A Reproducible HPLC Method for Phenolic and Carbohydrate Analysis in Juneberry (Amelanchier alnifolia Nutt.)

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Abstract

Amelanchier alnifolia Nutt. (Juneberry) is a native North American fruit that has gained increasing interest for its horticultural potential and presumed food values. Despite this growing interest, comprehensive nutritional and phytochemical characterization of Juneberry cultivars remains limited. This study describes the development of a high-performance liquid chromatography (HPLC) method for the extraction and quantification of phenolic compounds and carbohydrates within freeze-dried *A. alnifolia* samples. The phenolic compounds were extracted from the samples using a 1:1 methanol to water solution. The extract was then analyzed on a C30 reverse-phase column with a C18 guard column under gradient elution with HPLC-grade aqueous 5% o-phosphoric acid and methanol as the mobile phase. Both hydroxycinnamic acids and hydroxybenzoic acids, subclasses of phenolic acids, were analyzed using PDA detection at 310 nm and 215 nm, respectively. The soluble sugars, glucose and fructose as well as the soluble sugar alcohol sorbitol were extracted using HPLC-grade water and separated via a Ca^{2+} monosaccharide column under isocratic conditions. Detection was performed by a refractive index detector (RID). This method provides a way to reproduce chromatographic separation, extraction, and quantification of high enough quality for comparative biochemical characterization of juneberry cultivars that can be applied to a broad range of fruits rich in phenolic compounds.

Introduction

The Juneberry (*Amelanchier alnifolia* Nutt., Family Rosaceae) is a small pome fruit comparable (Fig. 1) to the size of a blueberry (approximately 0.5–1.0 cm in diameter) with a tender exocarp enclosing numerous small seeds [1]. The fruits are rich in phytochemical compounds, including hydroxycinnamic acids, hydroxybenzoic acids (Fig. 2), and various forms of soluble sugars positioning Juneberry among phenolic-rich berry matrices of nutritional and analytical relevance [2]Click or tap here to enter text.. Outside of their biochemical attributes, Juneberry has long held cultural, nutritional, and subsistence importance for Indigenous peoples of the US Northern Plains, particularly the Mandan, Hidatsa, and Arikara (MHA) Tribal Nations. Traditionally consumed fresh, dried, or incorporated into foods such as *pemmican*, Juneberries were a critical seasonal food source that supported winter survival and were embedded in cultural practices and intergenerational knowledge systems [1, 3]. Natural Juneberry stands along the Missouri River were extensively reduced during the mid-20th-century construction of the Garrison Dam and the subsequent creation of Lake Sakakawea on the Fort Berthold Reservation, resulting in the loss of access to this culturally significant plant. Natural Juneberry stands along the Missouri River were extensively reduced during the mid-20th-century construction of the Garrison Dam and the subsequent creation of Lake Sakakawea on the Fort Berthold Reservation, resulting in the loss of access to this culturally significant plant. As a result, the reintroduction and cultivation of Juneberry have become priorities within Tribal communities for reasons that include cultural revitalization, food sovereignty, and nutritional health [3].

Despite this renewed interest, compared to commercial berries, Juneberries are under-researched in terms of varietal differences, nutrient composition, and their integration into traditional food matrices

like *pemmican*. Production is also inconsistent across cultivars, and yearly yield is unpredictable, perhaps due to hybridization and polyploidy, complicating taxonomic identification. Despite this renewed interest, analytical studies characterizing Juneberry phytochemical and carbohydrate profiles remain limited, underscoring the need for reproducible and validated analytical methods [4].

Phenolic compounds in Juneberries consist of hydroxylated aromatic structures that contribute to the flavor, color, and oxidative stability and are commonly classified as flavonoids, phenolic acids, tannins, stilbenes, and lignans [5, 6]. Phenolic compounds and content can significantly vary among cultivars. A standardized analysis protocol, especially a high-performance liquid chromatography (HPLC) method, essentially ensures reproducible quantification, enables comparisons across cultivars, and provides a robust foundation for biochemical investigations [7, 8]. There is no published literature showcasing a validated HPLC method for the extraction and quantification of both phenolic acids and soluble carbohydrates in Juneberry samples.

Here, we present a validated HPLC method for the extraction and quantification of individual phenolic acids and soluble carbohydrates from freeze-dried juneberry samples. The method integrates optimized solvent extraction procedures with chromatographic separation using a reverse phase C30 column and a photodiode array (PDA) detection for phenolic compounds, along with a Ca^{2+} monosaccharide column with refractive index detection (RID) for carbohydrate analysis. These methods offer a reproducible and reliable framework for HPLC-based analysis of phenolic compounds and soluble carbohydrates in Juneberry samples.

Experimental Section

Reagents and Materials

Analytical-grade methanol, 5% o-phosphoric acid, and HPLC-grade water were obtained from Fisher Scientific (Waltham, MA, USA). The phenolic standards: chlorogenic, caffeic, p-coumaric, ferulic, gallic, and vanillic acids were all obtained from MilliporeSigma (Sigma-Aldrich) (Burlington, MA, USA). The carbohydrate standards, including glucose, fructose, sucrose, and sorbitol, were also purchased from MilliporeSigma.

Instrumentation

Phenolic compounds were analyzed using the Shimadzu LC-40D HPLC system (Kyoto, Japan). This system includes the Nexera HPLC/UHPLC Degasser (DGU-403/405), System Controller (CBM-40), Column Oven (CTO-40C), and Autosampler (SIL-40/SIL-40C). UV detection was performed by the Nexera Photodiode Array Detector (SPD-M40). Chromatographic separation of phenolics was carried out using a C30 column (150 mm x 4.6 mm) fitted with a C18 guard column (Phenomenex, Torrance, CA, USA) and maintained at 40°C.

Carbohydrates and sugar alcohol (sorbitol) were also analyzed using the same Shimadzu LC-40D HPLC system but the detector used was a Nextera Refractive Index Detector (RID-20A), replacing the (SPD-M40) PDA detector. Separation of the carbohydrates was performed on a Phenomenex Rezex RCM-Monosaccharide Ca^{2+} column (300mm x 7.8mm). The column temperature was maintained at 80°C, with the flow rate of 0.6mL/min for carbohydrate analysis.

Methods

Chromatographic Conditions

Phenolic compounds were separated using a Phenomenex C30 reverse-phase column (150 mm x 4.6 mm) fitted with a C18 guard column. The column oven was set and maintained at 40°C. The mobile phase consisted of solvent A (5% o-phosphoric acid) and solvent B (methanol), delivered under gradient elution over 25 minutes of run time at a flow rate of 0.9 mL min^{-1}. The gradient began at 70% solvent C and 30% solvent D and then transitioned to 50:50 (C to D) by the 20-minute mark. From 20 to 25 minutes, the concentration moved back toward the initial 70:30 composition. An injection volume of 10 µL was used for all phenolic standards and berry samples. Detection was performed using a Nexera Photodiode Array Detector (PDA) (SPD-M40). The chromatographs (Fig. 3) revealed that hydroxycinnamic acids (chlorogenic, caffeic, p-coumaric, and ferulic acids) exhibit absorption maxima at 310 nm, while hydroxybenzoic acids (gallic and vanillic acids) had maxima at 215 nm. Absorption spectra were acquired at both 310 nm and 215 nm for the best quantification of the chromatograph peaks and peak areas. The conditions described provided reproducible retention times and distinct peaks suitable for phenolic content quantification. The phenolic acids peak retention times ranged from 6–7, 8–9, 11–12, 12–13, 4–5, and 8–9 minutes for chlorogenic, caffeic, p-coumaric, ferulic, gallic, and vanillic acids, respectively.

Carbohydrate separation was conducted on a Phenomenex Rezex RCM-Monosaccharide Ca^{2+} column (300mm x 7.8mm). The column oven was set and maintained at 80°C. HPLC-grade water was used as the mobile phase under isocratic elution at a flow rate of 0.6mL/min. An injection volume of 10 µL was used for all the carbohydrate standards and the juneberry samples. Detection was performed using a Nextera (RID-20A), replacing the (SPD-M40) PDA detector previously used for phenolics. This method created reproducible retention times that were suitable for the quantification of glucose, fructose, and sorbitol within a 25-minute run time.

Standard and Stock Solution Preparation

Phenolic standard solutions were prepared for the standards of chlorogenic, caffeic, p-coumaric, ferulic, gallic, and vanillic acids. Each phenolic standard was dissolved in a 50:50 methanol-water solvent (50% methanol). To obtain a 1:100 standard solution concentration, ~ 0.1 g of standard was dissolved in 100 mL of the 50% methanol solvent. Leaving the standard solutions at a concentration of ~ 1 mg/mL. The

solutions were vortexed thoroughly until completely dissolved. The solutions were then filtered through 0.40 µm Phenomenex syringe filters to clean the solution of any particles before injection. Standard solutions were then analyzed through HPLC to determine the retention times. A stock solution was also made by combining ~ 0.1 g of each standard into the same 100 mL 50% methanol solution. From the stock solution, a 10-point calibration curve was created from 10 to100µg/mL (Fig. 4). After filtration and dilution, the vials for the calibration were then used to construct the calibration curve. A 10 µL injection volume and a flow rate of 0.9 mL/min-1 was employed on the C30 column with a C18 guard column for phenolics.

The carbohydrate standard solutions were prepared in a similar manner. Glucose, fructose, sucrose, and sorbitol were each dissolved in HPLC-grade water. To obtain a 1:100 standard solution, 1 g of standard was dissolved in 100 mL of HPLC-grade water. Each solution was thoroughly vortexed until completely dissolved and then filtered through a 0.40 µm Phenomenex syringe filter to clean the solution of any particles before injection. The peak retention times for glucose, fructose, sucrose, and sorbitol ranged between 11–12, 13–14, 7–8, and 20–21 minutes, respectively.

After filtration, the calibration solutions were injected 50 µL injection volume with a flow rate of 0.6mL/min-1 on the Phenomenex Rezex RCM-Monosaccharide Ca^{2+} column (300mm x 7.8mm).

Sample Preparation

Ripened berries of *Amelanchier alnifolia* representing four cutivars grown on the Fort Berthold Indian Reservation in north central North Dakota were collected on June 16, 2024. Following collection, the berries were stored on dry ice to preserve their biochemical integrity before arriving at the laboratory. After arriving at the laboratory, the berries were stored at -20°C until freeze-drying could be completed. To prepare for phenolic and carbohydrate analysis, all Juneberry fruits were freeze-dried to ensure preservation of the phenolic compounds, flavonoids, anthocyanins, and other thermally sensitive compounds. Freeze-drying was selected over hot air drying as it is significantly better than hot air drying at preserving thermally sensitive compounds, which tend to break down at higher temperatures [9]. They were freeze-dried at -80°C with a pressure of 0.001 bar for 72 hours using a Labconco (Kansas City, MO, USA) bench-top model freeze dryer with a 4.5 L chamber capacity. The resulting sample moisture content was measured at less than 0.1%.

Following lyophilization, the dried Juneberries were ground manually into a fine powder using a mortar and pestle. The powdered samples were stored in 15 mL centrifuge tubes covered in tinfoil to protect them from UV light. Only whole, unprocessed juneberries were used, as food processing can substantially alter phenolic and carbohydrate concentrations [10]. All materials that were prepared for HPLC analysis were stored at -20°C until analysis. Samples were prepared for analysis using the same method as used for the standards. Samples, for phenolics, were analyzed at two time points: right after extraction (15 minutes) and after 24-hours extraction, in 50% methanol. Similarly, sugar being instantly soluble, only 15 minutes extractions in HPLC-grade water were subjected to carbohydrate analysis.

Results and Discussion

Drying and Extraction of Juneberry Samples

The method used freeze-drying as a way to remove moisture from the juneberries. Freeze-drying is reported to effectively remove moisture from berries while preserving phenolic compounds and minimizing degradation associated with more thermally intensive methods [11]. The preservation shown by freeze-drying is also present within several other studies that show it has a higher capacity for preserving phenolic content and antioxidant capacity than hot-air or thermal drying [12, 13]. Low temperature lyophilization minimizes the oxidative and thermal degradation that would have otherwise occurred if using methods such as sun or salt drying [14]. The low temperature lyophilization method minimizes the loss of hydroxybenzoic and hydroxycinnamic acids, which were the target of analysis in this study [12, 14].

Freeze-drying also forms ice crystals that rupture cell walls, which facilitates the solvent penetration of the 50% methanol used, improving the release of phenolics into solution. The slower the rate of freezing, the larger the ice crystals created, the more damage done to the cells of the fruit, which facilitates the penetration of solvent and release of phenolic compounds [15]. In contrast, air and oven drying usually lead to a significant loss in the total phenolics of berries as well as other plant tissues when analyzed. Freeze-drying likely contributed substantially to the total phenolics that was quantified through HPLC analysis [12, 14, 15].

Carbohydrate preservation is also improved through the method of freeze-drying, particularly glucose, fructose, and sorbitol. Monosaccharides are chemically stable under cold conditions, and the implementation of freeze-drying prevents thermal degradation and browning of the sugars which are reactions that can occur during thermally intensive methods. Fructose can be especially sensitive to heat undergoing caramelization or Maillard reactions when exposed to elevated temperatures. Hot air drying has been shown to reduce the amount of measurable monosaccharide content within fruit tissues due to the transformation of those monosaccharides under heat [16].

Chromatographic Separation and Detection of Phenolics and Carbohydrates

Till date, very few studies have reported chromatographic detection of phenolics in Juneberry fruits; however, all of them have followed different extraction methods [10, 17, 18]. The HPLC method developed through this study provided clean separation of six phenolic acid standards using a C30 column. The separation provided distinct retention times, which allowed for the identification of phenolic acids within the Juneberry samples via HPLC analysis. Separation and quantification of Phenolic compounds are challenging due to the inherent structural complexity and associated separation complexities [19]. Hydroxycinnamic acids (chlorogenic, caffeic, p-coumaric, and ferulic acids) exhibited absorption maxima at 310 nm while hydroxybenzoic acids (gallic and vanillic acids) showed maxima at 215 nm. The methanol and 5% o-phosphoric acid method created sharp symmetrical peaks across the

25-minute run time. This agrees with the effectiveness of reverse phase HPLC methods for berry phenolic compounds shown in other studies; for example, studies related to blueberries and other closely related berries have sorted both hydroxybenzoic and hydroxycinnamic acids with similar chromatographic methods [20, 21] Click or tap here to enter text..

PDA detection allowed for the section of wavelength maxima specific to each class of phenolics being 310 nm for hydroxycinnamic acids and 215 nm for hydroxybenzoic acids. The dual wavelength approach taken allows for increased selectivity and sensitivity for phenolics that are structurally different from one another. A dual or multiple wavelength approach is commonly used in studies that quantify phenolic compounds within fruit samples [21, 22] Click or tap here to enter text..

Juneberry samples were analyzed right after extraction and then 24-hours after initial extraction, allowing the 50% methanol to extract phenolics from the berries for 24-hours. The 50% methanol remained in contact with the freeze-dried samples during the 24-hour timeline allowing the solvent to penetrate deeper into the tissues, facilitating the release of tightly bound phenolic acids. The profiles for initial and 24-hour extraction were compared, and changes in phenolic concentrations were noted.

The Ca^{2+} column with RID detection also produced distinct retention times which allowed for the identification of carbohydrates within the juneberry samples via HPLC analysis. Retention times matched the standard solutions that were run through HPLC analysis prior to the Juneberry samples. The elevated column temperature of 80°C led to peaks similar to that of phenolics in terms of being sharp and symmetrical with minimal trailing. HPLC-RID methods are commonly used to identify, analyze, and quantify carbohydrates within foods [23–26]Click or tap here to enter text.. The glucose, fructose, sucrose, and sorbitol concentrations were found in the ranges of 121.59-179.22, 130.87-217.69, 21.61–36.31, and 96.18-177.26 mg/g, respectively. The higher fructose compared to glucose, as shown by glucose:fructose < 1, aligns with earlier findings of Saskatoon fruits by Rogiers and Knowles [17]. The total sucrose content observed to be less than one-tenth of all the analyzed sugars is in agreement with that reported, in a different species of Juneberry (*A. lamarckii*), by Mikulic-Petkovsek et al. [10].

Comparison with Related Berry Matrices

Juneberries should be viewed as a phenolic-rich fruit that extends beyond the six acids targeted in the present method, because mature juneberries contain anthocyanins, flavanols, and hydroxycinnamate-type phenolics, and genotype work has identified 29 polyphenolic compounds, including 9 phenolic acids, 9 flavanols, 7 flavan-3-ols, and 4 anthocyanins [27, 28]. The current method captures a defined phenolic-acid subset of chlorogenic, caffeic, p-coumaric, ferulic, gallic, and vanillic acids rather than the full juneberry polyphenolic profile (Table 1). However, this targeted analysis remains important because chlorogenic and neochlorogenic acids were reported as the major phenolic acids across Saskatoon berry genotypes, accounting for most of the phenolic-acid concentration [28]. In the context of this study, the observed increase in chlorogenic acid after the 24-hour extraction is consistent with the broader literature on juneberries and supports the use of chlorogenic acid-centered profiling as a way to compare cultivars.

Comparison with other related berries helps give context to these findings. In blueberries, chlorogenic acid is reported as the predominant phenolic acid in both low and highbush fruit in an HPLC-MS comparison of two predominant blueberry species on the US market [29]. This suggests that Juneberry and blueberry share phenolic-acid features, more specifically chlorogenic acid-rich fraction, even though both fruits contain a much broader flavonoid range [29]. The chokeberry is another great contrast because the black chokeberry fruit is recognized as an especially polyphenol-and anthocyanin-rich berry [30]. At the same time, however, chlorogenic acids also remain important in chokeberry, and were reported as the dominant phenolic compound in undigested black chokeberry fruit samples in a recent comparative study [31]. Altogether, these comparisons suggest that juneberries are chemically more similar to blueberries, while chokeberries contain a higher overall level of phenolics, especially pigment compounds like anthocyanins.

A similar contextual comparison can be made for the carbohydrate fraction, where the current method has captured the glucose, fructose, sucrose, and sorbitol concentrations (Table 2). Saskatoon berry genotypes have been reported to contain fructose and glucose as the dominant sugars, with sorbitol also present as a major soluble carbohydrate [28]. A study carried out by Mikulic-Petkovsek et al. in *A. lamarckii* likewise identified glucose, fructose, and sorbitol as the main sugars, which matches the analyte panel selected in the present RID method [32]. By comparison, black chokeberry can be more sorbitol-driven, because sorbitol was reported as the main carbohydrate in fresh aronia fruit and represented 61%–68% of the low-molecular-weight carbohydrate fraction in Bulgarian samples [30]. This makes the current glucose, fructose, and sorbitol panel useful not only for within Juneberry cultivar comparisons, but also for distinguishing Juneberry from other dark-fruit carbohydrate profiles that are weighed differently across individual sugars and sugar alcohols.

Table 1
Phenolic compound concentrations (μg/g) in Juneberry samples immediately after extraction (Initial) and following 24-hour extraction, with percentage change.

Phenolic Compound	Extraction Time	Sample 1 (μg/g)	Sample 2 (μg/g)	Sample 3 (μg/g)	Sample 4 (μg/g)
Chlorogenic acid	Initial	56.42	77.70	44.75	92.99
	24 h	80.73	92.59	64.45	128.84
	Percent change (%)	43.10	19.16	44.03	38.54
Ferulic acid	Initial	13.84	13.60	0.00	1.04
	24 h	16.40	15.95	2.32	1.18
	Percent change (%)	18.54	17.26	N/A	13.94
Caffeic acid	Initial	11.40	2.60	5.25	25.00
	24 h	11.21	1.87	5.03	33.40
	Percent change (%)	−1.67	−28.20	−4.13	33.62
p-Coumaric acid	Initial	2.12	3.62	15.57	23.20
	24 h	2.71	4.41	20.71	25.27
	Percent change (%)	27.47	21.78	33.02	8.92
Vanillic acid	Initial	55.52	24.30	31.97	118.83
	24 h	102.49	28.53	37.28	127.04
	Percent change (%)	84.59	17.43	16.62	6.91
Gallic acid	Initial	89.04	86.72	71.69	112.35
	24 h	149.78	108.73	102.74	132.58
	Percent change (%)	68.23	25.37	43.31	18.01

We observed that with the increment in extraction time, the individual phenolic concentration increased by at least 8%, except for caffeic acid—decreased in—samples 1–3.

Table 2
Carbohydrate concentrations (mg/g) in Juneberry samples immediately after extraction.

Carbohydrates (mg/g)	Sample 1	Sample 2	Sample 3	Sample 4
Glucose	179.22	151.26	121.59	161.83
Fructose	217.69	175.45	130.87	163.59
Glucose: Fructose	0.82	0.86	0.93	0.99
Sucrose	21.61	22.14	31.59	36.31
Sorbitol	173.43	96.18	148.19	177.26

Method Validation and Performance

Calibration curves constructed from the 10-point dilution of the mixed phenolic stock solution showed a linearity of $R^2 > 0.99$ for all phenolic compounds (Fig. 4). This aligns with the validation parameters that have been reported in other fruit related phenolic HPLC methods [23, 25]Click or tap here to enter text..

Prior to the calibration curve being constructed, standards of each phenolic acid were run through HPLC analysis. When running the standards through HPLC, the retention time was taken note of to compare to the stock solution from which the calibration curve was created and actual juneberry samples. The peaks and retention times of standards correlated directly with the peaks of the combined stock solution.

Conclusion

The HPLC method developed in this study provides a reliable and sensitive protocol for the extraction, separation, and quantification of both phenolic compounds and soluble carbohydrates in freeze-dried juneberry (*A. alnifolia*) fruit. Phenolics were extracted via 50% methanol and analyzed using a reverse phase C30 column with PDA detection and a mobile phase of 5% o-phosphoric acid and methanol. Carbohydrates were extracted and separated via HPLC-grade water with a Ca^{2+} monosaccharide column and RID detection. Both methods resulted in strong linearity with precision and chromatographic resolution.

Through freeze-drying, the method maximized the amount of preserved phenolic and carbohydrate structures. This ensured greater and more consistent yields while limiting structural degradation that is associated with more thermally intensive drying methods.

This method relies only upon HPLC instrumentation and commonly used detectors. Aside from minor column and detector modifications, the method can be readily adopted in most biochemistry laboratories. While MS can provide valuable confirmatory information, it is not required for routine

analysis in the presented method. As a result, HPLC offers a practical and substantially more cost-effective alternative to MS-based methods, particularly for high-throughput or bulk sample analysis.

Abbreviations

HPLC: High-performance liquid chromatography

PDA: Photodiode array detector

RID: Refractive index detector

Declarations

The authors declare no conflict of interests.

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Authors Contributions

Conceptualization: KH, MPN and AKN: Investigation: GS, BA and SP, Data Analysis and Interpretation: GS, ANP, MPN. Writing of Original Draft GS and MN, Review & Editing: All authors. All authors have read and approved the final version of the manuscript and agreed to its submission to Chromatographia

Data Availability

All data supporting the findings of this study are available upon request from the corresponding author.

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Figures

Figure 1

Fruiting twigs of Juneberry shrub bearing ripened berries (photo by Nathan Bronson, Menan Idaho).

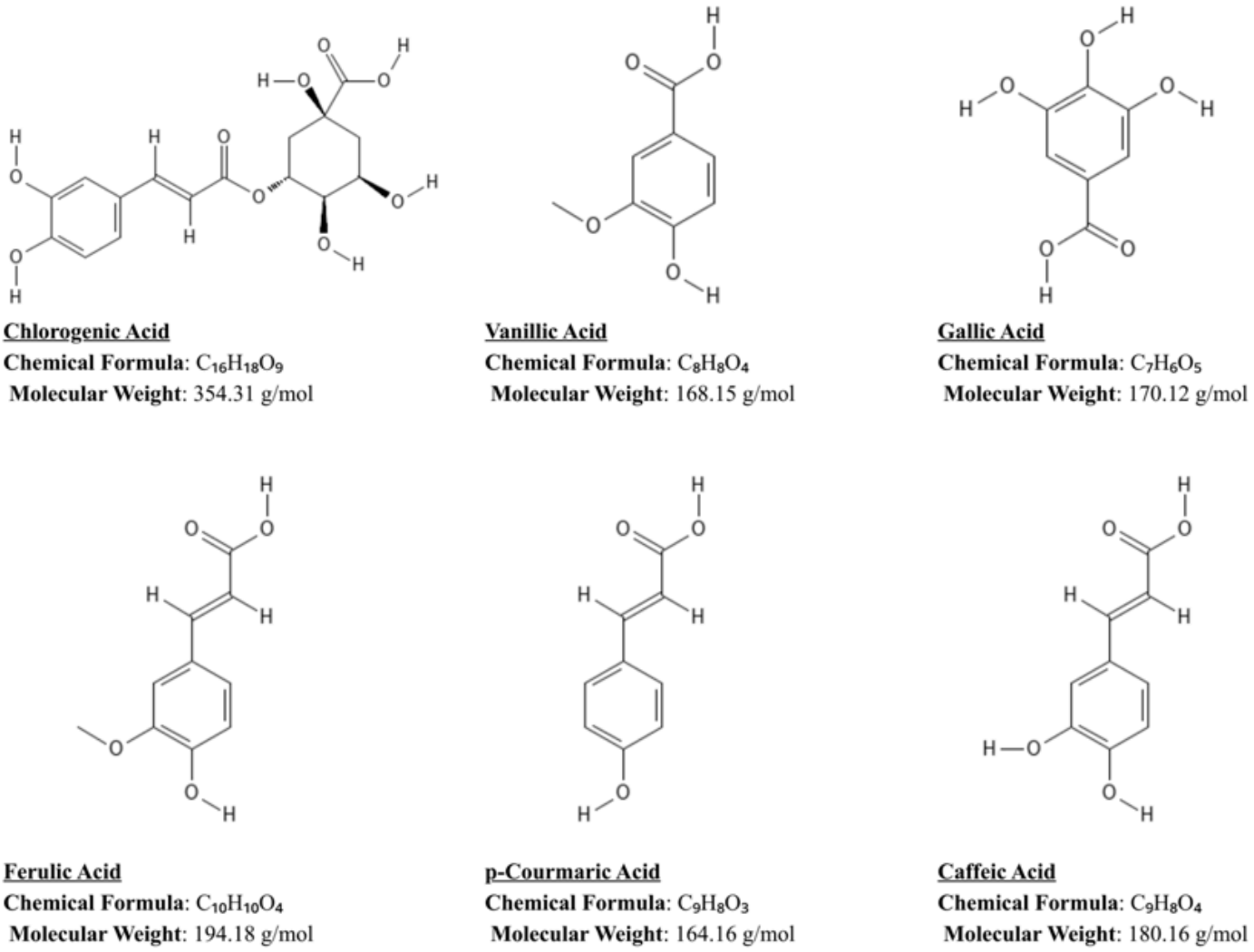

Figure 2

Chemical structure of phenolic acids targeted in the present HPLC method for Juneberry. Shown are hydroxycinnamic acids (chlorogenic, caffeic, p-coumaric, and ferulic acids) and hydroxybenzoic acids (gallic and vanillic acids). Chemical structures were drawn using PubChem Sketcher.

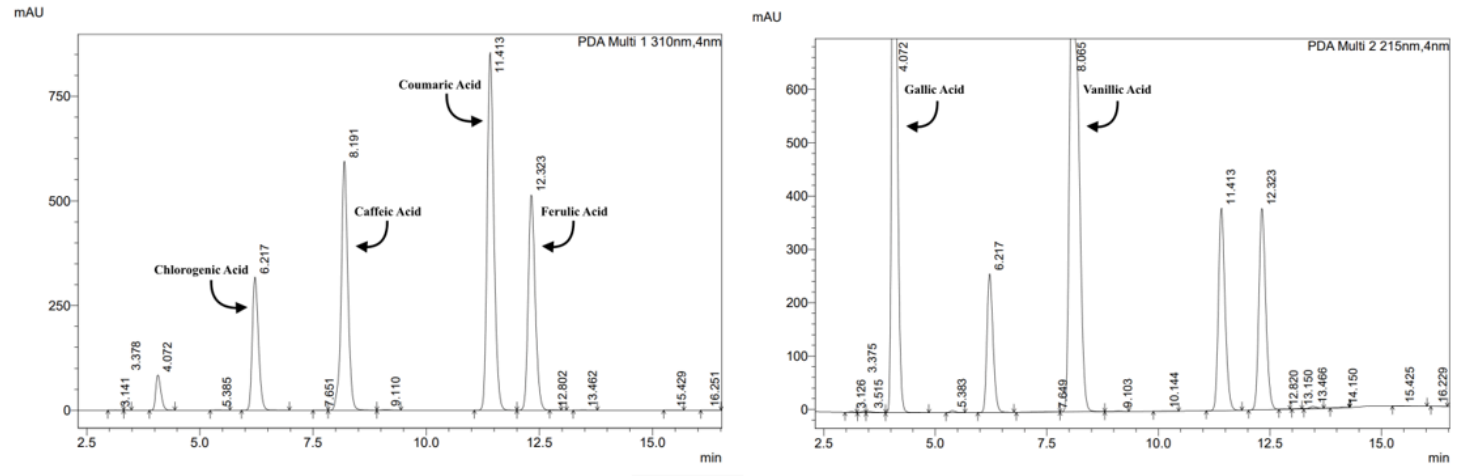

Figure 3

Representative HPLC chromatograms of phenolic acid standards detected by PDA at 310 nm (left; hydroxycinnamic acids) and 215 nm (right; hydroxybenzoic acids).

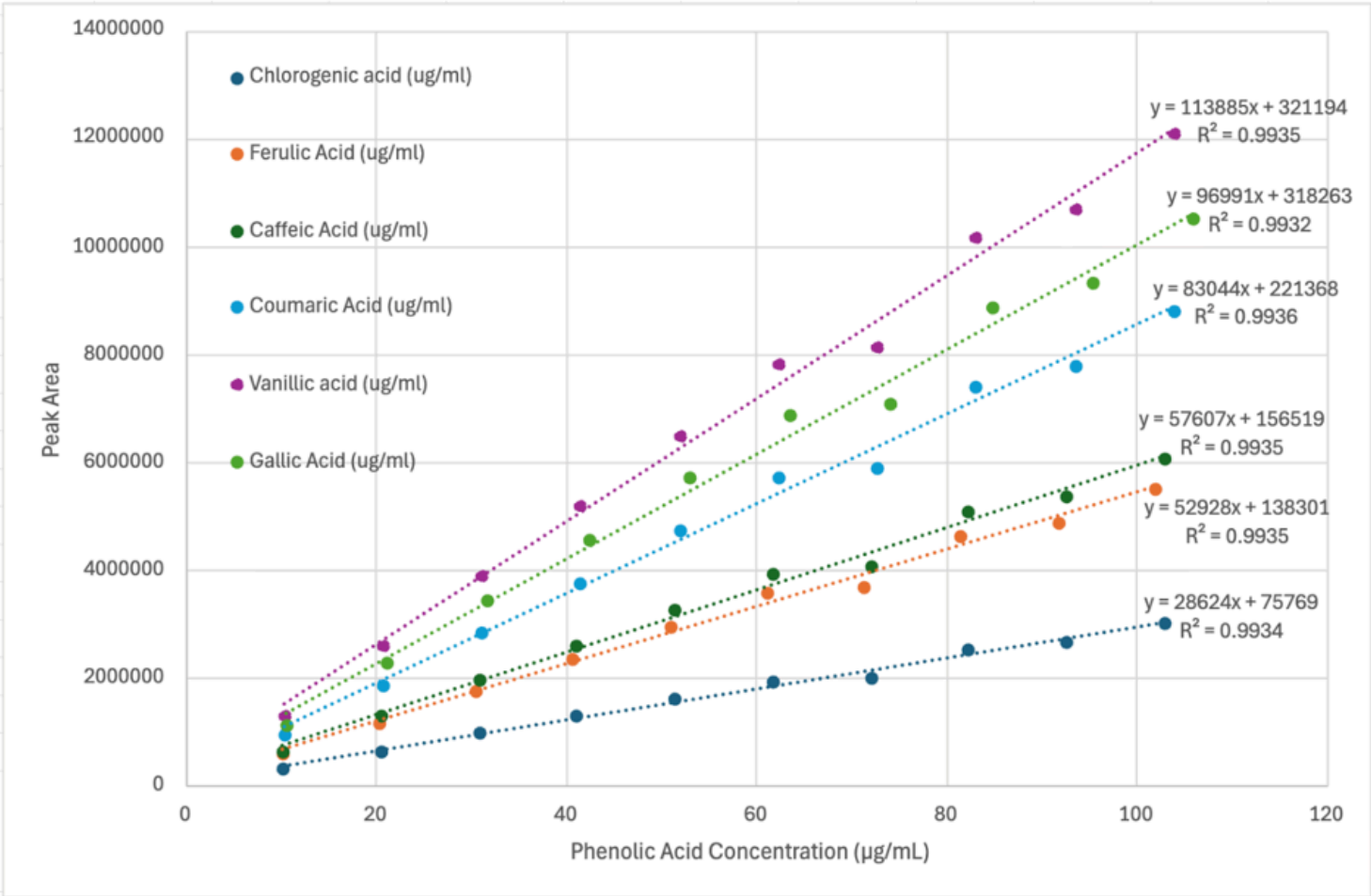

Figure 4

Calibration curves for individual phenolic acid standards used for HPLC quantification.