int.* **2013**, *96(1)*, 12–19.
8. AOAC Guidelines for Single Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals. *AOAC International* **2019**.


Return to Contents Section

# Appendix

**Table 2.** Ginsenosides with their corresponding HorRat values, mean values in mg/g, and percent coefficient of variation (% CV).

| Matrix | Analyte | HorRat | mg/g ($\bar{X}$) | % CV |
|---|---|---|---|---|
| *P. quinquefolius* Powdered Root | Notoginsenoside R1 | NA | NA | NA |
| | Rg1 | 0.82 | 8.15 | 3.39 |
| | Re | 1.06 | 5.65 | 4.60 |
| | Rf | NA | NA | NA |
| | Rb1 | 1.14 | 15.36 | 4.27 |
| | Rc | 0.62 | 2.66 | 3.02 |
| | Rb2 | 1.78 | 0.63 | 10.78 |
| | Rd | 0.94 | 3.92 | 4.31 |
| *P. ginseng* Powdered Root | Notoginsenoside R1 | NA | NA | NA |
| | Rg1 | 1.30 | 3.84 | 6.02 |
| | Re | 0.81 | 1.17 | 4.49 |
| | Rf | 0.47 | 0.74 | 2.77 |
| | Rb1 | 1.60 | 4.68 | 7.19 |
| | Rc | 1.02 | 2.13 | 5.13 |
| | Rb2 | 1.26 | 2.42 | 6.23 |
| | Rd | 1.07 | 1.26 | 5.86 |
| *P. notoginseng* Powdered Root | Notoginsenoside R1 | 1.02 | 7.77 | 4.23 |
| | Rg1 | 0.71 | 42.73 | 2.29 |
| | Re | 0.62 | 6.68 | 2.63 |
| | Rf | 1.26 | 0.96 | 7.18 |
| | Rb1 | 1.32 | 41.87 | 4.26 |
| | Rc | NA | NA | NA |
| | Rb2 | NA | NA | NA |
| | Rd | 0.83 | 7.41 | 3.49 |
| *P. quinquefolius* Root Extract | Notoginsenoside R1 | NA | NA | NA |
| | Rg1 | 1.61 | 1.64 | 8.45 |
| | Re | 0.61 | 12.54 | 2.35 |
| | Rf | NA | NA | NA |
| | Rb1 | 0.81 | 43.54 | 2.61 |
| | Rc | 0.42 | 13.24 | 1.61 |
| | Rb2 | 0.41 | 2.26 | 2.04 |
| | Rd | 0.59 | 16.73 | 2.18 |
| *P. ginseng* Root Extract | Notoginsenoside R1 | NA | NA | NA |
| | Rg1 | 1.65 | 8.59 | 6.77 |
| | Re | 1.52 | 3.93 | 7.00 |
| | Rf | 1.42 | 1.57 | 7.51 |
| | Rb1 | 1.61 | 13.62 | 6.15 |
| | Rc | 1.92 | 5.37 | 8.41 |
| | Rb2 | 1.60 | 5.79 | 6.93 |
| | Rd | 1.56 | 2.79 | 7.58 |
| *P. notoginseng* Root Extract | Notoginsenoside R1 | 0.55 | 26.60 | 1.88 |
| | Rg1 | 0.58 | 112.10 | 1.62 |
| | Re | 0.62 | 12.52 | 2.39 |
| | Rf | 0.37 | 1.95 | 1.88 |
| | Rb1 | 0.64 | 98.08 | 1.80 |
| | Rc | NA | NA | NA |
| | Rb2 | 0.93 | 2.56 | 4.58 |
| | Rd | 0.72 | 32.60 | 2.40 |

Return to Contents Section

| Matrix | Analyte | HorRat | mg/g ($\bar{X}$) | % CV |
|---|---|---|---|---|
| *P. ginseng* Tablet | Notoginsenoside R1 | NA | NA | NA |
| | Rg1 | 0.97 | 0.95 | 5.53 |
| | Re | 1.12 | 2.18 | 5.63 |
| | Rf | 1.10 | 0.42 | 7.12 |
| | Rb1 | 1.17 | 5.25 | 5.17 |
| | Rc | 1.04 | 3.28 | 4.92 |
| | Rb2 | 0.97 | 3.09 | 4.61 |
| | Rd | 1.37 | 2.97 | 6.56 |
| *P. ginseng* Hard-Shelled Capsule | Notoginsenoside R1 | NA | NA | NA |
| | Rg1 | 0.75 | 0.66 | 4.50 |
| | Re | 1.48 | 0.92 | 8.47 |
| | Rf | NA | NA | NA |
| | Rb1 | 1.07 | 1.30 | 5.81 |
| | Rc | 1.22 | 0.62 | 7.44 |
| | Rb2 | 1.51 | 0.65 | 9.09 |
| | Rd | 0.92 | 0.71 | 5.45 |

**Table 3.** Spike-and-recovery (mean percent), LOD, and LOQ results.

| Ginsenosides | LOD (µg/mL) | LOQ (µg/mL) | Level 1 ($\bar{X}$) % Recovery | Level 2 ($\bar{X}$) % Recovery | Level 3 ($\bar{X}$) % Recovery |
|---|---|---|---|---|---|
| Notoginsenoside R1 | 0.32 | 0.52 | 93.92 | 107.01 | 109.66 |
| Rg1 | 0.52 | 1.34 | 87.18 | 101.60 | 102.86 |
| Re | 0.47 | 0.70 | 110.78 | 105.12 | 101.76 |
| Rf | 0.34 | 0.72 | 83.49 | 91.93 | 97.28 |
| Rb1 | 0.80 | 1.39 | 111.17 | 106.48 | 102.21 |
| Rc | 0.57 | 0.95 | 110.20 | 111.99 | 109.38 |
| Rb2 | 0.18 | 0.53 | 81.67 | 95.61 | 94.31 |
| Rd | 0.86 | 1.37 | 109.74 | 98.66 | 95.73 |

Return to Contents Section

# Identifying and Quantifying Select Kavalactones and Flavokavains in Kava Root Raw Materials and Finished Products

## Using UHPLC/UV and an Agilent InfinityLab Poroshell 120 SB-C18 column

Application Note

Food and Beverage Testing

**Authors**

Ronan Yu, Ying Liu, and Paula Brown
BCIT, Natural Health and Food Products Research Group
Burnaby, British Columbia, Canada

Udo Huber
Agilent Technologies, Inc.
Waldbronn, Germany

## Abstract

Levels of kavalactones and flavokavains are important factors when determining the quality of kava and kava products. With an ongoing interest in the safety and quality of kava products, it is necessary to develop a method for the determination of kava chemical composition to ensure confidence in assessing product quality. This method can identify and quantify select kavalactones (methysticin, dihydromethysticin, DL-kavain, dihydrokavain, yangonin, and desmethoxyyangonin) and flavokavains (flavokavain A, flavokavain B, and flavokavain C) in a single chromatography run. The separation of analytes was achieved in 10 minutes using an Agilent InfinityLab Poroshell 120 SB-C18, 3.0 × 75 mm, 2.7 µm column.

## Introduction

Kava (*Piper methysticum* Forst. F.; family: Piperaceae) has become popular in the western world as an herbal supplement for treating anxiety symptoms and as a recreational drink for its mild inebriating effect.[1,2] The consumption of kava beverages made from its root or rhizome originated in the south Pacific region.[3–5] However, there is an ongoing debate regarding the safety of consuming kava. While research into kava grew considerably in the early 2000s, everything changed when concerns over toxicity led to a European ban of kava sales in 2002.[6,7] Other countries, such as the United States and Canada temporarily followed suit. Health Canada issued a stop-sale order in early 2002, and the FDA issued a warning that use of kava might potentially cause liver toxicity.[6,7] However, more recent studies have shown that kava can be consumed with an acceptably low health risk. The FDA accepted kava as a dietary supplement with serving limits, and Health Canada has licensed kava products under the natural health

Return to Contents Section

products regulations.[4,8,9] On 21 December 2017, the 15 year ban of kava in Europe was lifted. Still, questions of adulteration, authenticity, and concerns about the supply chain continue to plague this popular herbal product.[10] Kavalactones and flavokavains are the two most important groups of chemicals for determining kava quality. Hence, we developed a UHPLC method as a tool for quantifying select kavalactones and flavokavains in kava raw materials and finished products.

## Experimental

The Agilent 1290 Infinity II LC System was composed of the following modules:

- Agilent 1290 Infinity II Flexible Pump (G7104A)
- Agilent 1290 Infinity II Multisampler with Integrated Thermostat (G7167B, option 101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with Agilent InfinityLab Quick Connect heat exchanger, standard flow
- Agilent 1290 Infinity II Diode Array Detector (G7117B) equipped with an Agilent InfinityLab Max-Light Cartridge Flow Cell, 10 mm

### Software

Agilent OpenLab software suite, version 2.3 or later

### Column

Agilent InfinityLab Poroshell 120 SB-C18, 3.0 × 75 mm, 2.7 µm (part number 687975-302T)

### Samples

Kava root powders were purchased from Gaia Garden Herbal Dispensary (BC, CAN). Kava commercial finished products were purchased from Whole Foods Market (BC, CAN). Reference materials of methysticin (purity: 98.98% HPLC), dihydromethysticin (purity: 94.02% HPLC), desmethoxyyangonin (purity: 100% HPLC), and dihydrokavain (purity: 100% HPLC) were purchased from Cerilliant (TX, USA). Reference materials of DL-kavain (purity: 95% HPLC), yangonin (purity: 95% HPLC), flavokavain A (purity: 95% HPLC), flavokavain B (purity: 95% HPLC), and flavokavain C (purity: 95% HPLC) were purchased from Extrasynthese (Lyon, FR).

**Sample preparation for powdered root materials:** A total of 200 mg of kava root powder was extracted with 25 mL of methanol in a 50 mL centrifuge tube. Using a wrist action shaker, the centrifuge tube was shaken for 30 minutes at room temperature. Then, the extract was centrifuged at 4,500 rpm for 5 minutes, and the supernatant was transferred to a 50 mL volumetric flask. The remaining residue in the 50 mL centrifuged tube was re-extracted with 25 mL of acetone, and then subjected to the same extraction procedure as previously mentioned. The volumetric flask was filled to the mark with methanol and was mixed thoroughly by inverting the flask several times. An aliquot of the extract was filtered through a syringe fitted with a 0.22 µm nylon filter into an amber HPLC vial for analysis.

**Sample preparation for capsules and phytocaps:** The content of 20 capsules or phytocaps were combined and thoroughly mixed. A 200 mg sample from the bulk mixture was extracted following the same procedure as powdered root materials.

Return to Contents Section

**Sample preparation for tinctures:** Tincture products were thoroughly mixed, and an aliquot was diluted 100× through serial dilution. First, the aliquot was diluted 10× by methanol (e.g., 100 mL tincture to 900 mL of methanol). Then, the 10× diluted tincture was further diluted with methanol (e.g., 100 mL of the 10× diluted tincture to 900 mL of methanol) to produce a 100× dilution. The 100× dilution was filtered through a syringe fitted with a 0.22 µm nylon filter into an amber HPLC vial for analysis.

## Compounds

The kavalactones and flavokavains of interest are shown in Figure 1 with their chemical structures. The UHPLC method used to separate these compounds is outlined in Table 1.

**Table 1.** LC method parameters for analyzing compounds in kava samples.

| Parameter | Value |
|---|---|
| Column | Agilent InfinityLab Poroshell 120 SB-C18, 3.0 × 75 mm, 2.7 µm |
| Solvent | A) 0.1% formic acid in water<br>B) isopropanol and acetonitrile (7:3, v/v) |
| Gradient | Time (min) %B<br>0.00 30<br>5.00 35<br>7.00 90<br>8.00 100<br>10.00 100<br>Stop time: 10.00 min<br>Post time: 5 min |
| Flow Rate | 0.4 mL/min |
| Temperature | 55 °C |
| Detection | 240, 4 nm; reference off<br>355, 4 nm; reference off |
| Injection | Injection volume: 2 µL<br>Sample temperature: 5 °C<br>Needle wash: off |



Return to Contents Section

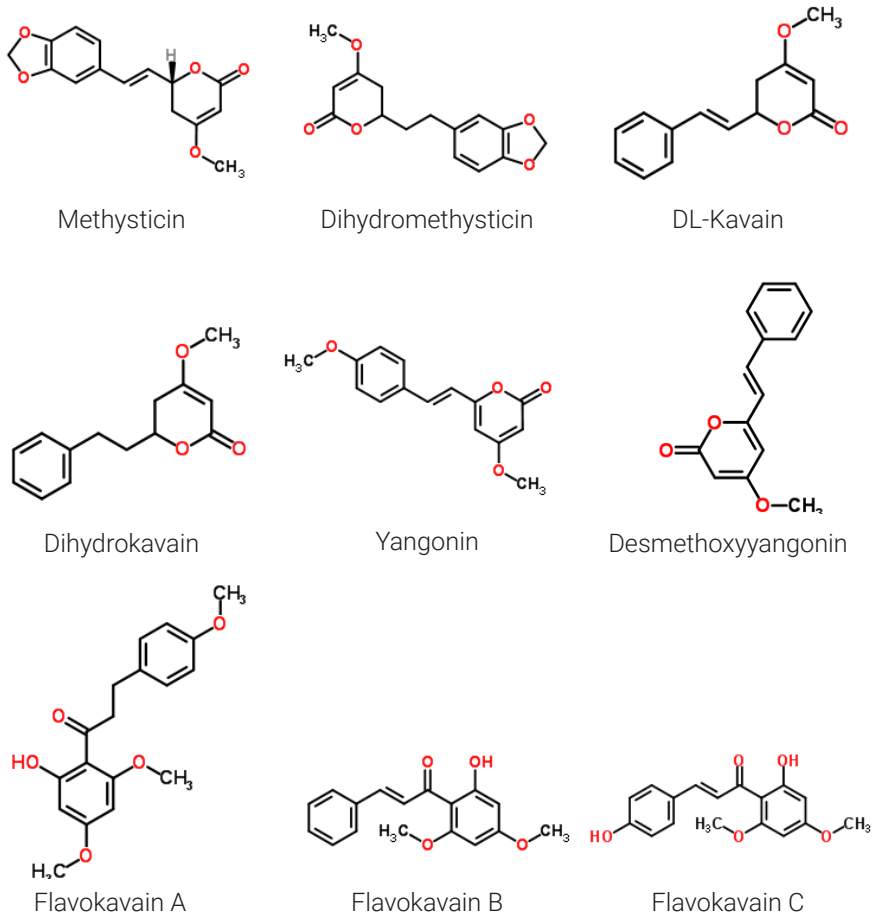



**Figure 1.** Chemical structures of the kavalactones and flavokavains of interest.

## Results and discussion

Analyte identification in the test materials was determined by comparing peak retention times and UV profiles to commercial reference standards. All six major kavalactones and three flavokavains were eluted separately in the following order: methysticin, dihydromethysticin, DL-kavain, dihydrokavain, yangonin, desmethoxyyangonin, flavokavain C, flavokavain A, and flavokavain B. A representative chromatogram of a mixed calibration standard, commercial root powder, and a liquid sample are shown in Figures 2, 3, and 4 respectively.

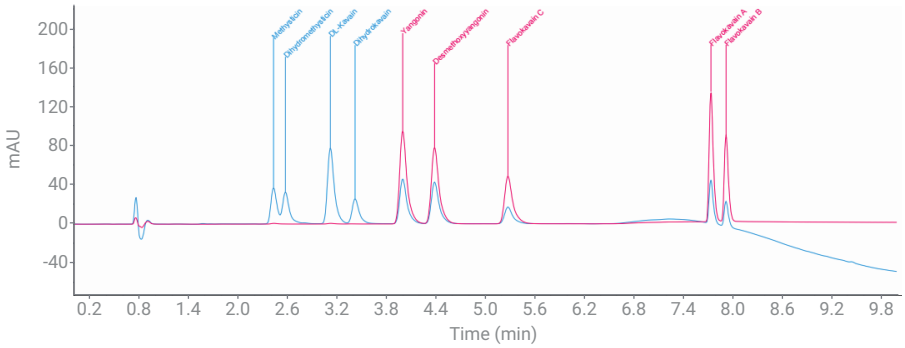



**Figure 2.** Chromatogram of a mixed standard that contains methysticin, dihydromethysticin, DL-kavain, dihydrokavain, yangonin, desmethoxyyangonin, flavokavain C, flavokavain A, and flavokavain B.

Return to Contents Section

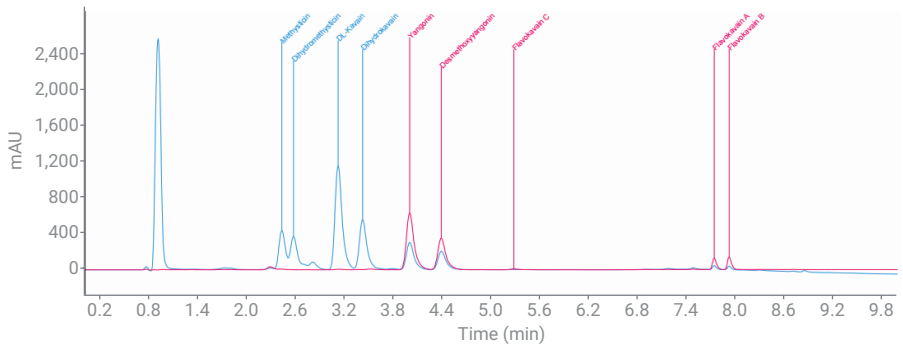



**Figure 3.** Chromatogram of a commercial kava powder sample from Fiji, which contains methysticin, dihydromethysticin, DL-kavain, dihydrokavain, yangonin, desmethoxyyangonin, flavokavain C, flavokavain A, and flavokavain B.

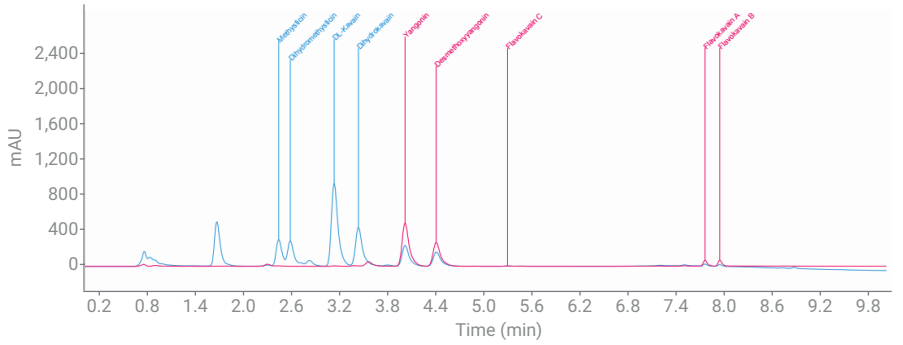



**Figure 4.** Chromatogram of a kava liquid sample that contains methysticin, dihydromethysticin, DL-kavain, dihydrokavain, yangonin, desmethoxyyangonin, flavokavain C, flavokavain A, and flavokavain B.

All compounds were separated with good resolution except for methysticin and dihydromethysticin. Despite the low resolution (Rs <1) of methysticin and dihydromethysticin, the method is still precise and accurate in quantifying both compounds, as demonstrated in a single-laboratory validation study.[11] Using an InfinityLab Poroshell 120 SB-C18, 3.0 × 75 mm, 2.7 µm column, the analysis can be accomplished in 15 minutes including post time.

## Conclusion

The UHPLC/UV method presented in this application note can determine nine selected kavalactones and flavokavains to assess the quality of kava root raw materials and finished products. Using an Agilent InfinityLab Poroshell 120 SB-C18 column, it was possible to run the separation in less time than currently published HPLC methods.[12–14]



Return to Contents Section

## References


1. Kava (Other Names: Ava, Intoxicating Pepper, Kawa Kawa, Kew, Sakau, Tonga, Yangona). US Drug Enforcement Administration, Diversion Control Division, Drug and Chemical Evaluation Section, **2019**.
2. Sarris, J. *et al.* A Comprehensive Review of Efficacy, Safety, and Psychopharmacology. *Aust N Z J Psychiatry* **2011**, *45*, 27–35.
3. Lebot, V. Kava (Piper methysticum Forst. f.): The Polynesian Dispersal of an Oceanian Plant. In: Cox, P.; *et al.* Islands, Plants, and Polynesians: An Introduction to Polynesian Ethnobotany. Dioscorides Press, **1991**.
4. Teschke, R.; Lebot, V. Proposal for a Kava Quality Standardization Code. *Food Chem. Toxicol*. **2011**, *49(10)*, 2503–16.
5. Kava: A Review of the Safety of Traditional and Recreational Beverage Consumption. Food and Agriculture Organization of the United Nations, *FAO Technical Report*, **2016**.
6. Lebot, V.; Do, T.; Legendre, L. Detection of Flavokavins (A, B, C) in Cultivars of Kava (*Piper methysticum*) Using High-Performance Thin Layer Chromatography (HPTLC). *Food Chem.* **2014**, *151*, 554–560.
7. Consumer Advisory: Kava-Containing Dietary Supplements May be Associated With Severe Liver Injury. *US Food and Drug Administration*, **2002**.
8. Coulter, D.; *et al.* Assessments of the Risk of Hepatotoxicity with Kava Products. World Health Organization, *WHO Document Production Services*, **2007**.
9. Ross-van Dorp, C. Besluit van 23 April 2003, houdende wijziging van het Warenwetbesluit Kruidenpreparaten (verbod op Kava kava in kruidenpreparaten). *Staatsblad van het Koninkrijk der Nederlanden, Sdu Uitgevers*, **2003**.
10. Kuchta, K.; Schmidt, M.; Nahrstedt, A. German Kava Ban Lifted by the Court: the Alleged Hepatotoxicity of Kava (*Piper methysticum*) as a Case of Ill-Defined Herbal Drug Identity, Lacking Quality Control, and Misguided Regulatory Politics. *Planta Med* **2015**, *81*, 1647–1653.
11. Liu, Y. *et al.* Single-Lab Validation for Determination of Kavalactones and Flavokavains in *Piper methysticum* (Kava). *Planta Med* **2018**, *84(16)*, 1213–1218.
12. Bilia, A. *et al.* Analysis of Kavalactones from *Piper methysticum* (Kava-Kava). *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **2004**, *812(1–2)*, 203–14.
13. Tang, Y.; Fields, C. A UHPLC-UV Method Development and Validation for Determining Kavalactones and Flavokavains in *Piper methysticum* (Kava). *Molecules* **2019**, *24(7)*, 1245.
14. Hu, L. *et al.* Determination of Six Kavalactones in Dietary Supplements and Selected Functional Foods Containing Piper Methysticum by Isocratic Liquid Chromatography with Internal Standard. *J. AOAC Int.* **2005**, *88(1)*, 16–25.


Return to Contents Section

# Detection of Punicalagins α and β in Pomegranate Extracts and Juices

## A single-laboratory validation column extension using UHPLC/UV and an Agilent InfinityLab Poroshell 120 SB-C18 column

Application Note

Food and Beverage Testing

**Authors**

Ronan Yu
BCIT, Natural Health and Food Products Research Group
Burnaby, British Columbia, Canada

Udo Huber
Agilent Technologies, Inc.
Waldbronn, Germany

## Abstract

This method can quantify punicalagins α and β in pomegranate powdered extracts and juice products. The compounds were chromatographically separated in 6.5 minutes, including a 2.5-minute post time for instrument equilibration, using an Agilent InfinityLab Poroshell 120 SB-C18, 4.6 × 150 mm, 2.7 µm column.

## Introduction

The pomegranate (*Punica granatum*) tree is from the family Lythraceae and is considered native to Iran. Its cultivation has extended to neighboring Mediterranean countries that extended throughout the Himalayas in northern India and drier parts of southeast Asia, Malaya, the East Indies, and tropical Africa.[1] The tree is commonly cultivated in semi-arid mild temperate to subtropical climates. It is sought after for its fruit that contains juicy edible seeds and arils that are eaten fresh, or the juice that is extracted by removing and passing the arils through a basket press.[2] Pomegranates contain ellagitannin compounds that are considered to have antioxidant and anti-inflammatory properties: punicalagins α and β.[3–5]

## Experimental

The Agilent 1290 Infinity II LC System was composed of the following modules:

- Agilent 1290 Infinity II High-Speed Pump (G7120A)
- Agilent 1290 Infinity II Multisampler with Integrated Thermostat (G7167B, option 101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with Agilent InfinityLab Quick Connect heat exchanger, standard flow
- Agilent 1290 Infinity II Diode Array Detector (G7117B) equipped with an Agilent InfinityLab Max-Light Cartridge Flow Cell, 10 mm

Return to Contents Section

### Software

Agilent OpenLab software suite, version 2.3. or later

### Column

Agilent InfinityLab Poroshell 120 SB-C18, 4.6 × 150 mm, 2.7 µm (p/n 683975-902)

### Samples

Ten pomegranate test materials were used in this validation study. Commercially available pomegranate extracts and juices were purchased from Verdure Sciences (ON, CAN), Organika (BC, CAN), LifeExtenstion (FL, USA), Nature's Way (WI, USA), Bremner Foods Ltd. (BC, CAN), Bolthouse Farms (CA, USA), and POM Wonderful (CA, USA). All samples were given IBRC numbers as identifiers to blind the identity label of the product but not the composition itself. Punicalagins α and β reference standards (lot 75676506-99002) were purchased from Cerilliant (CA, USA). Methanol (HPLC grade) and acetic acid (ACS grade) were purchased from VWR International (AB, CAN). A Thermo Fisher Scientific Barnstead Smart2Pure was used to provide ultrapure water.

**Preparation of the stock solution and calibration curve:** A 1 mg/mL (1,000 ppm) punicalagins α and β mixed stock solution was prepared by diluting the reference standard with 2% acetic acid in water. An eight-point calibration curve of the mixed punicalagins was prepared by serial dilution from 400 to 3.13 ppm using the mixed stock solution with 2% acetic acid in water as the diluent.

**Sample preparation for powdered extracts:** 100 mg (± 1 mg) of the ground test material was weighed into a 50 mL centrifuge tube. 25 mL of extraction solvent (2% acetic acid in water) was added using a volumetric pipette, vortexed for 60 seconds, and centrifuged at 4,500 rpm for 5 minutes. Approximately 1 mL of the solution was filtered through a 0.22 µm nylon syringe filter into an HPLC vial.

**Sample preparation for juices and juice concentrates:** The bottle was thoroughly mixed by inverting it about 20 times. A 1 mL liquid sample was transferred into a 2 mL microcentrifuge tube and centrifuged at 4,500 rpm for 5 minutes. 500 µL of the liquid was aliquoted and diluted with the extraction solvent using a dilution factor of 1:1 (1 part juice or juice concentrate to 1 part of extraction solvent) into a microcentrifuge tube. It should be noted that the 1:1 dilution factor is a recommendation. If the sample is more concentrated, then a higher dilution might be required to quantify the phytochemical compounds. Approximately 1 mL of the diluted solution was filtered through a syringe fitted with a 0.22 µm nylon filter into an HPLC vial.

Return to Contents Section

## Compounds

The ellagitannin punicalagins α and β are shown in Figure 1 with the chemical structure.

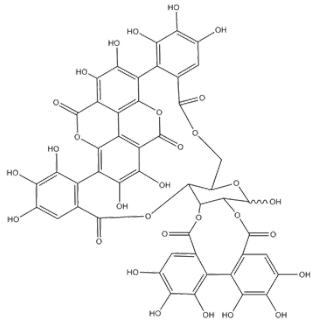


**Figure 1.** Chemical structure of the punicalagins α and β.

The aim of this single-laboratory validation (column extension) study is to use a more efficient column and provide a shorter analysis time than a previous in-house validation study completed on punicalagins α and β in pomegranate samples.[6] Therefore, this validation study only requires confirmation on repeatability and reproducibility regarding the application of the new column. Each sample was prepared in quadruplicate, and an eight-point standard curve of mixed standards was used to quantify the punicalagins over the course of three different days. The UHPLC method that was used is detailed in Table 1. The results were evaluated using the Horwitz Ratio (HorRat) and percent relative standard deviation (% RSD) to indicate method performance.

**Table 1.** LC method parameters for analyzing compounds in pomegranate samples.

| Parameter | Value |
|---|---|
| Column | Agilent InfinityLab Poroshell 120 SB-C18, 4.6 × 150 mm, 2.7 µm |
| Solvent | A) 2% acetic acid in water<br>B) 2% acetic acid in methanol |
| Gradient | Time (min) %B<br>0.00 8<br>5.00 12<br>5.50 75<br>6.00 75<br>Stop time: 6.50 min<br>Post time: 2.5 min |
| Flow Rate | 1.25 mL/min |
| Temperature | 30 °C |
| Detection | 378, 4 nm; reference off |
| Injection | Injection volume: 3 µL<br>Sample temperature: 5 °C<br>Needle wash: off |



Return to Contents Section

## Results and discussion

The method performance of the InfinityLab Poroshell 120 SB-C18, 4.6 × 150 mm, 2.7 µm column in quantifying punicalagins α and β is acceptable with HorRat values ranging from 0.55 to 1.93 and % RSD values from 1.64 to 5.75. Peaks were adequately separated with a resolution of at least 1 from each other and other compounds. See Table 2 for a summary of the average concentration of punicalagins α and β in the 10 test materials with their respective HorRat and % RSD values. Sample chromatograms are shown in Figures 2 through 4.

**Table 2.** Summary of the precision study of punicalagins α and β in the different test samples.

| Matrix | Punicalagin α | | | Punicalagin β | | |
|---|---|---|---|---|---|---|
| | Mean (mg/g, mg/L) | HorRat | % RSD | Mean (mg/g, mg/L) | HorRat | % RSD |
| IBRC 2801 Pomegranate Extract Capsule | 39.70 | 1.75 | 5.70 | 38.56 | 0.99 | 3.24 |
| IBRC 2803 Blueberry and Pomegranate Extract Capsules | 74.83 | 0.55 | 1.64 | 77.07 | 1.39 | 4.10 |
| IBRC 2804 Pomegranate Extract Capsules | 85.07 | 1.24 | 3.60 | 82.46 | 1.97 | 5.75 |
| IBRC 2857 Pomegranate Extract | 210.67 | 1.37 | 3.47 | 202.38 | 1.93 | 4.91 |
| IBRC 2858 Pomegranate Extract | 9.64 | 1.30 | 3.10 | 301.72 | 0.87 | 2.08 |
| IBRC 2859 Pomegranate Extract | 132.01 | 1.35 | 3.67 | 126.25 | 1.51 | 4.13 |
| IBRC 2806 Pomegranate Juice | 563.36 | 1.29 | 2.82 | 557.48 | 0.97 | 2.12 |
| IBRC 3308 Pomegranate Juice | 696.15 | 1.57 | 3.31 | 703.80 | 1.42 | 2.98 |
| IBRC 3309 Pomegranate Juice | 170.65 | 0.95 | 2.49 | 175.22 | 1.36 | 3.54 |
| IBRC 3310 Blueberry and Pomegranate Juice | 127.42 | 0.92 | 2.50 | 132.07 | 1.11 | 3.00 |

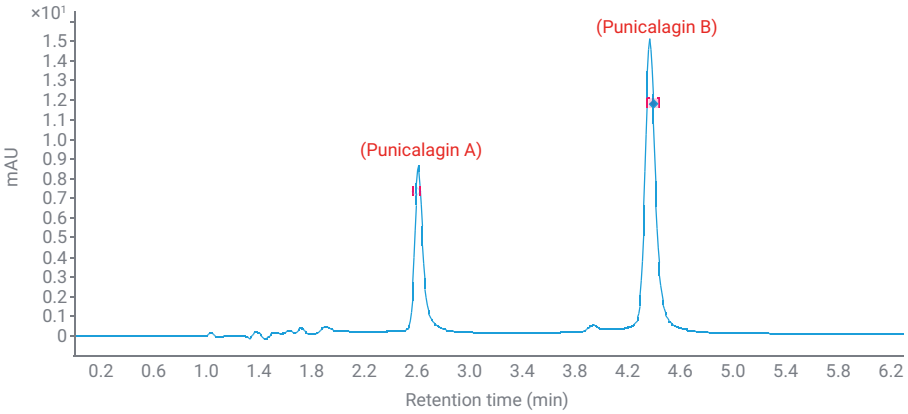



**Figure 2.** Chromatogram of 100 ppm mixed standard of punicalagins α and β.

Return to Contents Section

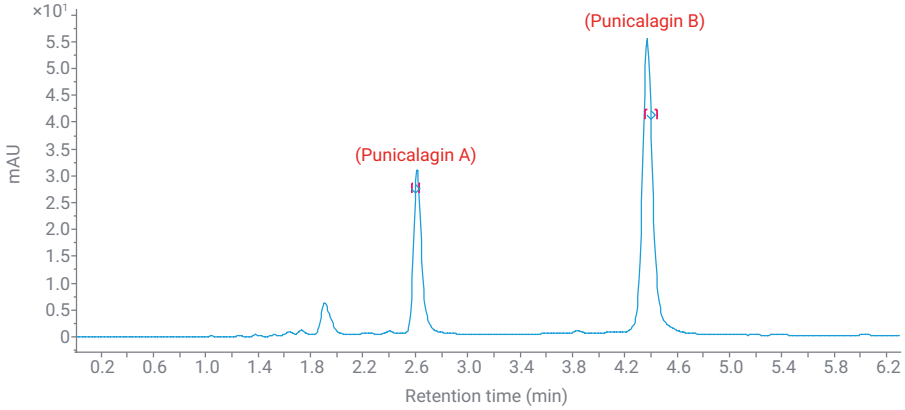



**Figure 3.** Chromatogram of IBRC 2803 blueberry and pomegranate extract.

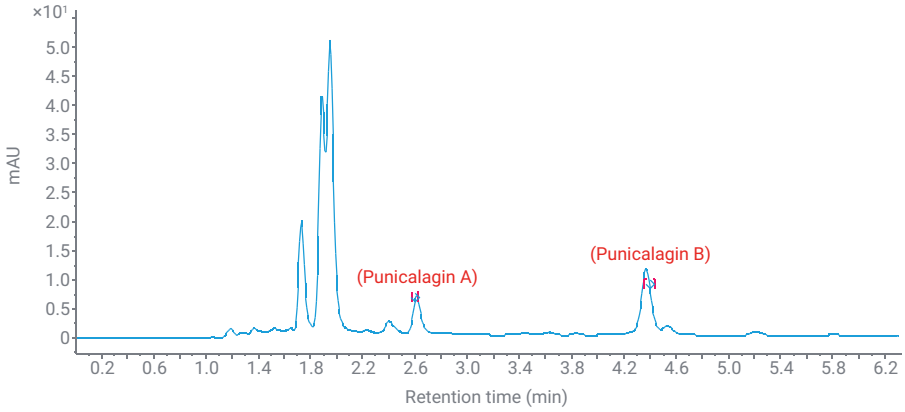



**Figure 4.** Chromatogram of IBRC 3310 blueberry and pomegranate juice.

## Conclusion

This validated method using an Agilent InfinityLab Poroshell 120 SB-C18, 4.6 × 150 mm, 2.7 µm column is suitable for the analysis of punicalagins α and β in pomegranate extracts and juices.



Return to Contents Section

## References


1. Morton, J. Pomegranate, Punica granatum L. Fruits of Warm Climates. *Purdue New Crops Profile*, **1987**, 352–5.
2. http://www.gourmet.com/food/testkitchen/2008/12/roberts_how_to_de-seed_pomegranate
3. Costantini, S. *et al.* Potential Anti-Inflammatory Effects of the Hydrophilic Fraction of Pomegranate (*Punica granatum* L.) Seed Oil on Breast Cancer Cell Lines. *Molecules* **2014**, *19(6)*, 8644–8660.
4. Seeram, N. *et al.* In Vitro Antiproliferative, Apoptotic and Antioxidant Activities of Punicalagin, Ellagic Acid and a Total Pomegranate Tannin Extract are Enhanced in Combination with Other Polyphenols as Found in Pomegranate Juice. *The Journal of Nutritional Biochemistry* **2005**, *16(6)*, 360–367.
5. Gómez Caravaca, A. *et al.* Determination of the Major Phenolic Compounds in Pomegranate Juices by HPLC–DAD–ESI-MS. *Journal of Agricultural and Food Chemistry* **2013**, *61(22)*, 5328–37.
6. Brown, P.; Chan, M. Determination of Punicalagins in Pomegranate Extract by HPLC: A Single Laboratory Validation Study. **2008**.


Return to Contents Section

# Detection of Six Phytochemicals in Cultivated Rhodiola Raw Materials and Finished Products

## Using UHPLC/UV and an Agilent InfinityLab Poroshell 120 SB-Aq column

Application Note

Food and Beverage Testing

**Authors**

Ronan Yu, Hazrah Moothoo, and Paula Brown
BCIT, Natural Health and Food Products Research Group
Burnaby, British Columbia, Canada

Udo Huber
Agilent Technologies, Inc.
Waldbronn, Germany

## Abstract

*Rhodiola rosea* has been used to promote good health and physical and mental performance. The method presented in this application note can quantify six phytochemicals commonly found in cultivated *Rhodiola rosea* raw materials and finished products, namely tyrosol, salidroside, rosin, rosarin, rosiridin, and rosavin (in order of elution). The phytochemicals were chromatographically separated in 17 minutes including post time using an Agilent InfinityLab Poroshell 120 SB-Aq, 3.0 × 50 mm, 2.7 µm column. The method was validated following AOAC single-laboratory validation guidelines. The LOQ for tyrosol, salidroside, rosin, rosarin, rosiridin, and rosavin are as follows: 1.46, 1.75, 1.28, 1.59, 2.39, and 1.95 µg/mL.

## Introduction

Rhodiola (family Crassulaceae) is a group of flowering plants that are found throughout northern Europe, North America, Russia, and some areas of China.[1] *Rhodiola rosea* has been used in traditional medicine to treat different illnesses including fatigue, depression, anxiety, infections, and nervous system disorders.[2] Most of its health benefits has been well studied in *Rhodiola rosea*. It is known to have adaptogenic properties that encourage homeostasis in the body.[3] The active constituents investigated in *Rhodiola rosea* are phenylethanoids (salidroside, tyrosol), phenylpropanoid glycosides (rosarin, rosavin, rosin), and a monoterpene (rosiridin).[4] However, it is the phenylpropanoids that are considered the main phytochemicals present in the plant.[1]



Return to Contents Section

## Experimental

The Agilent 1290 Infinity II LC System was composed of the following modules:

- Agilent 1290 Infinity II Flexible Pump (G7104A)
- Agilent 1290 Infinity II Multisampler with Integrated Thermostat (G7167B, option 101)
- Agilent 1290 Infinity II Multicolumn Thermostat (G7116B) with Agilent InfinityLab Quick Connect heat exchanger, standard flow
- Agilent 1290 Infinity II Diode Array Detector (G7117B) equipped with an Agilent InfinityLab Max-Light Cartridge Flow Cell, 10 mm

### Software

Agilent OpenLab software suite, version 2.3 or later

### Column

Agilent InfinityLab Poroshell 120 SB-Aq, 3.0 × 50 mm, 2.7 µm (part number 689975-314T)

### Samples

Rhodiola root powder and powdered extracts samples were provided by Alberta Rhodiola Rosea Growers Association (ARRGO), AB, Canada. Commercial capsule products were purchased from Advanced Orthomolecular Research (AOR), AB, Canada. Two of the rhodiola reference standards (tyrosol and salidroside) were obtained from Extrasynthese, Lyon, FR, and the others (rosin, rosarin, rosiridin, and rosavin) were from Cerilliant, TX, USA. Methanol and acetonitrile (HPLC grade) were purchased from VWR International. An in-house Thermo Fisher Scientific Barnstead Smart 3 UV/UF was used to provide ultrapure water.

**Sample preparation for raw materials and powdered extracts:** Rhodiola root samples were ground to <60 mesh size. 100 mg (+1 mg) of the ground test material was weighed into a 50 mL centrifuge tube. 40 mL of extraction solvent (methanol and water, 70:30, v/v) was added to the sample, and the mixture was vortexed for 10 seconds. Then, the mixture was transferred onto a wrist-action shaker and shaken for 60 minutes, after which it was vortexed for 10 seconds and centrifuged at 4,500 rpm for 3 minutes. Approximately 1 mL of the supernatant was filtered through a 0.22 µm polytetrafluoroethylene (PTFE) syringe filter into an HPLC vial.

**Sample preparation for tinctures:** The tincture was thoroughly mixed by inverting the container about 20 times. 10 mL of tincture was transferred into a 15 mL centrifuge tube and centrifuged at 4,500 rpm for 3 minutes. An aliquot of the supernatant was diluted 1:10 with the extraction solvent. It should be noted that the 1:10 dilution factor is a recommendation. If the tincture is more concentrated, then a higher dilution might be required to quantify the phytochemical compounds. Approximately 1 mL of the diluted solution was filtered through a syringe fitted with a 0.22 µm PTFE filter into an HPLC vial.

**Sample preparation for capsules:** The content of 20 emptied capsules was combined and transferred into a 15 mL centrifuge tube. 100 mg (+1 mg) of the material was used for analysis and 40 mL of extraction solvent was added. The same preparation for raw materials was followed.

The diluent solvent for the reference standards was 100% methanol.

Return to Contents Section

## Compounds

The rhodiola phenylethanoids and phenylpropanoid glycosides are shown in Figure 1 with their chemical structures.

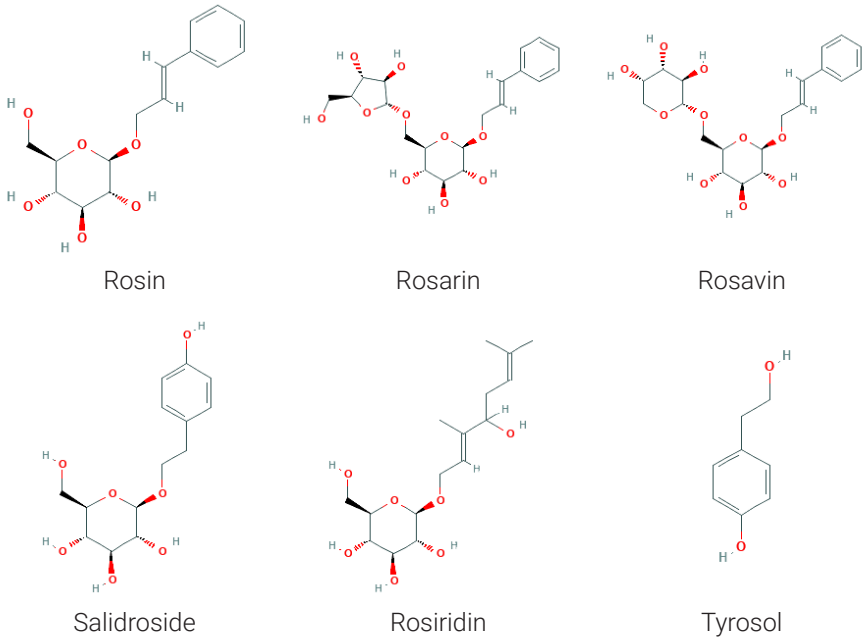



**Figure 1.** Chemical structures of the phenylethanoids and phenylpropanoid glycosides of interest.

A seven-point standard curve of mixed standards (166.7, 83.3, 41.7, 20.8, 10.4, 5.2, and 2.6 µg/mL) was used to quantify the six phytochemical compounds. The UHPLC method details are outlined in Table 1.

**Table 1.** LC method parameters for analyzing compounds in rhodiola samples.

| Parameter | Value |
|---|---|
| Column | Agilent InfinityLab Poroshell 120 SB-Aq, 3.0 × 50 mm, 2.7 µm |
| Solvent | A) water<br>B) acetonitrile |
| Gradient | Time (min) %B<br>0.00 2<br>2.00 2<br>14.00 6<br>14.10 90<br>15.10 90<br>Stop time: 15.1 min<br>Post time: 2.00 min |
| Flow Rate | 2.1 mL/min |
| Temperature | 35 °C |
| Detection | 205, 4 nm; reference off<br>220, 4 nm; reference off |
| Injection | Injection volume: 2 µL<br>Sample temperature: 5 °C<br>Needle wash: off |

To test for precision, all test samples were prepared in quadruplicate. Sample preparation and analyses were conducted on three different days. Precision was evaluated using the Horwitz Ratio (HorRat).[5] The accuracy of the method was tested by spiking a known amount of analyte into a blank matrix at three different levels and then calculated for percent recovery. The LOD and LOQ were calculated using the definition of the International Union of Pure and Applied Chemistry (IUPAC).[6]

Return to Contents Section

## Results and discussion

All compounds were separated with acceptable resolution (Rs ≥1.5) within 14 minutes as seen in Figures 2, 3, and 4. The single-laboratory validation demonstrated that the method is precise and accurate (Tables 2 and 3) with respect to AOAC Guidelines for Single-Laboratory Validation of Chemical Methods for Dietary Supplements and Botanicals, Appendix K, 2013.[7] For precision, the HorRat values ranged from 0.31 to 1.62, which are deemed acceptable and showed the reproducibility of the method. The percent recovery of the method ranged from 90 to 109% at different levels, demonstrating the method has a similar response to the standard curve in a different sample matrix. The LOD and LOQ for the examined compounds were below 2.5 µg/mL and are summarized in Table 4.

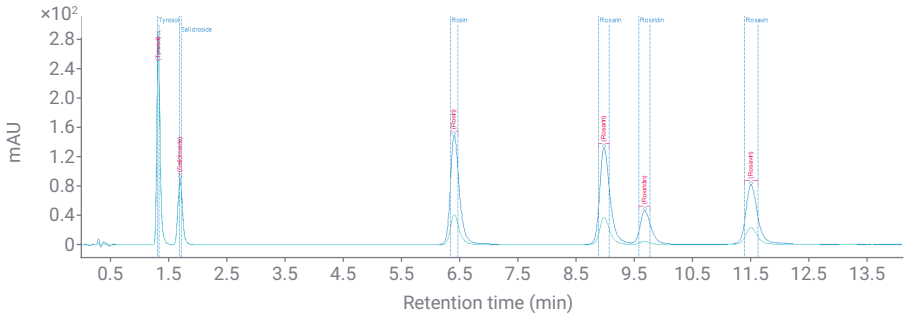



**Figure 2.** Chromatogram of the rhodiola reference standard.

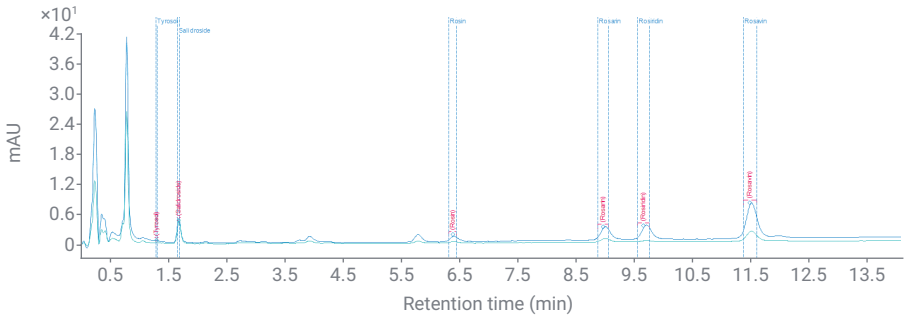



**Figure 3.** Chromatogram of a rhodiola powdered root material.

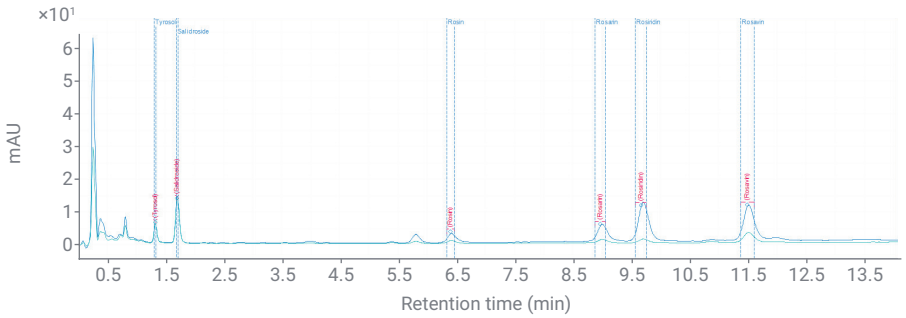



**Figure 4.** Chromatogram of a rhodiola extract from a capsule product.

Return to Contents Section

**Table 2.** Summary of the precision study for the different test materials (n = 4).

| Matrix | Analyte | HorRat Value | Mean (mg/g) | % CV |
|---|---|---|---|---|
| ARRGO *Rhodiola rosea* Root | Tyrosol | <LOD | <LOD | NA |
| | Salidroside | 0.78 | 3.33 | 3.68 |
| | Rosin | <LOQ | <LOQ | NA |
| | Rosarin | 1.20 | 1.84 | 6.18 |
| | Rosiridin | 0.91 | 4.43 | 4.13 |
| | Rosavin | 1.08 | 6.26 | 4.64 |
| AOR Relax and Recharge, Rhodiola + Ashwaganda Vegetarian Capsule | Tyrosol | 1.22 | 1.79 | 6.35 |
| | Salidroside | 0.74 | 8.73 | 3.02 |
| | Rosin | 0.57 | 1.80 | 8.28 |
| | Rosarin | 0.74 | 3.29 | 3.49 |
| | Rosiridin | 0.82 | 16.58 | 3.04 |
| | Rosavin | 0.94 | 8.98 | 3.83 |
| Botanica Rhodiola Tincture (*Rhodiola rosea*) | Tyrosol | 0.52 | 330.15 | 3.49 |
| | Salidroside | 0.35 | 521.51 | 2.20 |
| | Rosin | 0.55 | 61.42 | 4.75 |
| | Rosarin | 0.56 | 497.12 | 3.55 |
| | Rosiridin | 0.47 | 631.44 | 2.84 |
| | Rosavin | <LOD | <LOD | NA |
| ARRGO *Rhodiola rosea* Tincture | Tyrosol | 0.69 | 44.08 | 6.26 |
| | Salidroside | 0.52 | 498.46 | 3.29 |
| | Rosin | 0.53 | 115.85 | 4.16 |
| | Rosarin | 0.46 | 310.01 | 3.07 |
| | Rosiridin | 0.50 | 683.12 | 2.97 |
| | Rosavin | 1.62 | 818.01 | 3.34 |
| ARRGO *Rhodiola rosea* Dry Powdered Extract | Tyrosol | <LOD | <LOD | NA |
| | Salidroside | 0.31 | 8.94 | 1.28 |
| | Rosin | 0.70 | 1.70 | 3.65 |
| | Rosarin | 0.48 | 3.79 | 2.21 |
| | Rosiridin | 0.75 | 9.35 | 3.02 |
| | Rosavin | 0.67 | 12.39 | 2.60 |
| AOR *Rhodiola rosea* Dry Herbs | Tyrosol | <LOD | <LOD | NA |
| | Salidroside | 0.88 | 2.83 | 4.26 |
| | Rosin | 1.35 | 0.84 | 7.87 |
| | Rosarin | 0.63 | 2.35 | 3.14 |
| | Rosiridin | 1.26 | 4.49 | 5.70 |
| | Rosavin | 0.88 | 7.90 | 3.66 |
| AOR Rhodiola Capsules | Tyrosol | 1.15 | 1.75 | 5.99 |
| | Salidroside | 0.32 | 12.88 | 1.24 |
| | Rosin | 0.48 | 4.92 | 2.14 |
| | Rosarin | 0.92 | 8.88 | 3.73 |
| | Rosiridin | 0.69 | 37.47 | 2.25 |
| | Rosavin | 0.68 | 13.59 | 2.61 |



Return to Contents Section

**Table 3.** Summary of the spike recovery study at three different levels (75, 100, and 125%) that represents the typical concentration of each analyte found in a powdered extract capsule.

| Level | Analyte | % Recovery | % CV |
|---|---|---|---|
| 1 (75%) | Tyrosol | 109 | 2.97 |
| | Salidroside | 100 | 5.76 |
| | Rosin | 101 | 4.31 |
| | Rosarin | 106 | 1.96 |
| | Rosiridin | 96 | 6.41 |
| | Rosavin | 98 | 6.49 |
| 2 (100%) | Tyrosol | 96 | 2.42 |
| | Salidroside | 109 | 0.92 |
| | Rosin | 99 | 1.14 |
| | Rosarin | 107 | 1.74 |
| | Rosiridin | 108 | 1.29 |
| | Rosavin | 103 | 0.53 |
| 3 (125%) | Tyrosol | 93 | 2.10 |
| | Salidroside | 107 | 0.37 |
| | Rosin | 90 | 2.61 |
| | Rosarin | 104 | 0.66 |
| | Rosiridin | 106 | 1.33 |
| | Rosavin | 100 | 1.04 |

**Table 4.** LOD and LOQ values calculated using the IUPAC method.

| Analyte | LOD (µg/mL) | LOQ (µg/mL) |
|---|---|---|
| Tyrosol | 1.43 | 1.46 |
| Salidroside | 1.73 | 1.75 |
| Rosin | 1.26 | 1.28 |
| Rosarin | 1.58 | 1.59 |
| Rosiridin | 2.35 | 2.39 |
| Rosavin | 1.92 | 1.95 |

## Conclusion

The UHPLC method presented in this application note quantifies tyrosol, salidroside, rosin, rosarin, rosiridin, and rosavin in *Rhodiola rosea* raw materials and finished products. Using an Agilent InfinityLab Poroshell 120 SB-Aq column, it was possible to shorten the separation time and reduce solvent consumption compared to conventional HPLC methods.

Return to Contents Section

## References


1. Mudge, E. *et al.* Purification of Phenylalkanoids and Monoterpene Glycosides from Rodiola rosea L. Roots by High-speed Counter-current Chromatography. *Phytochemical Analysis* **2013**, *24(2)*, 129–134.
2. Brown, R.; Gerbarg, P.; Ramazanov, Z. *Rhodiola rosea*: A Phytochemical Overview. *HerbalGram.* **2002**, *56*, 40–52.
3. Mudge, E. *et al.* Purification of Phenylalkanoids and Monterpene Glycosides from Rhodiola rosea L. Roots by High-speed Counter-Current Chromatography, *Phytochemical Analysis* **2012**.
4. Ma, Y. *et al.* Rapid Resolution Liquid Chromatography (RRLC) Analysis for Quality Control of Rodiola rosea Roots and Commercial Standardized Products. *Natural Product Communications* **2010**, *6(5)*, 645–650.
5. Horwitz, W.; Albert, R. The Horwitz Ratio (HorRat): A Useful Index of Method Performance with Respect to Precision. *Journal of AOAC International* **2006**, *89(4)*, 1095–1109.
6. Limit of detection in analysis. IUPAC, Compendium of Chemical Terminology, 2nd ed. (the "Gold Book"), Blackwell Scientific Publications, Oxford, **1997**.
7. Appendix K: Guidelines for Dietary Supplements and Botanicals. AOAC International, *AOAC Official Methods of Analysis*, **2019**.

## Agilent CrossLab: supporting your success

CrossLab is an Agilent capability that integrates services and consumables to support workflow success, improve productivity, and enhance operational efficiency. Through CrossLab, Agilent strives to provide insight in every interaction to help you optimize the return you get on your instrument investment and achieve your business goals. Agilent CrossLab supports Agilent instruments and select non-Agilent instruments as well. We also provide consultative support for workflow enablement, lab analytics, regulatory compliance, inventory management, and asset management, including relocation services.

Learn more about CrossLab at www.agilent.com/crosslab

## Complete end-to-end solutions for the best scientific insights and operational outcomes

Agilent OpenLab software and Agilent CrossLab services combine with the Agilent InfinityLab LC family to provide you with complete end-to-end solutions and support to ensure the best analytical outcomes. All components are a perfect fit and integrate seamlessly together in your lab. And, they are all available from a single vendor—Agilent.

Learn more about OpenLab and CrossLab at www.agilent.com.

## Agilent Food and Beverage Testing Solutions: helping you achieve food safety, quality, and authenticity

Ensuring food safety and quality is increasingly challenging. Whether you're analyzing for pesticide residues, ensuring that label claims are correct and accurate, or profiling for food authenticity, Agilent works with you, helping you deliver what your customers demand. Agilent Technologies food and beverage testing products and services keep you at the forefront of the latest trends, ahead of issues, and focused where you need to be – delivering highest quality and value to your customers.

Learn more about Food and Beverage Testing Solutions:
www.agilent.com/en/solutions/food-beverage

DE39842622


Published in the USA, June 29, 2022
5994-5001EN

Agilent
Trusted Answers