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Chemical Fingerprinting Profile and Targeted Quantitative Analysis of Phenolic Compounds from Rooibos Tea (*Aspalathus linearis)* and Dietary Supplements Using UHPLC-PDA-MS

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Abstract: *Aspalathus linearis* (Burm.f.) R. Dahlgren, commonly known as rooibos tea, was consumed traditionally by the indigenous South African inhabitants as an herbal remedy. Beside antioxidant properties, it displays antiallergic, antispasmodic, and hypoglycemic activities. An ultra-high-performance liquid chromatography method coupled with photodiode array and mass spectrometry detectors were developed for the determination of 14 phenolic constituents from leaves and stems of *A. linearis*. The efficient separation was performed within 30 min at a temperature of 30 °C by using C-18 column as the stationary phase and water/acetonitrile with 0.05% formic acid as the mobile phase. Method validation for linearity, repeatability, limits of detection, and limits of quantification was achieved. The limits of detection from 0.2–1 µg/mL were reported for the standard compounds. Their total content varied substantially (1.50–9.85 mg/100 mg sample) in 21 dietary supplements. The presence of regioisomers and diastereomers which co-elute on a variety of stationary phases make separation for quantification purposes challenging. This method was found to be efficient in providing low retention times and excellent resolution for this type of phytochemicals. The established method is suitable for chemical fingerprint analysis of *A. linearis* and cost-effective for quality control of rooibos tea products.

Keywords: *Aspalathus linearis*; rooibos; Leguminosae; dietary flavonoids; aspalathin; nothofagin

1. Introduction

The *Aspalathus* genus belongs to the second largest plant family, Leguminosae, and comprises more than 270 plant species, of which *Aspalathus linearis* (Burm.f.) R. Dahlgren is utilized as rooibos tea. It is a leguminous shrub endemic to the western parts of the Cape region in South Africa [1,2]. It grows naturally in the Cederberg Mountains encompassing the Citrusdal, Clanwilliam and Nieuwoudtville regions. These regions are considered as the home of rooibos tea and the heart of the rooibos industry. The leaves and stems

are used to produce rooibos herbal tea, sought-after for its caffeine-free status and flavor. Besides antioxidant properties, it displays antiallergic, antispasmodic, and hypoglycemic activities [3–7]. Rooibos tea is enjoyed in over 37 countries, and elicited attention in the developed countries and the scientific community as a health beverage. Additionally, a report by the Swiss Business Hub South Africa stated that rooibos appears to be headed towards becoming the second most commonly consumed beverage tea in the world after ordinary tea (*Camellia sinensis*) [1]. Two types of rooibos, i.e., 'unfermented' (green) and 'fermented' (red) rooibos tea, are available. 'Fermentation' of the plant material (stems and leaves), mainly an oxidation process and an essential step in the production of the traditional red rooibos, alters the phytochemical profile, nutritional value, and yields [1,8,9].

The main secondary metabolites in the leaves and stems of *A. linearis* are the phenolic constituents including chalconoids, flavonoids, and phenylpropanoids [10]. Currently, there is no standardization for the aspalathin content. Some companies use the total orientin and isoorientin content as a standard to be higher than 0.5%. However, quantitative analysis for rooibos flavonoids often deals with the major constituents and relies heavily on four compounds; aspalathin, nothofagin, orientin, and isoorientin [11]. Others limited the quantification to the major compounds alongside with total polyphenolic content and total antioxidant activity which are exploited as quality indicators in some countries such as the USA market [12]. Taking into account that the flavone-6-*C*-glycoside has a specific clear loss of water in mass spectrometry in the negative mode [13], elutes earlier than flavone-8-*C*-glycoside [14], and has a distinctive UV absorbance spectrum (soulder) at 267 nm [15], some studies distinguished between the regioisomers such as orientin and isoorientin or vitexin and isovitexin, metabolites of aspalathin and nothofagin respectively, leading to efficient separation [16,17]. However, these studies could not resolve the overlapping peaks for the diastereomers such as hyperoside and isoquercitrin leading to incomplete separation and total quantification for these diastereomeric flavones.

Moreover, it is demonstrated that rutin content in these studies has been misquantified since the rooibos has quercetin-3-*O*-robinobioside (bioquercetin) which is a diastereomer to the rutin and not discriminated during the quantification [18]. Others have focused their work on the characterization of the phenolic compounds in aqueous and ethanolic extracts based on proposed structures interrogated by tandem mass [15]. A two-dimensional chromatographic method was conducted to overcome the one-dimensional counterpart issues, such as the difficulty of separation and characterization of phenolic compounds in rooibos tea leading to efficient phenolic profiling with a total analysis time of 17 h [19]. New technique such as the near-infrared (NIR) spectroscopic method has been recruited to analyze the rooibos tea phenolics to discriminate effectively between the fermented and unfermented rooibos tea [11]. Also, a cost-effective capillary electrophoresis method has been exploited for quantitative analysis with low sensitivity and robustness compared with the conventional HPLC method [20].

On the other hand, different ecotypes have been investigated from two species, *A. linearis* and an endangered species, *A. pendula* [21]. To the best of our knowledge, two comprehensive quantitative analyses on rooibos tea were conducted by the same research group utilizing a set of standard compounds [22,23]. However, the semi-quantitave analysis was applied for these studies for the flavanones and quercetin-3-*O*-robinobioside affecting the validity and reliability of the method to quantify these major compounds for rooibos tea products. Herein, lacking the standards, specifically the most abundant compounds of fermented rooibos tea, hampered the suitability for the accurate quantitative analysis. In addition, the aqueous extract was mostly used to simulate the cup of the tea ingested. However, a hydroalcoholic solvent deemed to be substantial because of their ability to extract compounds from the rooibos tea with higher abundance values. Exploiting an array of well-characterized pure compounds as quality markers, isolated previously from an authenticated sample of *A. linearis*, will develop a robust method which serves as a guideline for chemical fingerprint profile and obtains an accurate quantitative analysis of rooibos tea samples.

Besides gaining popularity all over the world, and the fermentation process which alters remarkably the chemical constituents of products, the assessment of the safety profile for the rooibos tea in our previous study highlighted that the extract and its secondary metabolites alter the activity of CYP450 isozymes, leading to potential herb-drug interaction [24]. In our continued quest to explore the quality and safety of botanical ingredients, we aim in this study to reexamine the phenolic profile of the rooibos tea via developing and validating robust, suitable, economical and straightforward analytical fingerprinting approaches which potentially aid in the identification and quantification of phytochemicals, and authentication of the rooibos tea samples for quality assurance.

The current study was intended for the qualitative and quantitative analysis of 14 phenolic glycosides; two dihydrochalcones, five flavones, four flavonols, two flavanones, and one phenyl propanoid of *A. linearis* using ultra-high-performance liquid chromatography-photodiode array-mass spectrometry (UHPLC-PDA-MS). The method was also applied to dietary supplements claiming to contain *A. linearis*.

2. Materials and Methods

2.1. Chemicals and Plant Samples

The 14 standard compounds used as chemical markers were syringin (**1**), (*S*)-eriodictyol-6-*C*-β-D-glucopyranoside (**2**), (*R*)-eriodictyol-6-*C*-β-D-glucopyranoside (**3**), isoorientin (**4**), orientin (**5**), aspalathin (**6**), vitexin (**7**), bioquercetin (**8**), hyperoside (**9**), isovitexin (**10**), rutin (**11**), isoquercitrin (**12**), nothofagin (**13**) and thermopsoside (**14**) as illustrated in Figure 1. Secondary metabolites (**1**–**14**) were previously isolated and elucidated from the authenticated plant material #16850 at the National Center for Natural Products Research (NCNPR). The purity of the components was determined based on the ^{1}H NMR spectrum and HPLC-PDA chromatogram [24].

The dried mature shredded leaves and stems of unfermented (green) *A. linearis* were received from South Africa as a gift from Rooibos Limited in Clanwilliam (Ms. Colette Cronje, sample #793) in October 2014. A specimen (NCNPR #16850) was prepared and deposited by Dr. Vijayasankar Raman in the herbarium of NCNPR, School of Pharmacy, University of Mississippi. The morphological and chemical properties of *A. linearis* plant material (NCNPR #16850) were compared with the reference sample (NCNPR #5488) at the NCNPR for authentication. Supplements containing *A. linearis* as an ingredient were identified by searching websites supplying such products. Twenty-one brands of products containing *A. linearis* were purchased from online sources, and the types of tea (red or green) were reported as claimed on the label (Table 1). All samples were assigned unique identifiers, and their representative samples were deposited in the botanical repository of NCNPR at the University of Mississippi. The solvents were purchased from Fisher Scientific (Fair Lawn, NJ, USA) as analytical grade solvents.

Table 1. NCNPR Code, product name, dosage form, serving size, type of tea, plant part of *A.linearis* supplements.

NCNPR Code	Dosage Form	Serving Size	Red/Green	Additional Notes	Plant Part
20750	Tea Bag	N/A	Red	N/A	leaves
19070	Tea Bag	1 tea bag	N/A	USDA organic	N/A
19069	Powder	N/A	N/A	N/A	N/A
19068	Powder	1 teaspoon	Red	USDA organic	N/A
19067	Tea Bag	N/A	green	USDA organic	N/A
19066	Powder	N/A	Red	N/A	N/A
19065	Powder	1 teaspoon	N/A	USDA organic	N/A
19064	Powder	N/A	Red	N/A	leaves
19063	Powder	2 g	N/A	USDA organic	leaves
19062	Powder	N/A	N/A	N/A	N/A
19061	Tea Bag	1 tea bag	N/A	USDA organic—contains other herbs	leaves

Table 1. *Cont.*

NCNPR Code	Dosage Form	Serving Size	Red/Green	Additional Notes	Plant Part
19060	Tea Bag	N/A	Red	N/A	N/A
19059	Powder	1.5 teaspoon	N/A	Contains other herbs	N/A
19058	Tea Bag	N/A	N/A	USDA organic	N/A
19057	Tea Bag	N/A	N/A	Contains other herbs	N/A
19056	Powder	N/A	Red	N/A	N/A
19055	Tea Bag	1 tea bag (2 g)	N/A	USDA organic	leaves
19054	Tea Bag	N/A	N/A	USDA organic	leaves
19053	Tea Bag	N/A	Red	N/A	N/A
19052	Powder	N/A	N/A	N/A	leaves
19051	Powder	N/A	N/A	USDA organic—contains other herbs	N/A

(N/A) indicates the lack of information.

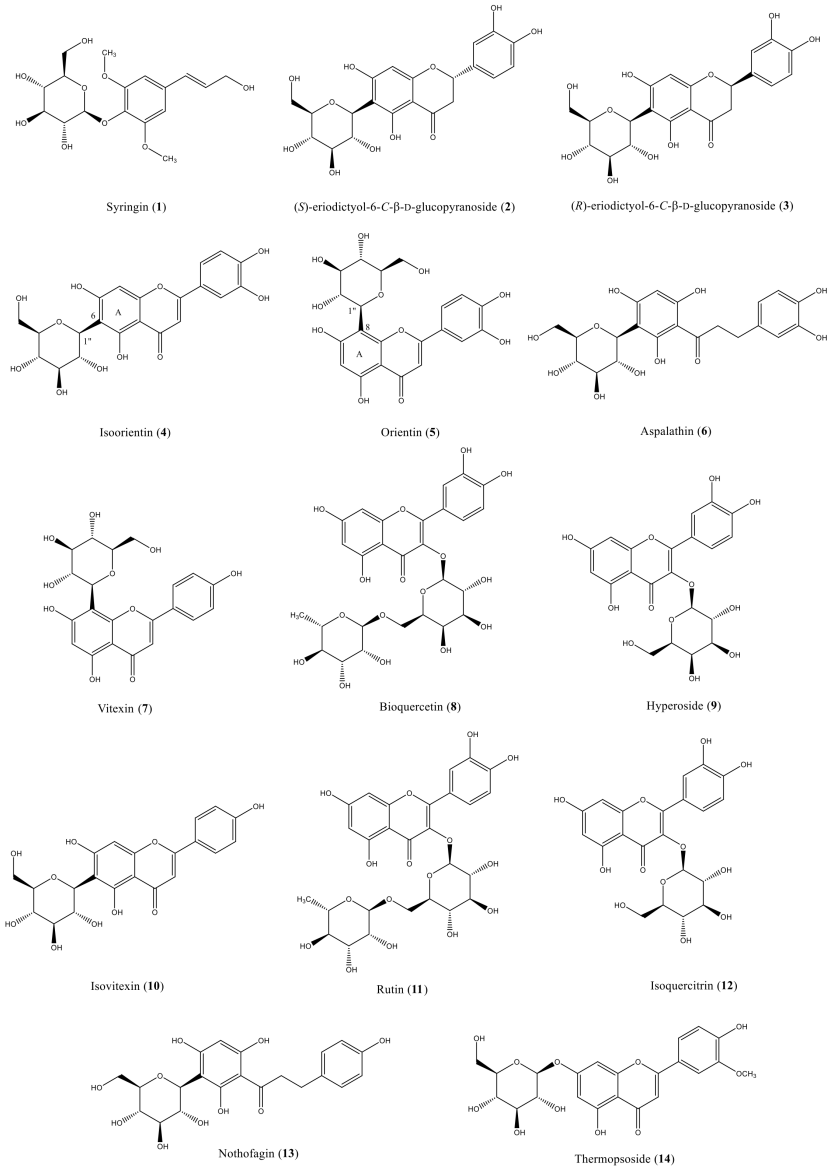

Figure 1. Chemical structures of isolated phytoconstituents **1–14** from *A. linearis*.

2.2. Preparation of Standard Solutions

Stock solutions containing the standard compounds were prepared separately at a concentration of 2 mg/mL in methanol. Seven different concentration levels were prepared

to obtain the calibration curves, and each level was injected in triplicate. The range of the calibration curves was 1–100 µg/mL for the compounds **1**–**3**, **7**–**8**, **10**–**12**, and **14** while 1–1000 µg/mL for compounds **4**–**6**, **9** and **13**, based on a pilot study using a UHPLC-PDA method (Figure S1).

2.3. Sample Preparation

Twenty-one products were purchased either as powder or tea bags. For tea bags, three representative sachets were randomly taken, emptied and mixed to represent the final product and improve sample non-homogeneity. Every product was ground to a fine powder using a Retsch PM 400 planetary ball mill. Two hundred milligrams of dried powdered plant samples were sonicated in 2.0 mL of 80% methanol (*v*/*v*) for 30 min, followed by centrifugation at 959× *g* for 15 min. The supernatant was transferred to a 10 mL volumetric flask. The procedure was repeated four times, and the respective supernatants were pooled, whereafter the final volume was adjusted to 10 mL using 80% methanol. The sample was shaken exhaustively, and an aliquot (2 mL) was filtered directly into an LC sample vial using a 0.45 µm polytetrafluoroethylene membrane syringe filter. The remaining volume was collected in a 10 mL vial and stored in the refrigerator.

2.4. Validation Procedure

The newly developed UHPLC method was validated regarding linearity, precision, and accuracy according to International Conference on Harmonization guidelines (ICH) [25]. The LOD and LOQ were determined by injecting a series of dilute solutions with known concentrations for each standard. A signal-to-noise ratio greater or equal to three and ten was defined for LOD and LOQ, respectively. For intra and inter-day assays, an analysis of three individual samples of *A. linearis* (#NCNPR 16850) was performed on three consecutive days. The samples were extracted and assayed for precision under optimized conditions. The method accuracy was determined in duplicate by a recovery experiment. The samples were exhaustively extracted and dried completely, then spiked with a concentration of 100 µg/mL of standards (Table S1). The samples were dried and extracted under optimized conditions similar to the extraction protocol mentioned in the sample preparation subsection above, then analyzed using the developed method. All samples and standards were injected in triplicate.

2.5. Instrumentation and Experimental Conditions

A Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA) was used to perform the analyses. UPLC system consisted of a quaternary solvent manager, sampler manager, column heater, and PDA detector connected to a Waters Empower 2 data station. Separation was achieved on a Waters CORTECS UPLC C18 column (1.6 µm, 2.1 × 100 mm). The temperatures for the column and sample were maintained at 30 °C and 10 °C, respectively. The mobile phase, at a flow rate of 0.25 mL/min, included water (A) and acetonitrile (B) both containing 0.05% formic acid were applied in the following gradient elution: 0–22 min, held as 90% A:10% B; 22–30 min, 90% A:10% B to 75% A:25% B; 30–32 min, 75% A:25% B to 100% B. Washing procedure for 2 min with 100% B was applied after separation followed by a re-equilibration period for 7 min. Strong and weak needle wash solutions (90/10 and 10/90; acetonitrile/water) were performed, respectively. The total run time for analysis was 32 min. The injection volume was 2 µL, and the compounds were detected at 288 nm. Spiking the samples with standard compounds and comparing the UV spectra (Figure S2) and retention times were necessary for peaks assignment. The mass spectrometric analysis of all samples was conducted using a single quadrupole detector (SQD) equipped with an ESI source operated in both positive and negative mode and a scan range of *m*/*z* 100–900 with operation parameters set at: capillary voltage 3.0 kV, cone voltage 35 V, source temp 150 °C, desolvation temp 350 °C, desolvation gas 650 L/h, and cone gas 25 L/h.

3. Results

3.1. Sample Preparation

The plant samples were composed of complex mixtures of components, and three extraction techniques were employed as preliminary testing to indicate the optimum extraction solvent. The authenticated plant sample (#16850) was extracted separately with methanol, 80% methanol, and 70% methanol in water. The abundance values for compounds **1–14** in the sample extracted with 80% methanol were higher than those extracted with pure methanol or 70% methanol in water. Therefore, 80% methanol in water was selected as an extraction solvent for analysis of the authenticated plant material and supplements accordingly.

3.2. Method Development and Optimization

Optimized chromatographic conditions were achieved after several trials with acetonitrile, methanol, and water in different proportions for the mobile phase. A mobile phase containing water and acetonitrile, both containing formic acid with a constant flow rate at 0.25 mL/min on a Cortecs UPLC C18, 1.6 um, 2.1 × 100 mm using gradient elution at a fixed column temperature of 30 °C were deemed the optimal separation condition for the determination of compounds **1–14** in various samples. The different columns tried were Acquity UPLC BEH Shield RP18, 1.8 um, 2.1 × 100 mm, and HSS T3, 2.1 × 100 mm. Each provided a different combination of silanol activity, hydrophobicity, hydrolytic stability, and chemical interaction with the analytes. Among these, the Cortecs UPLC C18 resolved peaks **1–14** adequately. Other columns could not resolve the positional isomers **7–10**, and diastereomers **8–11**, and **9–12** satisfactorily under the same conditions. The standard compounds in the positive ion mode were observed at m/z 395.22 $[M + Na]^+$ (**1**), 451.05 $[M + H]^+$ (**2**), 451.11 $[M + H]^+$ (**3**), 449.18 $[M + H]^+$ (**4**), 449.24 $[M + H]^+$ (**5**), 452.92 $[M + H]^+$ (**6**), 433.17 $[M + H]^+$ (**7**), 611.20 $[M + H]^+$ (**8**), 465.19 $[M + H]^+$ (**9**), 433.17 $[M + H]^+$ (**10**), 611.11 $[M + H]^+$ (**11**), 465.31 $[M + H]^+$ (**12**), 437.11 $[M + H]^+$ (**13**), and 463.23 $[M + H]^+$ (**14**), respectively (Figure S3). On the contrary, the standard compounds in the negative ion mode were detected at m/z 417.27 $[M - H+ HCO_2H]^-$ (**1**), 449.26 $[M - H]^-$ (**2**), 449.26 $[M - H]^-$ (**3**), 447.24 $[M - H]^-$ (**4**), 447.35 $[M - H]^-$ (**5**), 451.17 $[M - H]^-$ (**6**), 431.13 $[M - H]^-$ (**7**), 609.34 $[M - H]^-$ (**8**), 463.11 $[M - H]^-$ (**9**), 431.19 $[M - H]^-$ (**10**), 609.36 $[M - H]^-$ (**11**), 463.25 $[M - H]^-$ (**12**), 435.28 $[M - H]^-$ (**13**), and 461.28 $[M - H]^-$ (**14**), respectively (Figure S4).

3.3. Method Validation

Quantitative determination of 14 compounds in various samples was achieved using the UHPLC-PDA method, and results were expressed as mg/100 mg on a dry weight basis. The developed method was validated regarding limits of detection [LOD] and limits of quantification [LOQ], linearity, intra-day and inter-day precision for three consecutive days and accuracy (Table S1). These validation parameters enabled us to investigate the suitability of the method for routine analysis.

The seven-point calibration curve exhibited a linear correlation between concentration and peak area. Calibration data indicated the linearity ($r^2 > 0.989$) of the detector response for compounds **1–14** from 1 to 100 μg/mL, with additional concentration points (200–1000 μg/mL) considered for compounds **4–6**, **9** and **13**. The limits of detection were below 1 μg/mL (0.01 mg/100 mg) for compounds **1–14**.

Intra- and inter-day variation for this study was determined for the authenticated *A. linearis* plant sample (#16850) and relative standard derivation (RSD) was lower than 6%. The analysis was conducted three times on three different days, and each run was repeated in triplicate. The intra-day RSD for the replicates for compounds **1–14** was between 0.14 and 4.26%, and the RSD for the day-to-day replicates was between 1.2 and 5.9%.

The method accuracy for the related compounds was assigned by spiking two exhaustively extracted authenticated plant material samples (#16850) with a concentration of 100 μg/mL of a mixture of standards **1–14**. The samples were extracted and analyzed

again under similar conditions. The percentage recovery of these samples ranged from 89.50 to 109.36%.

4. Discussion

According to WHO, the described risks associated with traditional medicines include direct adverse events, side effects or unwanted treatment interactions, exposure of the individual to misleading or unreliable information, and the use of poor quality, adulterated or counterfeit products. It is necessary to develop and validate analytical fingerprinting approach for identification and quantification of the phenolic compounds as markers from rooibos tea samples, bearing in mind the economic impact, safety concerns, and fermentation process which determines the chemotaxonomic profile. Although several analytical methods have been applied for the qualitative and quantitative analysis of rooibos, some have poor resolution, longer analysis time, and/or reduced sensitivity that hamper their suitability for routine applications [11,12,15–17,20,21]. This highlights the necessity for a robust method with high sensitivity that can be used to quantify phenolic compounds in rooibos herbal products for quality control purposes. The presence of regioisomers or diastereomers which co-elute on a variety of stationary phases make separation challenging for quantification purposes.

The main chemical classes reported are chalconoids, flavonoids, and phenylpropanoids, along with traces of lignans and phenolic acids [10]. Unlike classical flavonoids (glycopyranosyloxy flavonoids), glycopyranosyl flavonoid derivatives have superior characteristic features. For instance, their glycone moiety is attached via the C-1″ directly to the flavonoid backbone, usually at the C-6 or C-8 position of the A ring as shown in isoorientin (**4**) and orientin (**5**) (Figure 1). This feature affords them the ability to resist hydrolysis and be absorbed intact in the colon or intestine. Therefore, *C*-glycosides have exclusive biological activities compared with *O*-glycosyl counterparts. During metabolism, they might also react differently with the liver enzymes [26].

Due to the ability to biosynthesize unique and rare secondary metabolites, rooibos tea is known for its potent hypoglycemic activity [5,27,28]. To the best of our knowledge, aspalathin (acyclic dihydrochalcone) has been found in *A. linearis* and in a closely related and endangered species, *A. pendula* (the golden tea) [7,21]. During the 'fermentation' process, aspalathin oxidation produces flavanone intermediates, which are also converted to flavones [29].

The UHPLC-UV separation of 80% methanol extracts of *A. linearis* shown in Figure 2. Optimization strategies were pursued by modifying the temperature, flow rate, mobile phase gradient and stationary phases. Although higher temperature and increased flow rate positively reduced the analysis time, they simultaneously affected the resolution (Figure S5). All 14 compounds were separated within less than 30 min. The identification of the compounds in all samples was based on the comparison of retention times and UV spectra with those of standards. The analysis was performed on an authenticated plant material (#16850) and 21 dietary supplements.

Figure 2 illustrates an example of the UHPLC-UV chromatograms at 288 nm. Of the 21 dietary supplement brands tested, all products contained phenolic compounds with varied contents between the fermented and unfermented rooibos tea products. The peaks of standard compounds in all samples were identified by comparing the UV (Figure S2) and MS spectra (Figures S3 and S4) obtained with the standards, which showed $[M + H]^+$ and $[M + Na]^+$ ions in the positive mode, and $[M - H]^-$ and $[M - H + HCO_2H]^-$ ions in the negative mode (Table 2). The total content of phenolic compounds analyzed (**1**–**14**) was found to be from 1.5 to 3.32 mg/100 mg for fermented rooibos tea samples, and from 4.58 to 9.85 mg/100 mg for unfermented rooibos tea samples (Figure 3).

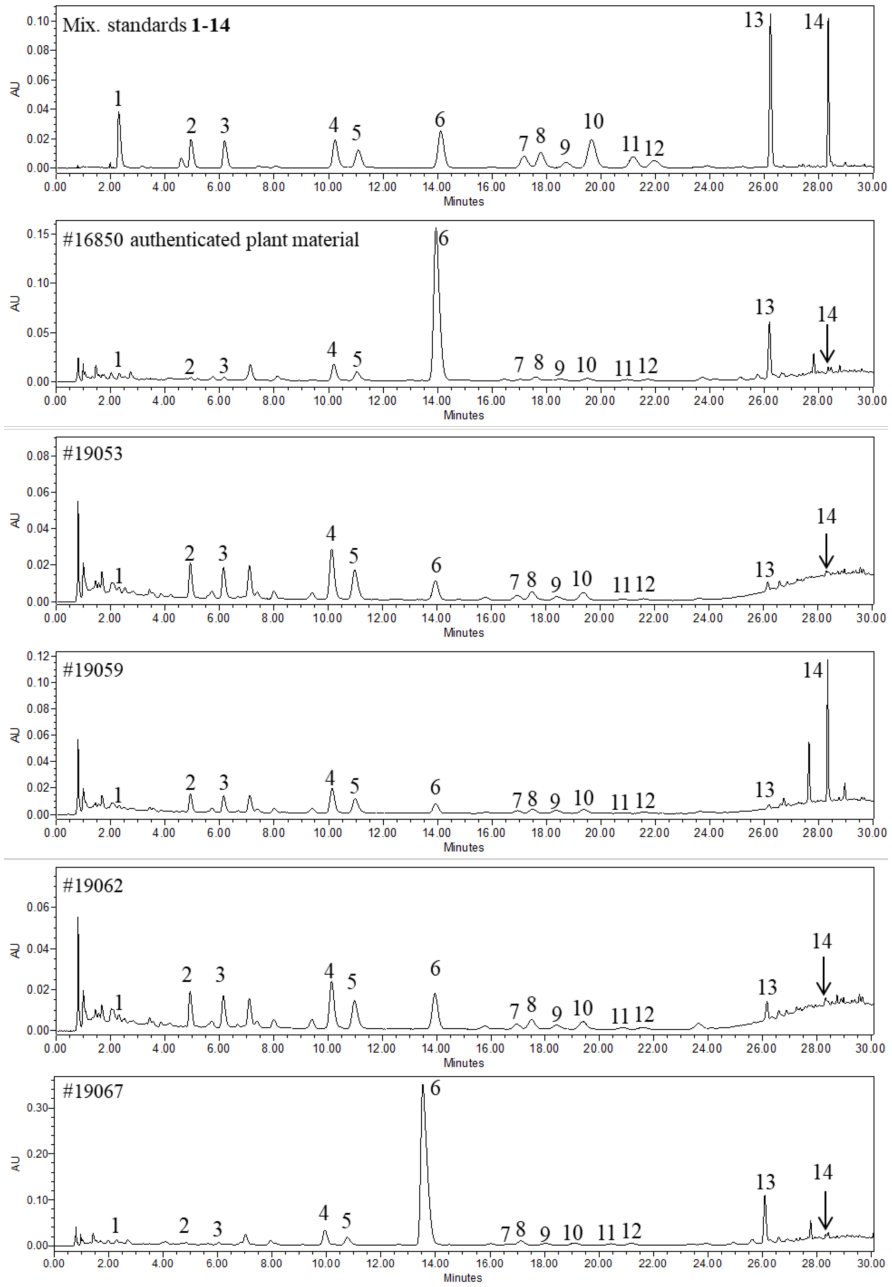

Figure 2. UHPLC-UV Chromatograms of a standards mixture (**1**–**14**), authenticated plant sample (#16850) and dietary supplements (#19053, #19059, #19062, and #19067) at 288 nm.

The characterization and confirmation of 14 compounds from an authenticated plant sample and dietary supplements claimed to contain *A. linearis* was achieved by liquid chromatography-mass spectrometry with electrospray ionization (LC-ESI-MS) hyphenated technique coupled with selective ion monitoring. The method involved using both positive and negative ion modes for compounds **1**–**14**. All compounds of interest displayed a superior response in negative mode ionization. The chromatogram shows no overlapping peaks at the retention time of interest. Also, the mass spectrum exhibited fragmentation behavior which was diagnostic in the characterization of the compounds (Table 2). The most abundant components detected were *C*-glycosides, and its neutral mass loss of hexose equals 120 Da usually observed compared with the 162 Da with the *O*-counterparts which means the fragmentation patterns could be useful for this type of compounds [30].

Table 2. Compound name, retention time (min), LOD, LOQ, UV spectra, M.wt, molecular formula, and MS of compounds used for analysis of *A. linearis*.

Sample #	Compound Name	^{t}R (min)	LOD (µg/mL)	LOD (mg/100 mg)	LOQ (µg/mL)	LOQ (mg/100 mg)	Molecular Weight	Molecular Formula	UV (nm)	*m/z* $[M + H]^+/[M + Na]^+$	*m/z* $[M - H]^-$ / $[M - H + HCO_2H]^-$
1	Syringin	2.295	0.5	0.003	2	0.012	372.37	$C_{17}H_{24}O_9$	220,264,383	395.22 $[M + Na]^+$	417.21 $[M - H + HCO_2H]^-$
2	(*S*)-eriodictyol-6-*C*-β-D-glucopyranoside	4.944	1	0.006	5	0.031	450.12	$C_{21}H_{22}O_{11}$	215,288,384	451.05 (frag., 433.17, 415.29, 331.01)	449.26 (frag., 329.05)
3	(*R*)-eriodictyol-6-*C*-β-D-glucopyranoside	6.187	1	0.006	5	0.031	450.12	$C_{21}H_{22}O_{11}$	215,288,384	451.11 (frag., 432.98, 415.16, 331.26)	449.26 (frag., 329.07)
4	Isoorientin	10.248	0.5	0.004	2	0.018	448.10	$C_{21}H_{20}O_{11}$	211,269,349	449.18 (frag., 329.13)	447.24
5	Orientin	11.106	1	0.010	5	0.050	448.10	$C_{21}H_{20}O_{11}$	211,269,349	449.24 (frag., 329.47)	447.35
6	Aspalathin	13.980	0.5	0.002	2	0.007	452.13	$C_{21}H_{24}O_{11}$	227,288	452.92 (frag., 434.98, 333.32)	451.17 (frag., 330.94)
7	Vitexin	17.164	1	0.010	5	0.050	432.10	$C_{21}H_{20}O_{10}$	214,268,337	433.17 (frag., 415.29, 313.01)	431.13
8	Bioquercetin	17.768	1	0.004	5	0.020	610.15	$C_{27}H_{30}O_{16}$	255,353	611.20 (frag., 465.05, 303.12)	609.34
9	Hyperoside	18.701	1	0.004	5	0.019	464.10	$C_{21}H_{20}O_{12}$	255,355	465.19 (frag., 303.04)	463.11
10	Isovitexin	19.630	0.5	0.006	2	0.025	432.10	$C_{21}H_{20}O_{10}$	214,268,337	433.17 (frag., 415.10, 312.87)	431.19
11	Rutin	21.156	1	0.007	5	0.030	610.15	$C_{27}H_{30}O_{16}$	255,353	611.11 (frag., 465.25, 303.11)	609.36
12	Isoquercitrin	21.916	1	0.004	5	0.019	464.10	$C_{21}H_{20}O_{12}$	255,350	465.31 (frag., 303.11)	463.25
13	Nothofagin	26.218	0.2	0.001	1	0.005	436.14	$C_{21}H_{24}O_{10}$	226,287	437.11 (frag., 419.29, 383.22, 341.14, 317.13)	435.28 (frag., 315.11)
14	Thermopsoside	28.338	0.5	0.005	2	0.020	462.12	$C_{22}H_{22}O_{11}$	252,348	463.23(frag., 301.09)	461.28

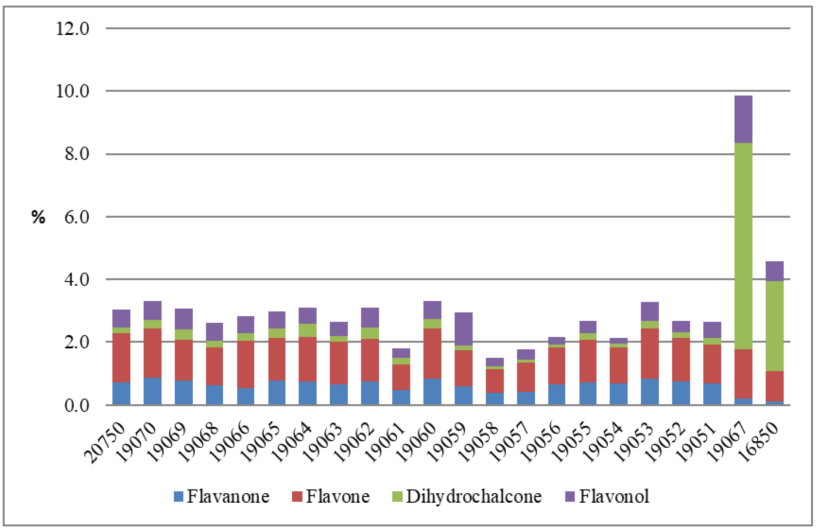

Figure 3. Quantitative analysis of flavonoids (**2**–**14**) in authenticated plant sample (#16850) and dietary supplements (green rooibos tea #19067), and red rooibos tea (#19051, #19052, #19053, #19054, #19055, #19056, #19057, #19058, #19059, #19060, #19061, #19062, #19063, #19064, #19065, #19066, #19068, #19069, #19070, and #20750) at 288 nm. The results are expressed as mg/100 mg of plant material and arranged according to the flavonoids subclasses; flavanone ((*S*)-eriodictyol-6-*C*-β-D-glucopyranoside (**2**) and (*R*)-eriodictyol-6-*C*-β-D-glucopyranoside (**3**)), flavone (isoorientin (**4**), orientin (**5**), vitexin (**7**), and isovitexin (**10**)), dihydrochalcone (aspalathin (**6**) and nothofagin (**13**)), flavonol (bioquercetin (**8**), hyperoside (**9**), rutin (**11**), isoquercitrin (**12**), and thermopsoside (**14**)).

The data suggests four compounds to be employed as optimal quality markers for nutraceuticals claiming to contain *A. linearis*; two dihydrochalcones, aspalathin and nothofagin, alongside two flavanones, (*S*)-eriodictyol-6-*C*-β-D-glucopyranoside and (*R*)-eriodictyol-6-*C*-β-D-glucopyranoside. According to this study, the red type of rooibos displayed the major compounds as the flavones isoorientin and orientin followed by the flavanones, (*S*)-eriodictyol-6-*C*-β-D-glucopyranoside and (*R*)-eriodictyol-6-*C*-β-D-glucopyranoside. On the contrary, aspalathin, isoorientin, orientin, and nothofagin, followed by hyperoside and bioquercetrin, were the major compounds detected and quantified in the unfermented rooibos tea (Table 3).

Table 3. Distribution and content of 14 phytochemicals (%, mg/100 mg sample weight) in authenticated green plant material (16850) and 21 herbal supplements claimed to contain *A. linearis*.

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**Total**
Code #															
16850	0.05	0.04	0.07	0.42	0.41	2.58	0.07	0.16	0.21	0.06	0.08	0.14	0.30	0.04	4.58
20750	0.02	0.36	0.36	0.67	0.65	0.16	0.15	0.20	0.22	0.10	0.04	0.08	0.03	0.03	3.05
19070	0.03	0.44	0.43	0.69	0.64	0.25	0.12	0.16	0.23	0.11	0.06	0.12	0.04	0.02	3.31
19069	0.02	0.40	0.38	0.56	0.56	0.30	0.08	0.16	0.35	0.10	0.03	0.08	0.03	0.02	3.06
19068	0.01	0.32	0.31	0.52	0.53	0.17	0.08	0.23	0.24	0.08	0.03	0.06	0.02	0.02	2.61
19067	0.09	0.08	0.11	0.74	0.65	6.05	0.08	0.45	0.47	0.12	0.18	0.34	0.52	0.05	9.85
19066	0.01	0.26	0.27	0.63	0.64	0.21	0.14	0.19	0.25	0.10	DUL	0.08	0.03	0.03	2.81
19065	0.02	0.39	0.39	0.58	0.57	0.25	0.12	0.17	0.23	0.09	0.05	0.07	0.04	DUL	2.96
19064	0.03	0.38	0.37	0.61	0.59	0.37	0.12	0.14	0.21	0.09	0.05	0.09	0.05	0.03	3.10
19063	0.02	0.34	0.33	0.59	0.57	0.15	0.12	0.13	0.20	0.09	DUL	0.07	0.02	0.02	2.63
19062	0.02	0.38	0.38	0.58	0.57	0.30	0.12	0.18	0.27	0.09	0.05	0.09	0.05	0.02	3.09
19061	0.03	0.21	0.27	0.34	0.33	0.18	0.07	0.10	0.13	0.07	0.03	0.04	0.02	DUL	1.79
19060	0.02	0.42	0.41	0.70	0.67	0.26	0.14	0.18	0.24	0.11	0.05	0.09	0.04	0.02	3.32
19059	0.02	0.31	0.30	0.47	0.48	0.14	0.11	0.13	0.30	0.08	0.03	0.11	0.02	0.50	2.96
19058	0.02	0.17	0.21	0.30	0.30	0.10	0.08	0.07	0.12	0.06	DUL	0.05	0.01	0.02	1.50
19057	0.05	0.21	0.21	0.39	0.39	0.08	0.10	0.09	0.15	0.06	DUL	0.05	0.01	0.02	1.78
19056	0.01	0.34	0.33	0.49	0.49	0.08	0.11	0.05	0.13	0.07	DUL	0.03	0.02	0.02	2.16
19055	0.01	0.36	0.35	0.58	0.57	0.18	0.13	0.10	0.20	0.09	DUL	0.05	0.03	0.02	2.67
19054	0.01	0.35	0.34	0.47	0.50	0.09	0.11	DUL	0.11	0.07	DUL	0.03	0.02	DUL	2.10
19053	0.02	0.42	0.41	0.70	0.67	0.20	0.14	0.18	0.27	0.11	0.04	0.09	0.03	0.02	3.27
19052	0.01	0.37	0.37	0.58	0.58	0.16	0.13	0.10	0.19	0.09	DUL	0.06	0.02	0.02	2.69
19051	0.03	0.33	0.36	0.52	0.50	0.18	0.13	0.15	0.19	0.10	0.04	0.10	0.03	0.02	2.64

Compounds are syringin (**1**), (*S*)-eriodictyol-6-C-β-D-glucopyranoside (**2**), (*R*)-eriodictyol-6-C-β-D-glucopyranoside (**3**), isoorientin (**4**), orientin (**5**), aspalathin (**6**), vitexin (**7**), bioquercetin (**8**), hyperoside (**9**), isovitexin (**10**), rutin (**11**), isoquercitrin (**12**), nothofagin (**13**) and thermopsoside (**14**). DUL: detected under LOQs.

Interestingly, the flavones such as orientin and isoorientin were suggested to be the potential responsible compounds for the antioxidant activity reduction and red color formation of the red tea as mentioned in previous studies because of the oxidation process for aspalathin [31]. The total flavone concentrations relative to the 100 mg plant material in this study ranged from 0.75 to 1.62 mg, and 0.96 to 1.59 mg for the fermented and unfermented rooibos tea, respectively. However, the percentage of the flavones to the total flavonoids analyzed was higher for the fermented rooibos tea ranging from 48.21 to 50.06%, compared to the unfermented type from 16.18 to 20.96% (Figure 4). The proximity in the percentage of flavones, orientin, isoorientin, vitexin, and isovitexin for red and green teas is interesting. Although the orientin and isoorientin are suggested to be the final products of the fermentation process of aspalathin, their contents were detected for the green tea as similar to the red tea products. It is possible that orientin and isoorientin form via another biosynthetic route independent of aspalathin. This resemblance also suggests that other oxidation products of aspalathin, such as yellow dibenzofurans and brown polymers, might play a substantial role in the browning mechanism of the fermented rooibos tea [31].

The variation between the herbal supplement labeled as green tea (#19067) and the authenticated plant material (#16850) is justifiable. While the former contains leaves entirely, as stated in the label, the latter encompasses composite parts of leaves and stems, which could be one of the aspects that affected the dihydrochalcone content in this study. According to prior studies, the leaf-to-stem ratio and changes in biotic and abiotic stress could be key factors for the content difference of dihydrochalcones in the plant and reflect the natural variation [32]. On the other hand, the high content of thermopsoside in the herbal supplement #19057 compared with the other brands could be attributed to other herbs listed on the product label (Table 1).

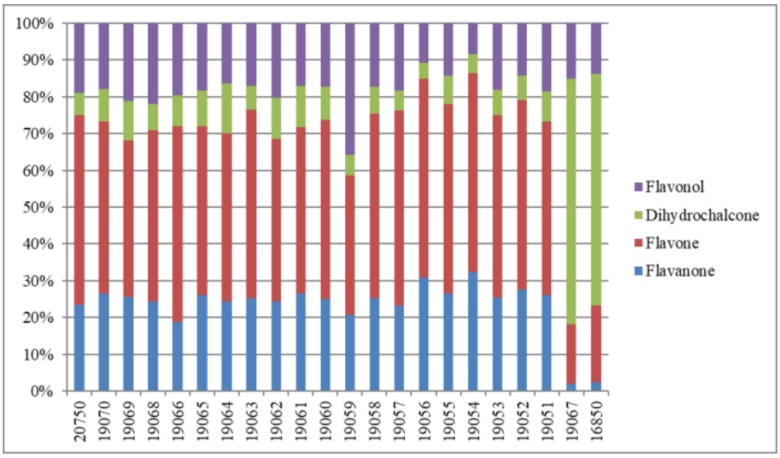

Figure 4. Percentage of flavonoid subclasses for compounds **2**–**14** to the total flavonoid content in authenticated plant sample (#16850) and dietary supplements (green rooibos tea #19067), and red rooibos tea (#19051, #19052, #19053, #19054, #19055, #19056, #19057, #19058, #19059, #19060, #19061, #19062, #19063, #19064, #19065, #19066, #19068, #19069, #19070, and #20750) at 288 nm.

In our recent in vitro safety assessment regarding the potential herb-drug interaction, the two major and structurally related components, aspalathin and nothofagin, increased PXR activity to 1.56 and 1.38 fold, respectively. Moreover, further mRNA expression studies in HepG2 cells displayed a remarkable increase in CYP3A4 gene expression after treatment with these phytochemicals (30 μM). This observation should be considered when standardizing the rooibos tea products indicating that green and red rooibos tea extract might interact differently with CYP450 isozymes owing to their distinctive chemical profiles and yields [24].

5. Conclusions

In summary, the newly developed method is suitable for the quality control and chemical fingerprint analysis of *A. linearis* plant samples and dietary supplements claimed to contain *A. linearis*. UHPLC-PDA-MS method for the determination of 14 phenolic compounds was found to be efficient in providing low retention times and excellent resolution simultaneously. It was validated, and 21 dietary supplements were analyzed accordingly. All tested dietary supplements contained the phenolic compounds that are also present in an authentical green rooibos sample. The total quantities of the compounds in these brands ranged from 1.50 to 9.85 mg/100 mg with a higher content observed for the unfermented products.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations9070159/s1, Figure S1: Calibration curves for compounds **1**–**14**; Figure S2: UV spectra for compounds **1**–**14**; Figure S3: The positive mode of Mass spectra for compounds **1**–**14**; Figure S4: The negative mode of Mass spectra for compounds **1**–**14**; Figure S5: Different chromatograms for different columns and flow rates tried in the method development and optimization at 280 nm; Table S1: Inter-day and Intra-day precision (% RSD) and recovery analysis for *A. linearis* #16850 samples.

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Conflicts of Interest: The authors declare no conflict of interest.

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