Waters™

應用手冊

Antioxidant Isolation Using the Prep 150 LC System

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Abstract

This application note demonstrates how the purity of chlorogenic acid which is isolated from Aronia berries can

be maximized by focusing the gradient to improve resolution of the compound of interest.

Benefits

- The Waters Prep 150 LC System, is an affordable, highly reliable, UV-directed system used for preparative chromatography. The straightforward design and uncomplicated software control, facilitates users to more efficiently process samples and thereby increase productivity.
- With knowledge of general chromatographic scaling principles, analytical methods can be scaled-up predictably and accurately for bulk isolation and purification of compounds in larger quantities.
- The Waters Prep 150 LC System is an ideal instrument for laboratories where chemists prefer to complete analytical method development, purity optimization, prep scale-up, and fraction isolation on a single system.

Introduction

Compounds and extracts isolated from natural products such as the Aronia berry (*Aronia Melanocarpa*), have found uses in medicine, agriculture, cosmetics and food in ancient and modern societies around the world.[1] The ability to access and purify these compounds through chromatographic separation has been a major driving force in research and discovery. Chromatographic methods can be developed on any scale, but when developed at a small scale and transferred to a larger scale, valuable sample material and solvents are conserved.

In this application note, the antioxidant chlorogenic acid (Figure 1) is purified from Aronia berries. The isolation is used to demonstrate method development, scale-up, and fraction collection using the Waters Prep 150 LC System. The application also demonstrates how purity of the isolate can be maximized by focusing the gradient to improve resolution of the compound of interest.

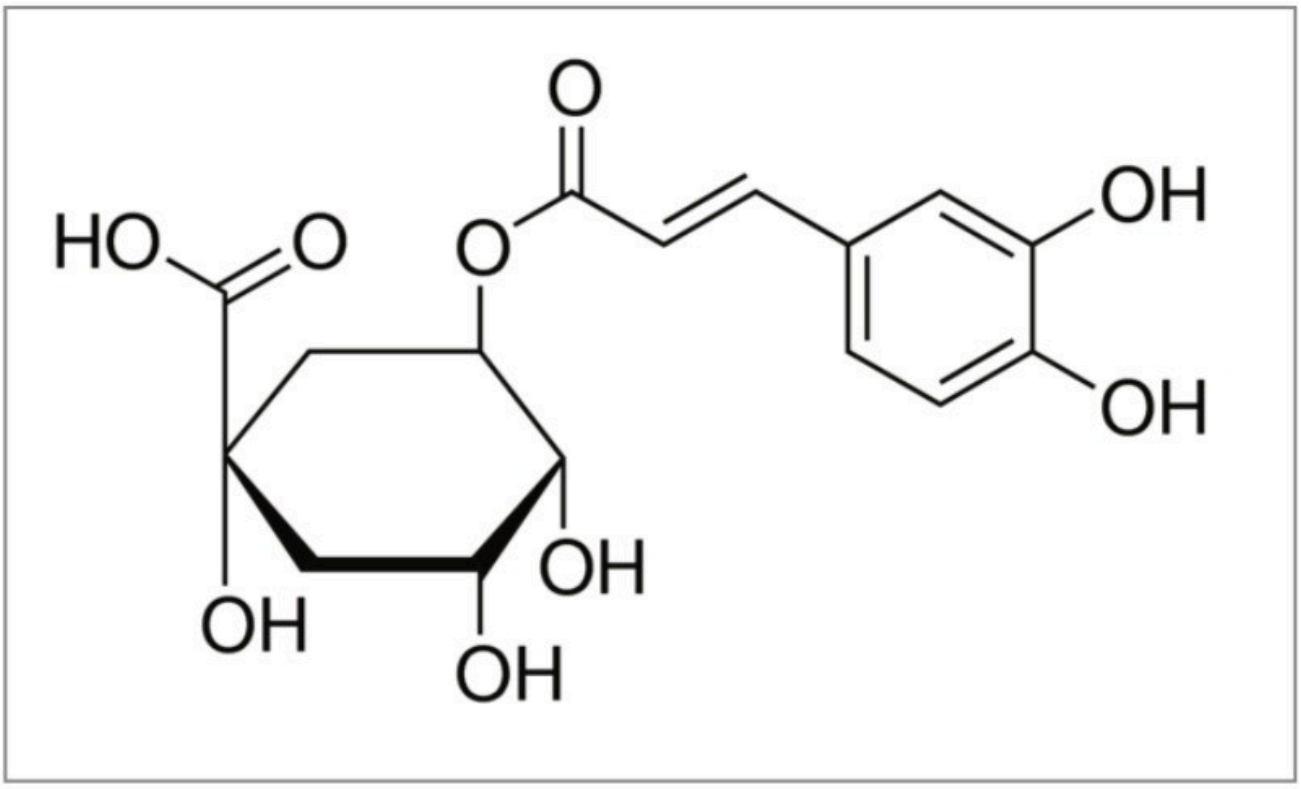

Figure 1. Structure of chlorogenic acid.

Experimental

Conditions

Analytical column:	SunFire C_{18}, 5 µm 4.6 x 50 mm
Analytical flow rate:	1.46 mL/min
Prep column:	SunFire C_{18} OBD Prep, 19 x 50 mm, 5 µm
Prep flow rate:	24.9 mL/min
Mobile phase A:	0.1% formic acid in water
Mobile phase B:	0.1% formic acid in acetonitrile

Detection:	2489 UV/V is at UV 324 nm (Autopurification flow cell)
Temperature:	Ambient
Pump:	2545 Binary Gradient Module
Injector:	FlexInject Manual Dual Injection Module configured with 100 µL loop (analytical) and 2000 µL loop (prep)
Collector:	Waters Fraction Collector III
Data collection:	ChromScope v1.6

Discussion

Prep Chromatography System

The Waters Prep 150 LC System (Figure 2) consisted of the 2545 Binary Gradient Module, a solvent delivery module capable of flow rates up to 150 mL/min; the 2489 UV/Visible Detector; the Waters Fraction Collector III; and the FlexInject Manual Dual Injector Module controlled by ChromScope v1.6, which manages UV-based collection triggers and tracks samples, fractions, and associated data through its straightforward browser (Figure 3). The system configuration is an entry level, easy-to-use modular design that is capable of purifying several samples daily.

Figure 2. The Waters Prep 150 LC System.

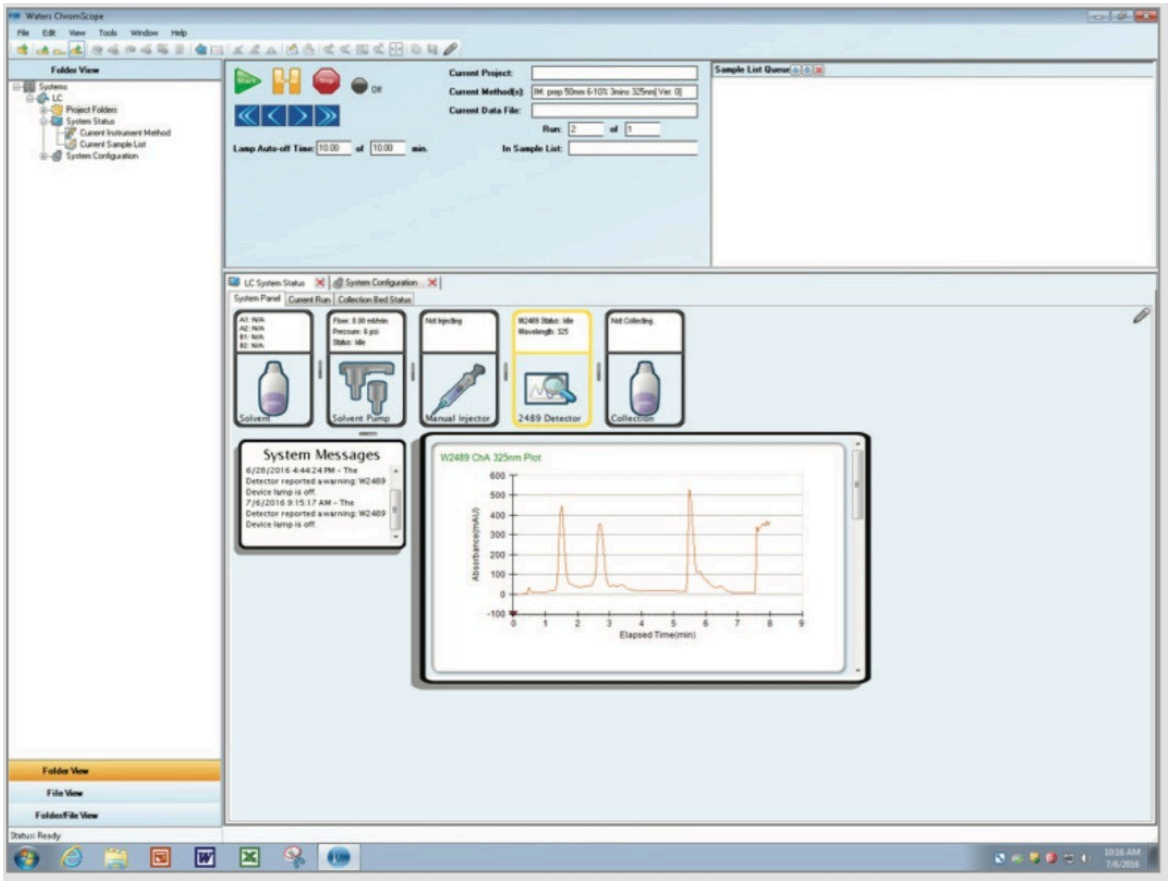

Figure 3. Screen shot of the ChromScope console.

Sample preparation

Fresh Aronia berries were obtained from an organic farm and dried through lyophilization. The dried berries were ground into a fine powder and subsequently 1.5 g reconstituted with 3.0 mL acidified water (1 M HCl). The sample solution was mixed well, centrifuged to remove solids, and the supernatant filtered through a 0.45 µm syringe filter.

A chlorogenic acid reference standard obtained from Sigma-Aldrich, part # C3878, was prepared by dissolving 5.0 mg in 20 mL acidified water (1 M HCl). The reference standard was used to confirm retention time of the chlorogenic acid isolated product.

Results and Discussion

The sample solution was analyzed at the analytical scale with four rapid scouting gradients. Each gradient started from a 2% organic concentration and rapidly increased linearly over 5 minutes to a 25%, 50%, 75%, or 95% organic concentration Figure 4). The scouting run from 2–25% provided the best resolution of chlorogenic acid and surrounding impurities, as determined by a reference standard injection.

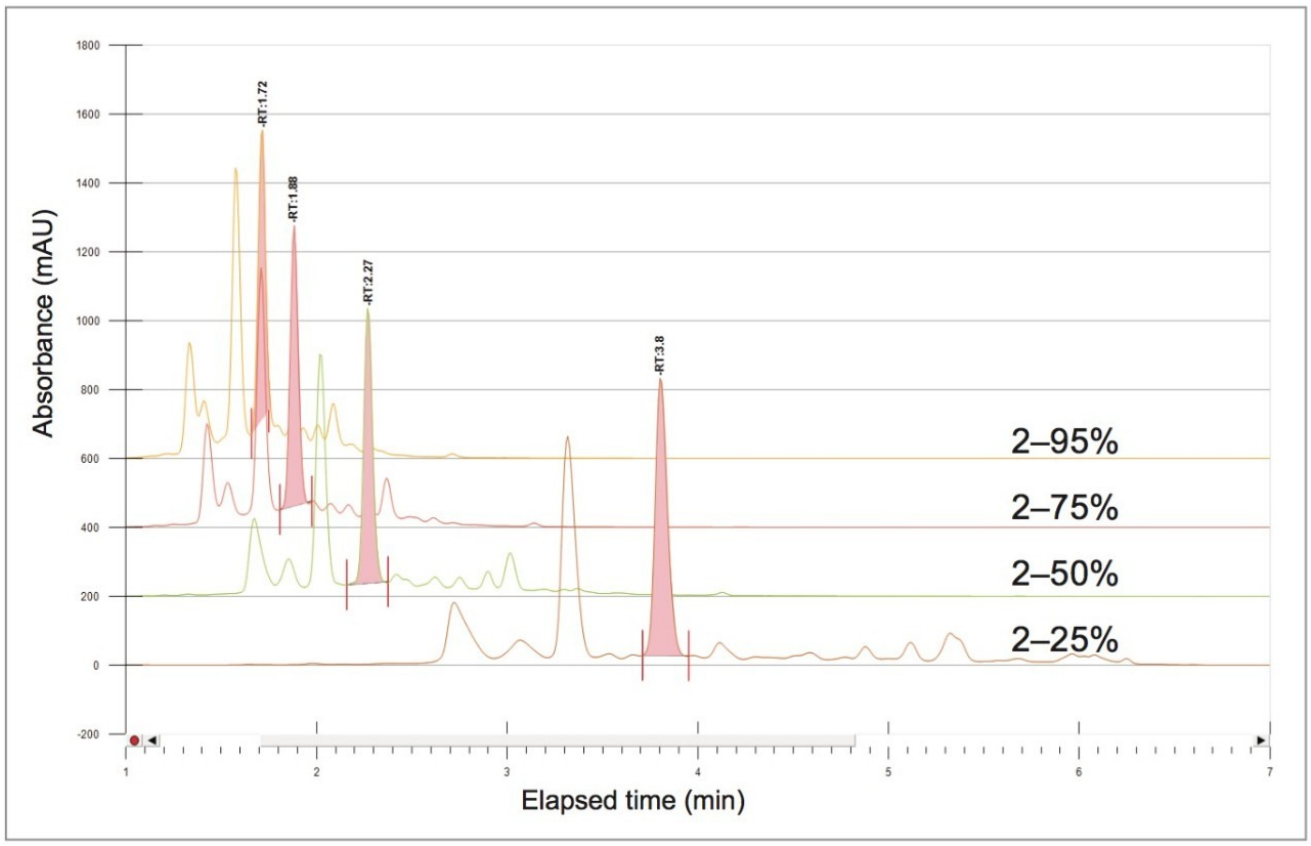

Figure 4. Results from the method scouting runs. The retention time of chlorogenic acid is shown in each chromatogram. A linear gradient was run for 5 minutes from 2% to 95%, 75%, 50%, and 25% organic concentrations, respectively.

Focused gradient

With most separations, to improve the resolution between the compounds of interest and close eluting impurities, a focused gradient can be used. The focused gradient typically begins at the initial organic concentration used in the scouting run and quickly ramps to an organic concentration just before the percentage of solvent that elutes the compound of interest. Then a shallow linear gradient is performed to elute the peak of interest, and finally the column is washed quickly with a high organic concentration.

Scouting run 4 provided the highest resolution between chlorogenic acid and the impurities therefore it was used to develop the focused gradient.

Time	Flow rate	%A	%B
0.00	1.46	98	2
5.00	1.46	98	2
6.00	1.46	75	25
6.50	1.46	5	95
7.00	1.46	98	2

Table 1. Gradient conditions for scouting run 4.

The following calculations were used to generate the focused gradient.

Column volume was calculated with the equation Π x $(r)^2$ x Length, with compensation for the volume occupied by packing material (66% as per the "Waters Optimum Bed Density (OBD) Prep Calculator").

- Column volume (mL) = Π x $(r)^2$ x Length x 66% x (1 mm^3/1000)
  cv = 3.14 x (4.6 mm/2)^2 x 50 mm x 0.66 x (1 mm^3/1000) cv = 0.548 mL
- Offset between the point of gradient formation and the detector = system volume* + column volume
  Offset = 0.949 mL + 0.548 mL Offset = 1.497 mL
  *Refer to the "Waters Optimum Bed Density (OBD) Prep Calculator" application for instructions on determining system volume instructions.
- Time to detector = (Offset in mL)/(Flow rate in mL/min)

  Time to detector =1.497 mL /1.46 mL/min Time to detector = 1.03 min
- Time when the elution concentration was formed= Peak retention time – Time to detector – Gradient hold
  Time elution concentration = 3.80 min – 1.03 min – 0.00 min
  Time elution concentration = 2.77 min
- % Elution concentration for the peak of interest = (Time elution concentration x Change scouting + Initial scouting gradient %)/(Length scouting gradient segment)
  % Elution concentration = (2.77 min)/(5 min) x 23% + 2%

% Elution concentration = 14.7% = 15%

NOTE: For all calculations involving percent, use absolute values only, i.e., 23 not 0.23 for 23%.

- # Column volumes (cv) = (1 col vol)/(mL) x (Flow mL)/(min) x gradient segment time

  # cv = (1 col vol)/(0.548 mL) x (1.46 mL)/(min)x 5 min

  # cv = 13.3

- Scouting gradient slope = (% change scouting gradient)/(# column volumes)

  Scouting gradient slope = (23%)/(13.3 cv) = (1.73%)/(cv)

- Focused gradient slope % per column volume = 1/5 x scouting gradient slope

  Focused gradient slope = (1)/(5) x (1.73%)/(cv)

  Focused gradient slope = 0.346%/cv

- Time focused gradient segment = % focused gradient range* x (1/focused grad slope) x column volume x (1/flow rate)

  Time focused grad segment = (18%–10%) x (1 cv)/(0.346%) x (0.548 mL)/(1 cv)x (1 min)/(1.46 mL)

  Time focused grad segment = 8.7 min

  *Range is typically between 3–5% greater than and less than the concentration that elutes the peak of interest.

Based upon the calculations, a focused gradient was developed to begin at the initial conditions of the scouting run (2%) with a quick ramp to the beginning concentration of the focused gradient (10%). The solvent concentration increased linearly for 8.7 minutes to elute chlorogenic acid, and finally the column was washed with high organic. Adequate resolution between chlorogenic acid (3.8 mins) and surrounding impurities was obtained by the focused gradient (Table 2, Figure 5).

Time	Flow rate (mL/min)	%A	%B
0.00	1.46	98	2
1.00	1.46	90	10
9.70	1.46	90	18
10.5	1.46	5	95
11.0	1.46	5	95
11.5	1.46	98	2

Table 2. Calculated focused gradient.

Time	Flow rate (mL/min)	%A	%B
0.00	1.46	98	2
1.00	1.46	90	10
4.00	1.46	90	13
4.50	1.46	5	95
5.00	1.46	5	95
5.50	1.46	98	2

Table 3. Abbreviated focused gradient.

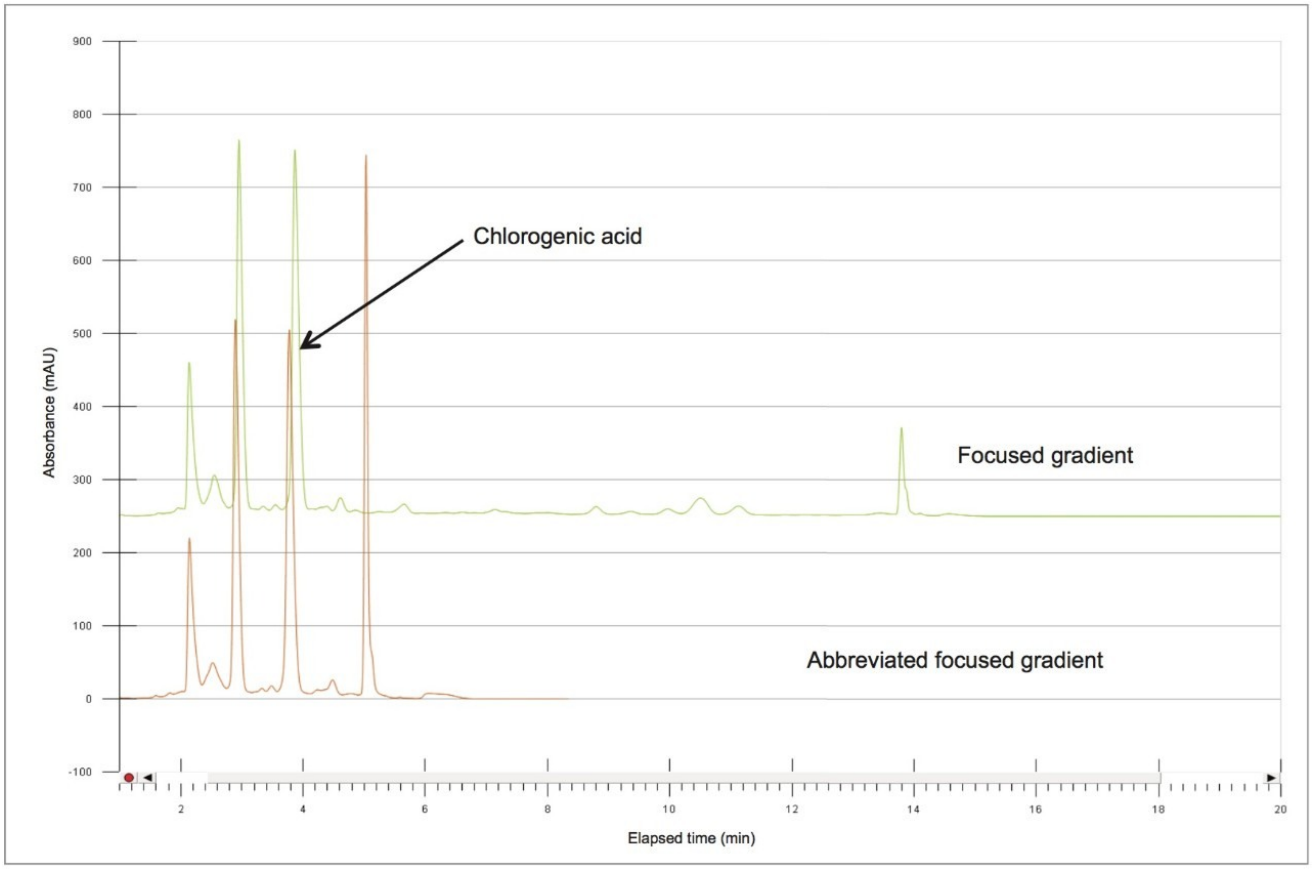

Figure 5. Overlay of the focused gradient (green) and the abbreviated focused gradient (orange).

To increase throughput and reduce run time by approximately 6 minutes, the column was washed with solvent immediately after the elution of chlorogenic acid, rather than executing the entire focused gradient (Figure 5). The purity of chlorogenic acid in the crude berry sample was determined by the ChromScope Software to be 32% by area calculation when separated by the abbreviated focused gradient (Figure 6).

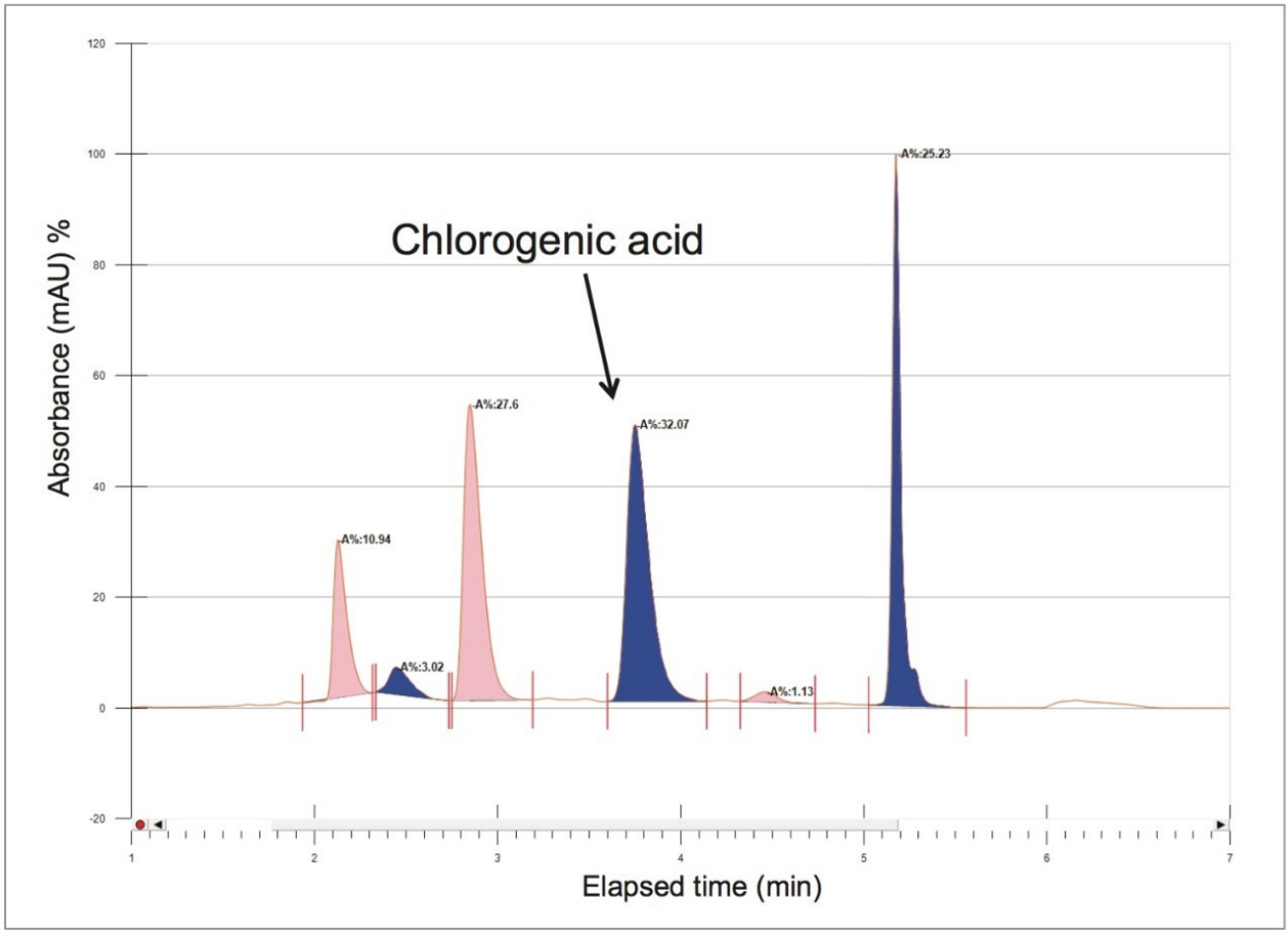

Figure 6. Analytical scale analysis of the crude berry mixture with a column wash programmed after the elution of chlorogenic acid (3.8 mins) to increase throughput. The purity of chlorogenic acid is 32%.

Injection volume and flow scale-up

To increase throughput, the abbreviated focused gradient was scaled-up from analytical to prep. The following calculations were used to determine the appropriate flow rate and injection volume for the 19 mm I.D. prep column. The calculations were based upon the flow rate and injection volume used for the 4.6 mm I.D. analytical column:

$$\text{Flow}_{\text{Prep}} = \text{Flow}_{\text{Analytical}} \times (\text{Diameter}_{\text{Prep}}/\text{Diameter}_{\text{Analytical}})^2$$

$$\text{Flow}_{\text{Prep}} = 1.46\ \text{mL/min} \times (19\ \text{mm}/4.6\ \text{mm})^2$$

$$\text{Flow}_{\text{Prep}} = 24.9\ \text{mL/min}$$

$$\text{Volume}_{\text{Prep}} = \text{Volume}_{\text{Analytical}} \times (\text{Diameter}_{\text{Prep}}/\text{Diameter}_{\text{Analytical}})^2 \times (\text{Length}_{\text{Prep}}/\text{Length}_{\text{Analytical}})$$

$$\text{Volume}_{\text{Prep}} = 100\ \mu\text{L} \times (19\ \text{mm}/4.6\ \text{mm})^2 \times (50\ \text{mm}/50\ \text{mm})$$

$$\text{Volume}_{\text{Prep}} = 1706\ \mu\text{L}$$

The 4.6 mm I.D. column flow rate of 1.46 mL/min and 100 µL injection volume scales-up to a 24.9 mL/min flow rate and 1706 µL injection volume for the prep column. Retention time was identical on both columns after scale-up using the abbreviated focused gradient (Figure 7).

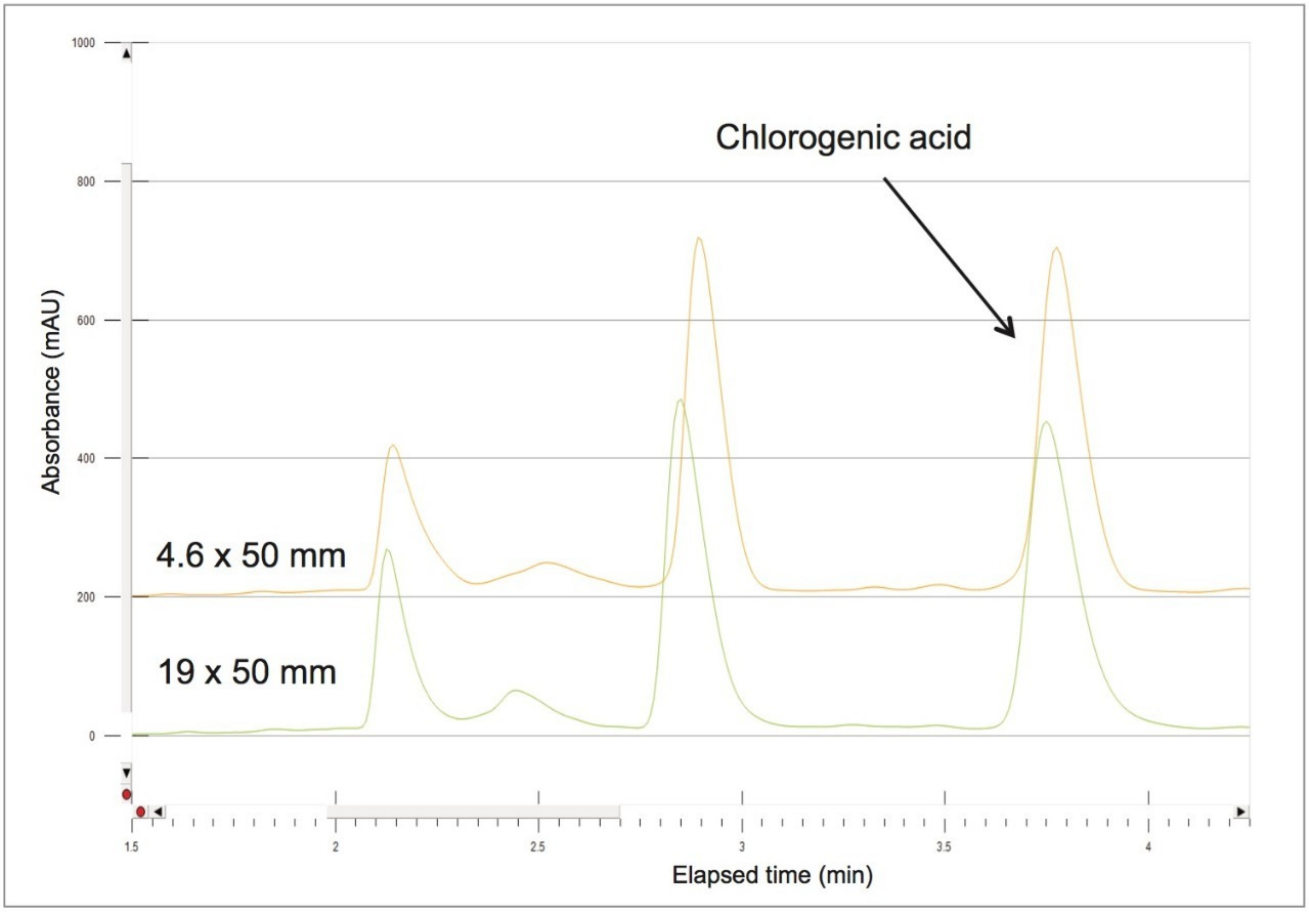

Figure 7. Overlay of the analytical (orange) and prep (green) separations obtained by the abbreviated focused gradient for chlorogenic acid (3.8 min).

A collection method using a µ absorbance threshold trigger was developed to collect the chlorogenic acid fraction at prep scale using the ChromScope fraction collection simulator. After collection (Figure 8), the retention time and purity of the fraction was compared to the reference standard (Figure 9, Table 4). Purity of chlorogenic acid increased from 32% in crude Aronia berries to 99% when compared to the chlorogenic acid reference standard at 324 nm. The isolate was also analyzed by the scouting gradient to demonstrate the increase in purity compared with the crude Aronia berry sample (Figure 10).

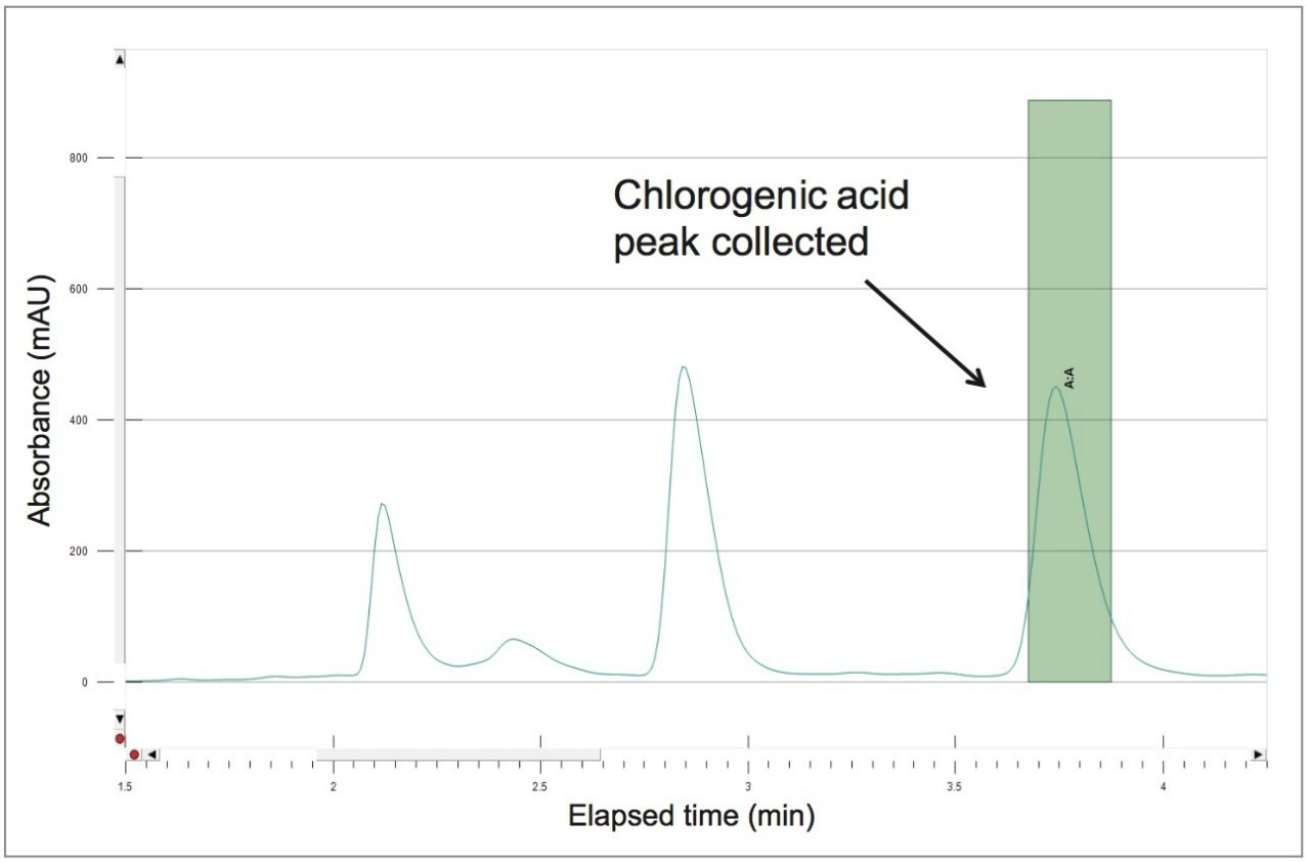

Figure 8. Chlorogenic acid peak collected at prep scale (19 mm x 50 mm) by absorbance threshold in the ChromScope Software.

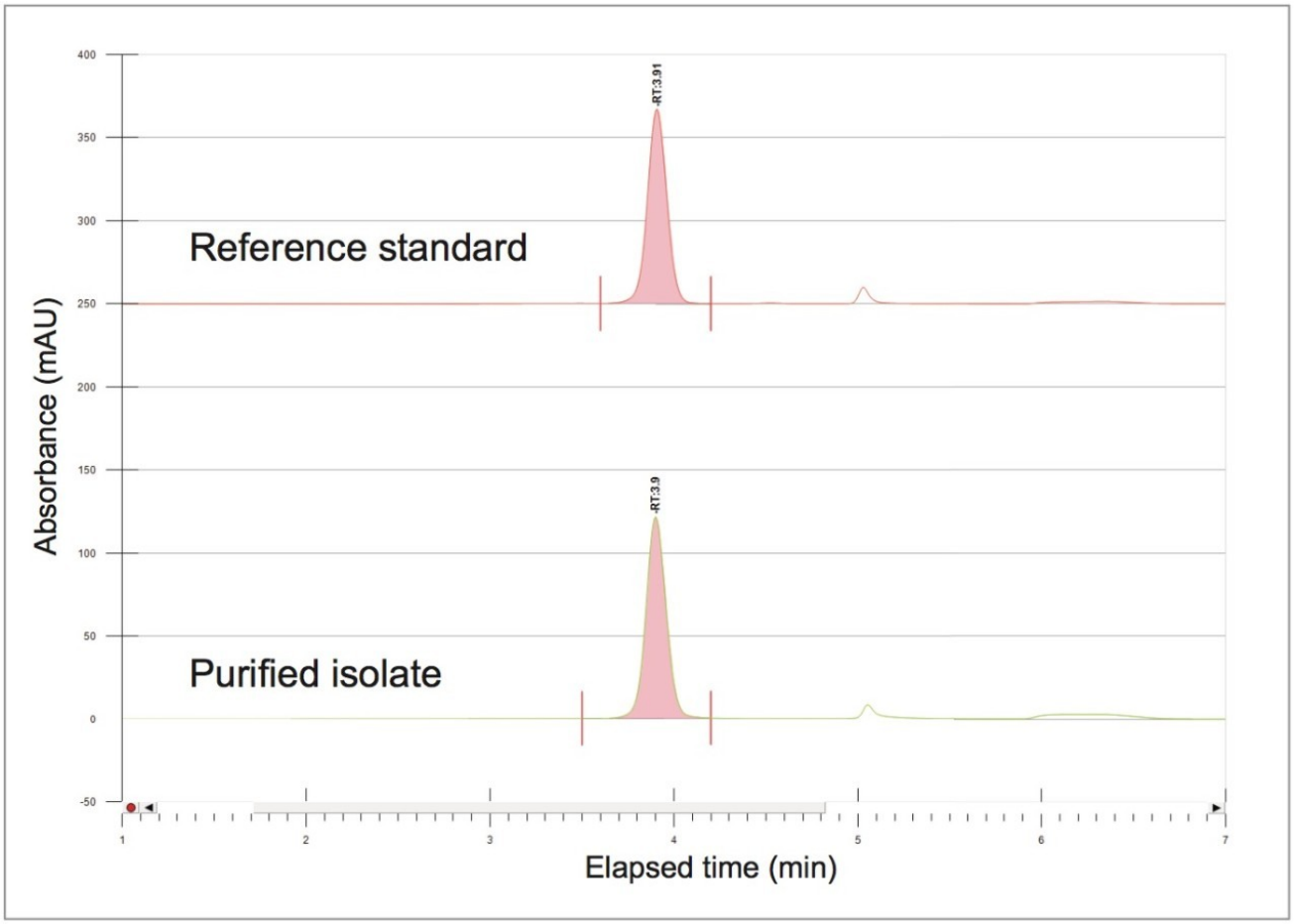

Figure 9. Overlay of the chlorogenic acid purified isolate (green) compared with a reference standard (red) analyzed by the focused gradient with the 4.6 x 50 mm analytical column.

Sample	Chlorogenic acid purity at 324 nm Compared to the reference standard
Crude Aronia berry sample	32%
Purified isolate	99%

Table 4. Purity of chlorogenic acid in crude Aronia berries compared with the purified isolate.

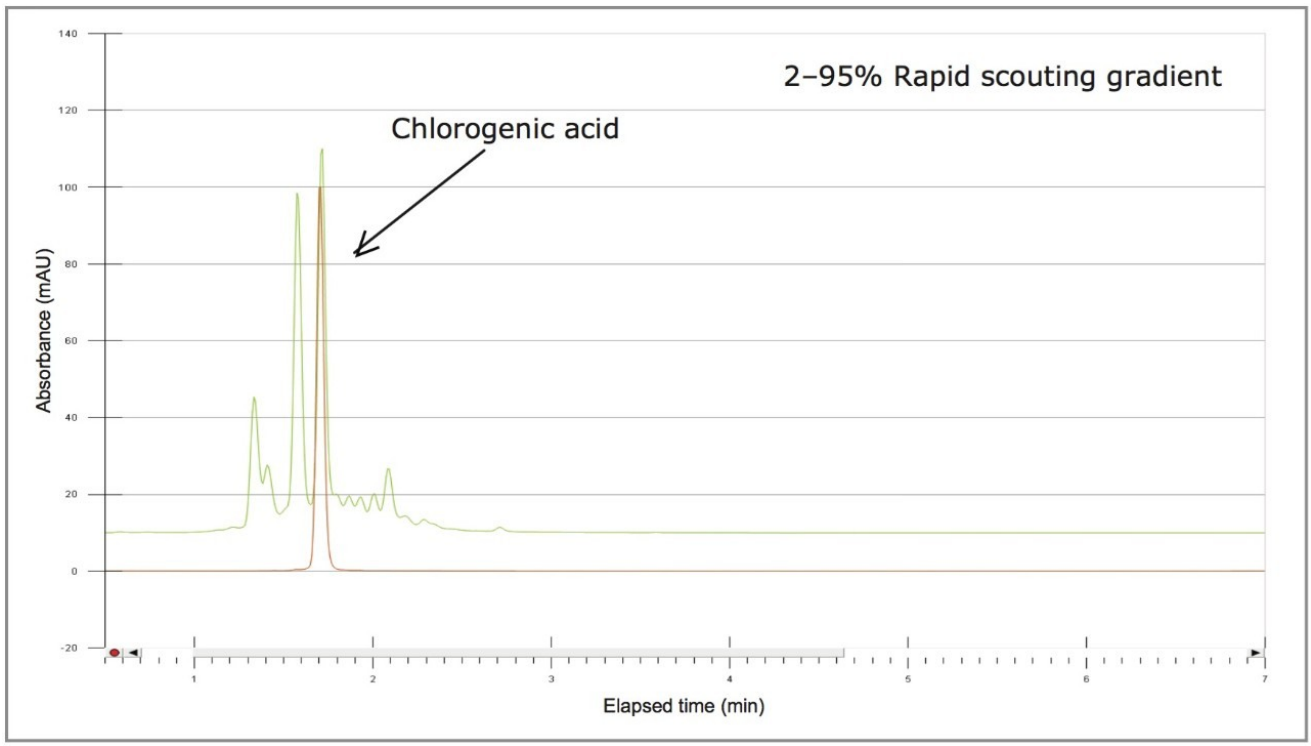

Figure 10. Overlay of the purified isolate (orange) and the crude Aronia berry sample (green) separated by the 2–95% rapid scouting gradient to compare the purity of chlorogenic acid before and after purification.

Conclusion

Here, fast and efficient isolation of the antioxidant chlorogenic acid from crude Aronia berries was demonstrated. Throughput was maximized by first generating a focused gradient, then optimizing the focused gradient for run time. The purification method effectively yielded a chlorogenic acid isolate comparable in purity to the reference standard at 324 nm. The entire purification when scaled from analytical to prep, was successfully accomplished using the Waters Prep 150 LC System.

References

1. “Aronia: The North American super berry with cancer-fighting properties,” http://www.foxnews.com/health/2013/06/07/ aronia-north-american-super-berry-with-cancerfighting-benefits.html. July 1, 2016.

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