Single-Laboratory Validation for the Determination of Flavonoids in Hawthorn Leaves and Finished Products by LC-UV

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Abstract

Suitably validated analytical methods that can be used to quantify medicinally active phytochemicals in natural health products are required by regulators, manufacturers, and consumers. Hawthorn (*Crataegus*) is a botanical ingredient in natural health products used for the treatment of cardiovascular disorders. A method for the quantitation of vitexin-2″-*O*- rhamnoside, vitexin, isovitexin, rutin, and hyperoside in hawthorn leaf and flower raw materials and finished products was optimized and validated according to AOAC International guidelines. A two-level partial factorial study was used to guide the optimization of the sample preparation. The optimal conditions were found to be a 60-minute extraction using 50 : 48 : 2 methanol : water : acetic acid followed by a 25-minute separation using a reversed-phased liquid chromatography column with ultraviolet absorbance detection. The single-laboratory validation study evaluated method selectivity, accuracy, repeatability, linearity, limit of quantitation, and limit of detection. Individual flavonoid content ranged from 0.05 mg/g to 17.5 mg/g in solid dosage forms and raw materials. Repeatability ranged from 0.7 to 11.7% relative standard deviation corresponding to HorRat ranges from 0.2 to 1.6. Calibration curves for each flavonoid were linear within the analytical ranges with correlation coefficients greater than 99.9%. Herein is the first report of a validated method that is fit for the purpose of quantifying five major phytochemical marker compounds in both raw materials and finished products made from North American (*Crataegus douglasii*) and European (*Crataegus monogyna* and *Crataegus laevigata)* hawthorn species. The method includes optimized extraction of samples without a prolonged drying process and reduced liquid chromatography separation time.

Abbreviations

EPA:	Environmental Protection Agency
LOQ:	limit of quantitation
MDL:	method detection limit
NHPs:	natural health products
PTFE:	polytetrafluoroethylene
THF:	tetrahydrofuran
USP:	United States Pharmacopeia

Introduction

Crataegus (commonly called hawthorn) is a large and taxonomically difficult genus in the Rose family (Rosaceae), with worldwide distribution in northern climates including Europe, North America, and Asia. In North America and Europe, the plant material is processed into NHPs as well as dietary supplements for the treatment of cardiovascular disorders [1]. Despite the great number of hawthorn species, *Crataegus monogyna* Jacq. and *Crataegus laevigata* (Poir.) DC. are the two species commonly used in European and North American NHPs [1–4]. The products can be made from the leaves, flowers, and berries of the hawthorn plant [1, 5–8].

To date, about 650 licensed hawthorn NHPs are approved for sale in Canadian markets [4]. Governmental agencies require manufacturers of NHPs to provide evidence for safety, efficacy, and quality of botanical materials used as active constituents of their products, while dietary supplement manufacturers are required to comply with current good manufacturing practices and demonstrate product quality. Following recent events demonstrating problems with quality and authenticity of dietary supplements, there is a need for updated, valid analytical methods that can

more efficiently and accurately evaluate product quality. The assay section of the existing USP monograph for European hawthorn defines high quality material based on the quantification of two compounds, hyperoside and vitexin [1,9].

Hawthorn is a phytochemically diverse plant, where the leaves and flowers are known to contain a high abundance of flavonoids [1,10]. A total of 49 flavonoids have been identified in hawthorn, but many are minor components not used in the determination [1]. The flavonoid contents and profiles may vary between different species and their finished products. For that reason, there is a need for validated methods that measure important phytochemicals to ensure the quality of hawthorn-based NHPs and dietary supplements [1]. Procyanidins have also been shown to be important phytochemicals in hawthorn, but are present in much lower abundance, which is reflected in the European Pharmacopeia where flavonoids are quantified in leaves and flowers, while procyanidins are analyzed in berries [11].

Current validated methods for quantifying the individual flavonoids in hawthorn leaves and flowers share some similar problems [2,3,12,13]. Most of them focus on Asian hawthorn varieties (e.g., *Crataegus pinnatifida* or *Crataegus cuneata*) and quantify different flavonoids than are present in North American and European varieties. In addition, many focus on the analysis of botanical raw materials without considering other matrices (extracts, capsules, tablets) that are available in the NHP and supplement industry. These methods usually require long sample preparation techniques not suitable for routine analysis, use old column technologies not suitable for separation and identification of multiple compounds, and consume large quantities of mobile phase and are thus neither economically nor environmentally friendly. Several of the available published methods are not adequately validated for accuracy and precision based on international guidance for demonstrating method performance either lacking a sufficient number of test materials or using matrix-free standards for precision, and not determining repeatability and intermediate precision using statistical analysis relevant to analyte concentrations such as HorRat values [2,3,13–16].

Because of the drawbacks of existing methods, a method was developed and validated for commercial products that contain the plants of interest, evaluates a wider variety of measurands that naturally occur in each plant species, and confirms that method performance exceeds that of previous methods and meets international performance criteria. Availability of proven methods enhances public health and consumer confidence by allowing industry, regulators, and research scientists to rigorously assess product quality.

This paper describes the optimization and validation of a method for the quantitation of five flavonoids in *C. monogyna, C. laevigata*, and *Crataegus douglasii* using statistically guided method development. The five major flavonoids quantified in North American and European hawthorn are vitexin-2″-*O*-rhamnoside, isovitexin, vitexin, rutin, and hyperoside, shown in **Fig. 1**. The method was subjected to a single-laboratory validation according to AOAC International guidelines [16] to evaluate the method's performance using several test materials including raw materials, capsules, tablets, and tinctures to ensure the method was suitable for a variety of sample matrices with less sample preparation steps and lower consumption of solvent.

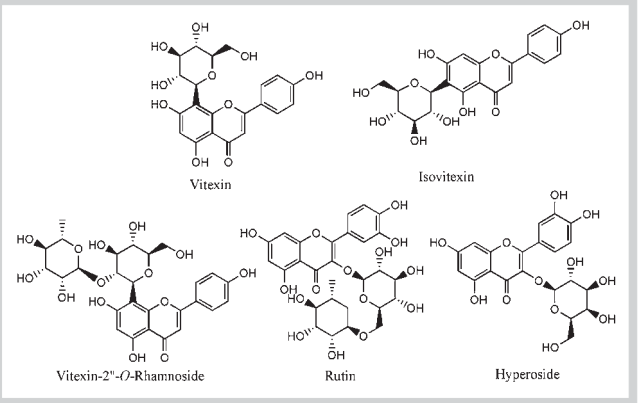

Fig. 1 Structures of the five major flavonoids in hawthorn leaves and flowers.

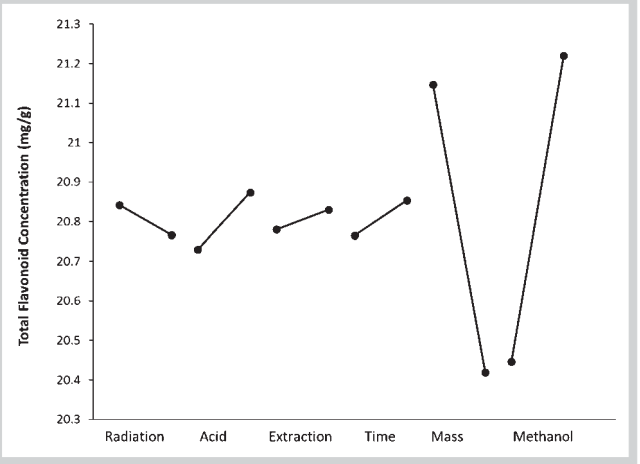

Fig. 2 Factor plots for the total flavonoids in hawthorn leaves using a two-level partial factorial design.

Results and Discussion

Past protocols for extraction of flavonoids from hawthorn have used solvents that ranged from 50 to 100% methanol with a variety of extraction methods (e.g., soxhlet, ultrasonic bath, orbital shaker), times, and solvent to volume ratios [2,3,12,13]. Optimization of the sample preparation was performed using a two-level factorial design with six factors: radiation (light/dark), acid percentage (1/4%), extraction type (sonication/shaking), extraction time (30 min/60 min), sample mass (100 mg/500 mg), and percent methanol (30/70%). Results indicated that the methanol percentage and sample mass had the most significant impacts on the total extracted flavonoid content as shown in **Fig. 2**; therefore, further optimization was performed. Methanol percentages varied from 30 to 70% methanol with either 0 or 1% acetic acid. There was no significant difference between the 50 and 70% methanol, but the addition of acid improved the yield of flavonoids. Acid concentration of 2% was optimal. Based on these results, 50% methanol containing 2% acetic acid was selected as the extraction solvent. The final step was to optimize the mass of sample; there were significantly lower levels of flavonoids found in 500 mg samples compared to 100 mg, indicating potential sat-

uration of the extraction by the analytes. Therefore, sample masses of 100 to 300 mg were evaluated. The flavonoid content determined was not significantly different in 100 or 200 mg, while lower concentrations of flavonoids were observed in the 300 mg samples. Therefore, the final extraction method used in the method section was performed on all samples for the method validation.

LC methods for flavonoids in hawthorn vary, and co-elution with other minor components has been shown to occur with some methods. The separation in this paper was performed using a Phenomemex C18 Kinetex column (4.6 × 150 mm, 2.6 µm). The THF content of the mobile phase appeared to have the most significant impact on the separation of the flavonoids, as the structures varied minimally. The optimal mobile phase was 8 : 4 : 1 THF : methanol:2-propanol, with a baseline resolution between the flavonoids of interest and co-eluting components in the raw materials. Some extracts appeared to have a component that co-elutes with vitexin-2″-*O*-rhamnoside; the resolution was > 1.2 and was deemed suitable for the quantitation. Although chromatograms were collected at 270, 340, and 360 nm, there was no significant difference in detector response between 340 and 360 nm, while at 270 nm there were co-eluting components with vitexin and isovitexin in some products. The quantitation wavelength chosen for all five flavonoids was therefore 340 nm.

Selectivity was confirmed by evaluating chromatograms of the five flavonoids at 340 nm. The flavonoids vitexin-2″-*O*-rhamnoside, isovitexin, vitexin, rutin, and hyperoside were well resolved from interfering components, as shown in **Fig. 3**. In most cases, the resolution was > 1.5 for the flavonoids, with the exception of vitexin-2″-*O*-rhamnoside in some commercial products where the resolution from an unknown co-eluting component was 1.2. According to AOAC guidelines, a resolution of 1.5 is desirable, while 1.0 is usable in natural product samples [15].

All standard curves produced throughout method development and validation appeared linear upon visual inspection with all calculated correlation coefficients (r^2) values above 99.9%.

The detection limits were evaluated using the EPA's MDL procedures [17]. The variance checks confirmed that the methodology was applicable for the analytes. The MDL and LOQ for each flavonoid are described in **Table 1**.

Method precision was evaluated by measuring the individual flavonoids in four replicates on three separate days. The within-day, between-day, and total standard deviations were used to determine the HorRat values, which describe the repeatability of each analyte in the test matrix evaluated. The HorRat values and relative standard deviations for each of the test samples are described in **Table 2**. Repeatability relative standard deviations (% RSDs) ranged from 0.43 to 12.1%, which, according to AOAC guidelines, are within acceptable ranges on sample concentration. In this case, flavonoids greater than 1% are expected to have % RSDs below 2%, while materials with lower concentrations can have upwards of 4 to 15%. The HorRat values, which evaluate the intermediate precision from three separate days of analysis, ranged from 0.16 to 1.58, where AOAC guidelines recommend an acceptable range from 0.5 to 2.0 [15]. HorRat values determine the ratio of the observed versus expected precision based on the sample concentration, as it is expected that low concentration analytes will have poorer precision. Some values were below the acceptable range, indicating that tighter precision was observed, which is considered acceptable under these circumstances.

Recovery of the flavonoids from a matrix blank control material made from methylcellulose and magnesium stearate ranged from 85.8 to 101.8%. Recoveries at low levels (0.2 mg/g) ranged from 85.8 to 91%, which are within the range of 85 to 110%. At higher concentrations, the recovery ranged from 97.4 to 101.9%. The data are summarized in **Table 3**. Extraction of the residual biomass indicated that a complete extraction of flavonoids was achieved in a single extraction procedure.

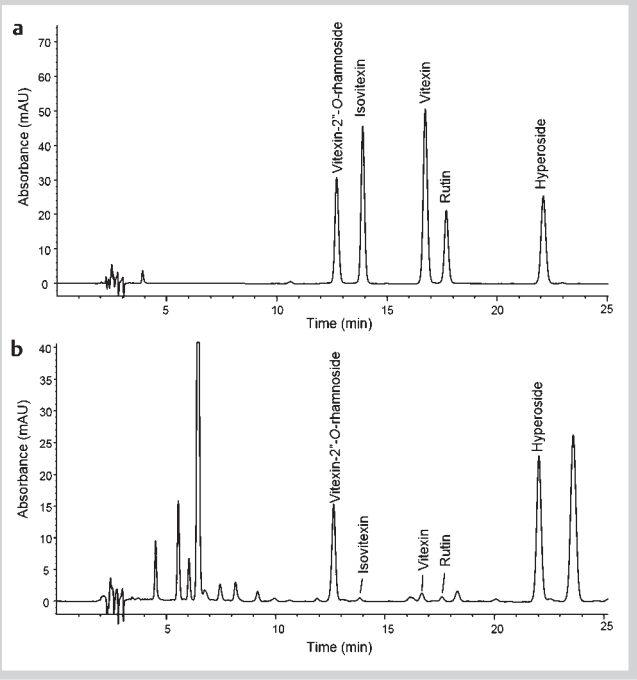

Fig. 3 Chromatographic separation of (**a**) flavonoid standards and (**b**) raw materials at 340 nm.

Table 1 MDL and LOQ for the five major flavonoids quantified in hawthorn leaves and flowers.

Flavonoid	MDL (µg/mL)	MDL (mg/g)	LOQ (µg/mL)	LOQ (mg/g)
Vitexin-2″-*O*-rhamnoside	0.45	0.03	1.2	0.09
Isovitexin	0.32	0.03	0.9	0.07
Vitexin	0.21	0.02	0.6	0.04
Rutin	0.41	0.03	1.1	0.08
Hyperoside	0.29	0.02	0.8	0.06

The optimized method for the analysis of five flavonoids in hawthorn was subjected to a single-laboratory validation, according to AOAC guidelines, by evaluating the accuracy, precision, linearity, selectivity, and detection limits of the method. All of the performance characteristics were within the limits established by AOAC International, therefore, this method is suitable for quantitation of selected flavonoids in raw materials, capsules, tablets, and tinctures containing hawthorn leaves and flowers.

The existing USP method is based on an assay of vitexin and hyperoside only [9]. In addition to these compounds, the method described herein is also suitable for vitexin-2″-*O*-rhamnoside, isovitexin, and rutin, all of which are major flavonoids of hawthorn. There are many published studies of hawthorn and its phytochemicals; however, most of them did not report the method validation for measuring phytochemicals [13, 18]. Previous

Table 2 Precision determinations for the individual flavonoids in hawthorn leaf and flower raw materials and finished products.

Sample ID	Flavonoid	Matrix	Content (mg/g)	Repeatability (% RSD)	Intermediate precision (% RSD)	HorRat
2.1	Vitexin 2″-*O*-rhamnoside	Raw material	3.27	1.47	1.87	0.40
	Isovitexin		0.05	12.1	12.18	1.39
	Vitexin		0.23	2.08	8.39	1.19
	Rutin		0.24	3.06	4.79	0.68
	Hyperoside		7.21	1.91	1.73	0.41
2.2	Vitexin 2″-*O*-rhamnoside	Raw material	0.42	1.87	3.55	0.39
	Isovitexin		<MDL			
	Vitexin		0.15	3.18	11.62	1.55
	Rutin		4.46	0.43	1.47	0.33
	Hyperoside		17.54	0.48	0.96	0.26
2.3	Vitexin 2″-*O*-rhamnoside	Raw material	14.12	1.95	2.04	0.54
	Isovitexin		0.51	1.74	3.63	0.58
	Vitexin		0.30	3.80	8.14	1.20
	Rutin		0.36	1.87	5.08	0.77
	Hyperoside		15.59	1.27	0.97	0.26
2.4	Vitexin 2″-*O*-rhamnoside	Capsule	4.34	0.62	2.01	0.44
	Isovitexin		0.15	3.80	7.03	0.94
	Vitexin		0.23	4.03	9.59	1.36
	Rutin		0.16	5.99	9.99	1.34
	Hyperoside		3.88	0.88	2.17	0.47
2.5	Vitexin 2″-*O*-rhamnoside	Capsule	11.07	1.90	1.94	0.49
	Isovitexin		0.24	6.86	4.99	0.71
	Vitexin		0.46	1.20	10.01	1.58
	Rutin		0.54	2.71	3.22	0.52
	Hyperoside		5.52	1.92	2.51	0.57
2.6	Vitexin 2″-*O*-rhamnoside	Tablet	6.63	0.75	0.67	0.16
	Isovitexin		0.16	6.20	10.79	1.44
	Vitexin		0.35	3.98	8.18	1.23
	Rutin		0.28	3.44	4.59	0.67
	Hyperoside		4.11	0.59	0.90	0.20
Liquid sample			Content (µg/mL)			
2.7	Vitexin 2″-*O*-rhamnoside	Tincture	1683.06	0.77	2.58	0.30
	Isovitexin		<LOQ			
	Vitexin		238.28	0.99	6.52	0.93
	Rutin		<MDL			
	Hyperoside		<LOQ			

validated methods for leaves and flowers were optimized for two of the most popular Chinese hawthorn varieties, *C. pinnatifida* Bge. Var Major N.E.Br and *C. pinnatifida* Bge [2,12]. The current method was optimized for European and North American hawthorn species, which are morphologically and chemically different than the Chinese hawthorn species [4]. This method is applicable to a wide range of materials, including raw leaves and flowers and finished products, and has been subjected to a rigorous validation study using international guidelines. These were not investigated in previous method studies [3,12,13,19].

The current method simplified the sample preparation steps and reduced the LC run time compared to previous methods. The solvent removal steps during sample preparation were eliminated using this method. Traditional methods dry and reconstitute extracts without internal standards, which leads to potential losses in flavonoids with the increased number of sample transfers. By performing a single extraction step followed by filtration, this issue is eliminated and sample preparation time is reduced. Several previous methods require multiple sample transfers and vacuum drying at 40 °C, increasing sample preparation time and the potential for losses [2,3,13]. The current method reduced the LC separation to 25 min without comprising the separation, and is shorter than previous methods with run times of 30–50 min [3, 12,13].

This is the first validated method fit for the purpose of quantifying five major phytochemicals in a wide range of materials, including both raw and finished products, from North American and European hawthorn species. The method described herein: 1) reduced the sample preparation time and is suitable for routine analysis; 2) used new column technology that allows separation and quantitation of five phytochemicals; and 3) reduced the LC run time, thus lowering the consumption of mobile phase. This method allows for multiple sample analyses in a short time frame with a lower cost, and is suitable for the needs of NHP industries.

Materials and Methods

Reagents and calibration standards

HPLC grade methanol, glacial acetic acid, acetonitrile, tetrahydrofuran, 2-propanol, and *o*-phosphoric acid were purchased from VWR International. Water was purified to 18 MΩ using a Barnstead Smart2Pure nanopure system (Thermo Scientific).

Calibration standards for each of the individual flavonoids were purchased from ChromaDex. Vitexin-2″-*O*-rhamnoside (purity 94%), isovitexin (96.6%), vitexin (95.9%), rutin (89.3%), and hyperoside (99.1%) were stored at room temperature and desiccated prior to use.

Test materials

C. douglasii and *C. laevigata* leaves were obtained from the Naturally Grown Herb & Spice Cooperative. Voucher specimens were deposited at the University of Toronto Herbarium, #TRT00019818 and #TRT00019820, respectively, under the supervision of Dr. Timothy A. Dickinson. *C. monogyna* leaves and flowers were obtained from American Herbal Pharmacopeia (AHP) voucher #2357. Several hawthorn leaf and flower products were obtained from commercial suppliers, including two capsule products (Lot 141515 and FC1567A2), one tablet product (Lot 509099), and one tincture (Lot 05). These seven materials were used in the method validation.

Liquid chromatography analysis

An Agilent 1200 LC system equipped with an autosampler, binary pump, and diode array detector (Agilent Technologies) was used. The separation of the flavonoids was achieved on a Kinetex C18 2.6 µm, 150 × 4.6 mm i.d. (Phenomenex). The mobile phase was composed of (A) 0.01% phosphoric acid and (B) THF/acetonitrile/2-propanol (8 : 4 : 1, % v/v/v), and elution was performed using gradient conditions at 0.4 mL/min. The gradient elution was as follows: 0–12 min, 15–18% B; 12–22 min, 18–20% B, 22–23 min, 20–75% B, 23–25 min, 75% B; 25–25.5: 75–15% B, 25.5–30.5 min 15% B with a post-run time of 5 min. The injection volume was 3 µL, the column was maintained at 25 °C using a column oven, and the UV absorbance detector monitored the effluent at 270, 340, and 360 nm, while quantitation was performed at 340 nm for all flavonoids.

Preparations of test materials

Raw materials: Leaves and flowers were ground to less than 80 mesh using a Retsch centrifugal mill (Verder Scientific). Two hundred mg of ground material were extracted with 15.0 mL of the extraction solvent (methanol/water/acetic acid; 50 : 48 : 2, % v/v/v) in a 50-mL polypropylene centrifuge tube. Samples were mixed for 1 min using a vortex mixer and then shaken on a wrist action shaker for 60 min at room temperature. Extracts were then centrifuged at 4600 *g* for 12 min and filtered through 0.22 µm PTFE filters into an autosampler vial and subjected to LC analysis.

Capsules and tablets: The contents of 15 capsules were combined and mixed thoroughly. The contents of 25 tablets were combined and ground using a mortar and pestle. Approximately 200 mg of ground material were extracted with 15.0 mL of methanol/water/acetic acid (50 : 48 : 2, % v/v/v) in a 50-mL centrifuge tube. Samples were vortexed for 1 min and shaken on the wrist action shaker for 60 min. Extracts were centrifuged at 5000 rpm for 12 min and filtered through 0.22 µm PTFE filters into an LC vial for analysis.

Tinctures: Tincture products were mixed by inversion of the product container a minimum of 20 times prior to sampling. One hundred µL of tincture were combined with 900 µL of methanol/water/acetic acid (50 : 48 : 2, % v/v/v) for a 1 : 10 dilution in a 2.0-mL microcentrifuge tube. Samples were vortexed for 1 min and filtered through 0.22 µm PTFE filters into an LC vial for analysis.

Table 3 Spike recovery for the flavonoids using matrix blank for all five major flavonoids in hawthorn leaves and flowers at three concentration levels.

Flavonoid	Level (mg/g)	% Recovery
Vitexin-2″-*O*-rhamnoside	0.2	88.1
	1.0	100.2
	2.0	99.9
Isovitexin	0.2	85.8
	1.0	101.9
	2.0	99.7
Vitexin	0.2	88.1
	0.4	97.4
	0.8	99.9
Rutin	0.2	87.8
	1.0	100.5
	2.0	101.8
Hyperoside	0.2	91.0
	1.0	99.0
	2.0	99.0

Single-laboratory validation parameters

The method was subjected to AOAC International single-laboratory validation guidelines for dietary supplements [15].

Preparation of calibration solutions: Individual 1000 µg/mL stock solutions were prepared for each flavonoid by accurately weighing 10 mg of each into separate 10 mL volumetric flasks and diluting with methanol. These stock solutions were stored at 4 °C in the dark throughout method optimization and validation and checked periodically for stability by evaluating the peak area change over time. Any change over 5% from the original preparation was considered degraded. Seven-point mixed standard calibration curves were constructed daily using the stock solutions. The calibration ranges for each flavonoid were as follows: vitexin-2″-*O*-rhamnoside 1–500 µg/mL, isovitexin 0.5–200 µg/mL, vitexin 0.5–50 µg/mL, rutin 0.5–200 µg/mL, and hyperoside 1–500 µg/mL. The calibration curves were plotted and the slope and y-intercept of each curve was determined using linear regression. Calibration curves were visually inspected and correlation coefficients were calculated. An $r^2 \geq 0.995$ was deemed suitable for quantitation.

Selectivity: The selectivity for flavonoids was achieved by developing an extraction solvent specific to the analytes and optimizing the separation to achieve resolution greater than 1.5, with 1.0 being the minimal usable resolution. Injections of the individual references standards and raw material extracts were performed to ensure chromatographic resolution between the analytes and any interferences. Chromatographic peaks were evaluated for peak purity using the diode array detector spectrum.

MDL and LOQ: The limits of detection and quantitation were determined using the U.S. EPA MDL protocol [17]. The MDL is defined as the minimum concentration of a substance that can be measured and reported with 99% confidence that the analyte concentration is greater than zero. Two standard mixtures containing low concentrations of the flavonoids were used to evaluate MDL/LOQ. Seven replicates were injected and the calculation for MDL was determined as the standard deviation of the calculated concentration between the seven replicates multiplied by the *t*-statistic at the 99% confidence interval. The second set of replicates was used to confirm the variance ratio; therefore, the MDL was valid. LOQ was determined as 10 times the standard deviation for the replicates to determine the MDL.

Precision: Repeatability was determined by preparing quadruplicate replicates on a single day. The intermediate precision was determined by repeating the analysis of quadruplicate samples on 2 additional days. The within-day, between-day, and total standard deviations were calculated for each of the flavonoids. HorRat values were also calculated to assess the overall precision of the method [14].

Accuracy: A matrix blank to mimic the capsule contents of dietary supplements was composed of 99% methylcellulose and 1% magnesium stearate. The matrix blank was spiked with three levels from 1 to 10% total flavonoids. Each level was prepared in triplicate. To evaluate the extraction efficiency of raw leaves, the residue obtained after the first extraction was re-extracted with a second aliquot of 15 mL extraction solvent using the same extraction parameters as the original material.

Data analysis

Individual flavonoids were quantified as mg/g and µg/mL in solid and liquid matrices, respectively. Microsoft Excel was used for calculations and statistical analysis of the validation data including the within-, between-, and total standard deviations and HorRat values. Minitab 16 (Minitab Inc.) was used to design and analyze the factorial study.

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Conflict of Interest

The authors declare no conflict of interest.

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