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The Chemical and Pharmacological Variability of Key Bio-Actives Present in Commercially Available *Angelica sinensis*, *Glycyrrhiza uralensis and Rhodiola rosea* Samples

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Abstract

There is global demand for better quality control (QC) of medicinal herbs including standardisation of bio-active chemical components and pharmacological testing. The quantitative variability of key chemical markers in *A. sinensis*, *G. uralensis* and *R. rosea* from several sources and the pharmacological activity was determined to confirm that the chemical markers' variability is linked to the biological activity.

To quantify the chemical variation, three novel, simple and rapid UPLC-PDA-ESI-MS/MS methods were developed and validated. The qualitative chemical variability of the bio-actives was further studied using ^{1}H NMR metabolomics and principal component analysis (PCA). The pharmacological anti-inflammatory activity of the commercials extracts and marker compounds was assessed using the Griess reagent NO scavenging assay.

The *A. sinensis* samples exhibited the greatest chemical fold-variation while *G. uralensis* showed the least chemical variability. *R. rosea* samples indicate the presence of other *Rhodiola* sub-species. The PCA clustering was consistent with observed trends and identified adulteration. The bioactivity of the selected marker compounds was linked to the extracts activity. The use of PCA analysis and *in vitro* anti-inflammatory testing improve and provide rationale for better QC of herbal extracts.

Keywords: *Angelica sinensis*; *Glycyrrhiza uralensis*; *Rhodiola rosea*; Chemometrics; UPLC; NO scavenging; PCA

Introduction

There is global demand for high quality herbal products. Quality control (QC) testing ensures that safety and efficacy is valued by consumers. Chemical quantification of marker compounds is routinely adopted for QC of herbal extracts and is essential to provide consistently. Beyond the marker compounds it is essential to confirm the ID of the herb and the markers selected are biologically relevant.

The rhizome (or root) of *Angelica sinensis* also known as Dang Gui, *Glycyrrhiza uralensis* also known as Gan Cao and *Rhodiola rosea* also known as Hong Jing Tian are used extensively in complementary traditional Chinese medicine (TCM) and European herbal medicine. There are few or no well validated, inexpensive ultra-performance liquid chromatography (UPLC) methods available for the quality control (QC) of the known bio-actives in these herbs. Most literature in relation to these herbs is about their pharmacological activity or analytical methods that are tedious for chemical standardisation [1-5]. The Herbal Chemical Marker Ranking System (Herb MaRS) should be used to determine appropriate markers and often multiple markers are required [6]. For routine QC testing of these three herbs simple yet adequate quantitative methods are needed. UPLC is a common technique employed for the quality control of herbal products. It does however have limitations, since it can only detect compounds with UV absorption and peak identity typically only tentative being based on retention time and UV spectrum in comparison to a reference standard. Therefore, the identity of the peak is typically confirmed by mass spectrometry.

Whilst the role of marker compounds for QC is important, the non-quantified minor components are also theorised to contribute to efficacy. It is therefore essential to ensure that the unquantified components of the herb are consistent. This is typically achieved by chromatographic comparison (LC or TLC) to a certified herbal reference standard. For routine repeat analysis, as typically performed by manufactures or QC testing laboratories, multiple peer samples and databases of previous certified samples may be available. PCA can cluster samples based on similarities of whole data sets. It is however limited by the chromatographic technique used to acquire the data. Complementary techniques should therefore be considered during validation to ensure a wide spectrum of compounds is covered. For example, fingerprinting by UPLC with PDA detection may be complimented by ^{1}H NMR which can detect non-UV absorbing compounds. Comprehensive fingerprinting coupled with quantification of marker compounds provides a robust basis for QC, however biological testing is required to determine the significance of chemical variation.

The appropriate biological testing should be selected based on the herbs use. The anti-inflammatory activity of commercial samples is studied using the Griess reagent NO scavenging assay. *A. sinensis* also known as 'female ginseng' is believed to increase blood circulation

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and prevent platelet aggregation [7]. *G. uralensis* or 'licorice' is used as an anti-ulcer agent that is claimed to function by detoxifying the digestive system and liver [8,9]; its use is reported in the treatment of various inflammatory diseases [10]. *R. rosea* or 'rose root' is reportedly a stimulant or adaptogen for the immune system, reducing stress and haematotoxicity [11-13]. The use of these herbs is broad, however many of these uses are linked to inflammation.

To improve and strengthen the QC of three commonly used herbs, commercial rhizomes of *A. sinensis*, *G. uralensis* and *R. rosea* samples were purchased and subjected to chemical and biological testing. To quantitate the chemical variation in the herbs, three improved simple reversed phase ultra-performance liquid chromatography with photodiode array detection (UPLC-PDA) methods were developed. The bio-active analytes selected for monitoring in each of the herbs are shown in Figure 1. The target analytes were selected using the Herbal Chemical Marker Ranking System (Herb MaRS) [6]. The analyte selection system takes into account the bioactivity, physiological activity and the bioavailability of each target analyte in each herb and is shown in Table 1. The peak identity was confirmed by electrospray ionization tandem mass spectrometry (ESI-MS/MS) and the peak purity checked using PDA.

The main goal of this study is to characterize the quantitative chemical variability of the selected herbs using reversed phase ultra-performance liquid chromatography with photodiode array detection (UPLC-PDA) and electrospray ionization (ESI-MS/MS). The qualitative chemical variability of these herbs is assessed using principal component analysis (PCA) comparison of UPLC chromatographic profiles and ^{1}H NMR spectra. Further, a measure of the biological anti-inflammatory activities of the samples was determined using the *in vitro* Griess reagent NO scavenging method.

Experimental

Apparatus

UPLC-PDA analysis was performed using a Waters Acuity™ H-Class UPLC system (Waters, NSW, Australia). UPLC-MS/MS analyte identity confirmation was achieved using a Waters Acuity™ Xevo TQ UPLC system (Waters, NSW, Australia) with electrospray ionisation ((+)/(-) ESI-MS/MS) and tandem triple quadrupole MS detector. Separation was achieved on a Kinetex reverse phase C-18 (150 mm x 2.10 mm, 1.7 µm packing) column. Chromatograms were processed using the Empower Pro manager. A Bruker Avance 400 MHz NMR spectrometer

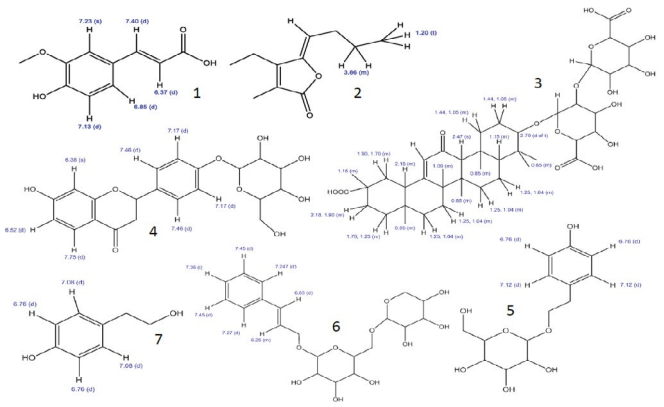

Figure 1: Chemical structures of ferulic acid (1) and *Z*-ligustilide (2) present in *A. sinensis*, glycyrrhizin (3) and liquiritin (4) present in *G. uralensis*, salidroside (5), rosavin (6) and tyrosol (7) present in *R. rosea* with ^{1}H NMR shifts of interest shown.

Botanical name	Traditional name	Analyte and molecular weight	Main bioactivity	Herb MaRS ranking
Angelica sinensis	Dang Gui	(1) Ferulic acid, 194	Anti-inflammatory, uterosedative	4
		(2) Z-ligustilide, 190	Suppressive and modulatory effect on nervous system	4
Glycyrrhiza uralensis	Gan Cao	(3) Glycyrrhizin, 822	Anti-ulcer, Anti-inflammatory	4
		(4) Liquiritin, 418	Anti-toxic	4
Rhodiola rosea	Hong Jing Tian	(5) Salidroside, 300	Anti-depressant, anti-inflammatory	4
		(6) Rosavin, 428	Anti-depressant, anti-inflammatory	4
		(7) Tyrosol, 138	Anti-ischemic	4

Table 1: Target analyte activities and ranking in accordance with the Herbal MaRS system, with a ranking of '0' being the least ideal and '5' being the most ideal for analysis.

(Bruker, MA, USA) was used for the ^{1}H NMR metabolomics. The FLUOstar Optima (BMG Labtech, Ortenburg, Germany) fluorescence plate reader was used in the anti-inflammatory testing.

Reagents and materials

Raw herb samples were purchased from different complementary medicine retailers. All of the Dang Gui samples were product labelled as belonging to the *A. sinensis* variety, while all Gan Cao and Hong Jing Tian samples were labelled as *G. uralensis* and *R. rosea* respectively. The commercial herbal samples were purchased from various retailers in Sydney (NSW, Australia). The samples as sliced root or an unsliced root and were assigned as DG-I to DG-XI for *A. sinensis*, GC-I to GC-VII for *G. uralensis* and HJT-I to HJT-VII for *R. rosea*. Ferulic acid (98.7%) and Z-ligustilide (98.5%), the analytical standards for *A. sinensis* were purchased from Sigma-Aldrich (CA, USA) and Chengdu Biopurify Chemicals (Sichuan, China) respectively. Glycyrrhizin (98.1%) and liquiritin (99.2%) for *G. uralensis* were purchased from Sigma-Aldrich (CA, USA) and Chengdu Biopurify Chemicals (Sichuan, China) respectively. Tyrosol (98.4%) was purchased from Sigma-Aldrich (CA, USA) with salidroside (99.3%) and rosavin (99.1%) being purchased from Chengdu Biopurify Chemicals (Sichuan, China) for *R. rosea*.

The LC grade methanol used for extraction was purchased from Fisher Scientific (VIC, Australia). LC grade acetonitrile mobile phase was obtained from Ajax Finechem (NSW, Australia). The analytical reagent grade formic acid was obtained from Univar (NSW, Australia). For UPLC-MS, the nitrogen nebulising and argon collision gas used for multiple reaction monitoring (MRM) were purchased from Coregas (NSW, Australia). The purified water used for herbal extraction procedure and LC mobile phase was obtained using a MilliQ ultra high purity water system supplied by ELGA Lab Water (NSW, Australia). Methanol-d4 and deuterated water (D_2O) used in ^{1}H NMR metabolomics were obtained from Cambridge Isotope Laboratories (MA, USA).

Preparation of the raw herb and standard solutions for analysis

The samples (100 g) were ground to pass through a 212 µm sieve to ensure sample homogeneity. They were then placed in a desiccator with P_2O_5 and dried for one week before use. The analytical standards were weighed using a micro analytical balance and the solutions stored in the refrigerator at 4°C. The stock calibration solutions are discarded after 72 h for *A. sinensis*, 96 h for *G. uralensis* and 120 h for *R. rosea* samples as these times were the analyte stability method validation parameter for which as the analyte peak area decreased by ≥ 2%. Initial decreases

in peak area may be caused due to the analyte coating the glass vials used for analysis, though the peak area of the selected analytes does eventually degrade ≥ 2% over time.

For *A. sinensis*, a mixed stock calibration solution containing 30.8 µg/mL of ferulic acid and 547.8 µg/mL of Z-ligustilide was prepared by accurately weighing 0.3082 mg and 5.4801 mg of ferulic acid and Z-ligustilide respectively into a 10 mL volumetric flask and made up to volume with 100% methanol. The working standard solutions contained (1.93, 3.85, 7.71, 15.41 and 30.82 µg/mL) of ferulic acid and (34.24, 68.48, 136.95, 273.90 and 547.80 µg/mL) of Z-ligustilide.

For *G. uralensis*, a mixed stock calibration containing 1912.4 µg/mL of glycyrrhizin and 752.2 µg/mL of Liquiritin was prepared by accurately weighing 19.1240 mg and 7.5221 mg of glycyrrhizin and liquiritin respectively into a 10 mL volumetric flask and made up to volume with 50% aqueous methanol. The working standard solutions contained (95.62, 191.24, 382.48, 478.10 and 956.20 µg/mL) of glycyrrhizin and (37.61, 75.22, 150.44, 188.05 and 376.10 µg/mL) of liquiritin.

For *R. rosea*, a mixed stock calibration containing 1051.9 µg/mL of salidroside, 1175.9 µg/mL of rosavin and 321.4 µg/mL of tyrosol was prepared by accurately weighing 10.5189 mg, 11.7592 mg and 3.2144 mg of salidroside, rosavin and tyrosol respectively into a 10 mL volumetric flask and made up to volume with 100% methanol. The working standard solutions contained (13.20, 26.30, 52.60, 105.19 and 210.38 µg/mL) of salidroside, (14.70, 29.40, 58.80, 117.59 and 235.18 µg/mL) of rosavin and (4.02, 8.03, 16.07, 32.14 and 64.28 µg/mL) of tyrosol.

Extraction and recovery of analytes from the raw herb

The concentration of the analytes in the unspiked samples were determined by weighing 100 mg (95.0 – 105 mg) of the ground sample into a 10 mL volumetric flask and made up to ~95 mL with 100% methanol for *A. sinensis* and *R. rosea*. For *G. uralensis*, 50% aqueous methanol was used as the extraction solvent. The flask was then sonicated for 2 x 30 min and cooled in-between sonications for 5 min inside a refrigerator. The flask was then made up to volume with the extraction solvent and mixed by multiple inversions. The solution was then filtered through a 0.22 µm PVDF filter into a 2 mL glass vial for LC analysis.

To evaluate extraction efficiency, known quantities of the target analytes were spiked into the herbal sample and analysed. The mixed spiking solution was prepared by accurately weighing 1.54 mg of ferulic acid and 27.39 mg of Z-ligustilide for *A. sinensis*, 76.48 mg of glycyrrhizin and 30.08 mg of liquiritin for *G. uralensis* and 10.46 mg of salidroside, 11.84 mg of rosavin and 3.16 mg of tyrosol for *R. rosea* each into a 10 mL volumetric flask. The spiking solution was then made up to volume using the respective extraction solvent. For the 50%, 100% and 200% spiking levels, 125, 250 and 500 µL of the spiking solution was added to accurately weighed 100 mg of each sample. The spiking solvent was then allowed to evaporate overnight. The spiked recovery study was carried out with seven replicates per spiking level. The unspiked samples were also analysed with seven replicates. Each sample was injected in triplicate.

UPLC-PDA and ESI-MS/MS conditions

The gradient mobile phase composition for each herb is shown in Table 2. Column temperature was set at 30 °C and the flow rate at 0.2 mL/min. The injection volume was 1.0 µL. The *A. sinensis* run was 18 min with a 4 min wash and equilibration. The *G. uralensis* run was 7 min with a 1 min wash and equilibration. The *R. rosea* run 16 min with a 9 min wash and equilibration. PDA detection and quantitation was carried out at 325 nm for *A. sinensis*, 254 nm for *G. uralensis* and 268 nm for *R. rosea*. The PDA was set to acquire between 190-450 nm and the peak purity checked by comparing the UV spectrum of the standard and sample peaks. The product ions used for ESI-MS/MS analysis in each case was determined by direct infusion of the pure standard prepared in the extraction solvent into the MS. The product ions were chosen based on the area curve of each product ion shown by the collision cell breakdown display of the operating software. The MS conditions were selected to produce at least two product ions from a precursor ion for identity confirmation as per the guidelines set by the European Commission Directorate for Agriculture (2002) [14]. The standard solution was directly infused directly into the MS using a motor driven syringe at a rate of 10 µL/min. Argon gas was used for multiple reactions monitoring (MRM). The ESI-MS/MS monitoring conditions for each analyte are shown in Table 3.

A. sinensis			*G. uralensis*			*R. rosea*		
Time (min)	% (A)	% (B)	Time (min)	% (A)	% (B)	Time (min)	% (A)	% (B)
Start	80.0	20.0	Start	80.0	20.0	Start	90.0	10.0
7.00	60.0	40.0	1.00	70.0	30.0	16.00	70.0	30.0
14.00	5.0	95.0	2.00	55.0	45.0	17.50	50.0	50.0
18.00	5.0	95.0	3.50	55.0	45.0	19.00	20.0	80.0
18.50	80.0	20.0	5.00	20.0	80.0	22.00	20.0	80.0
22.00	80.0	20.0	7.00	20.0	80.0	22.50	90.0	10.0
			8.00	80.0	20.0	25.00	90.0	10.0

Table 2: Gradient program for the LC analysis of the extracts of *A. sinensis*, *G. Uralensis* and *R. Rosea*. A=0.1% aqueous formic acid, B=0.1% formic acid in acetonitrile and the flow rate=0.2 mL/min.

NMR metabolomics

^{1}H NMR metabolomics was performed specifically for PCA analysis comparison. Around 200 mg ± 10 mg of herbal sample was weighed into a 2 mL Eppendorf tube. For *A. sinensis* and *R. rosea* 1 mL of 100% methanol-d_4 was added, while 50% aqueous D_2O methanol-d_4 with a phosphate buffer (pH 6) was added for *G. uralensis*. The samples were then extracted by sonication for 15 min and vortexed for 1 min followed by centrifugation at 12131 × *g* (14000 rpm) for 10 min. The supernatant (800 µL) was placed into 5 mm NMR tubes for metabolomics analysis. The pure standards of each target analyte was analysed to ascertain the ^{1}H NMR regions most suited for PCA analysis and their relevant shifts are shown in Figure 1**.** The samples were analysed at 25°C. The parameters used are as follows, standard proton pulse, 60° flip angle, 1.5 s relaxation delay, 16000 complex points, 4 dummy scans, 64 scans, 64 K zero filling and 0.3 Hz line broadening. The NMR spectrometer was auto-tuned and shimmed before the acquisition of each sample. The data was acquired in triplicate for each sample and was then processed to yield a ^{1}H NMR spectral profile for each herbal extract.

PCA analysis

The qualitative chemical variability of the herbal samples was studied using the PCA data generated from the UPLC-PDA chromatograms and ^{1}H spectra of each sample. The raw data from the chromatograms and spectra were converted into a comma-separated value (CSV) file. The data files were then loaded into a folder and accessed using the R (v.2.14.2) 'chemometrics' package written by Varmuza and Filzmoser [15]. The graphics wrapper for the displays was provided by the 'ChemoSpec' package written by Hanson [16].

Analyte	ESI mode	Cone voltage (V)	Parent ion *m/z*	Collision energy (eV)	*m/z* Pure standard (%)	*m/z* Sample (%)	Relative difference (%)
Ferulic acid	(-)	38	$[M-H]^-$ 193	4	166 (39.5)	166 (37.5)	5.2
				10	81 (60.4)	81 (62.2)	2.9
***Z*-ligustilide**	(+)	28	$[M+H]^+$ 191	24	117 (50.0)	117 (46.2)	7.9
				20	91 (50.0)	91 (54.5)	8.6
Glycyrrhizin	(+)	26	$[M+H]^+$ 823	14	647 (16.2)	647 (15.9)	1.9
				26	453 (83.7)	453 (84.0)	0.4
Liquiritin	(-)	26	$[M-H]^-$ 417	20	255 (70.5)	255 (71.9)	2.0
				32	135 (29.3)	135 (28.7)	2.1
Salidroside	(-)	24	$[M-H]^-$ 299	14	119 (46.9)	119 (47.5)	1.3
				14	89 (53.1)	89 (52.8)	0.6
Rosavin	(-)	26	$[M-H]^-$ 427	8	293 (59.6)	293 (61.2)	2.6
				22	89 (40.6)	89 (40.1)	1.2
Tyrosol	(-)	30	$[M-H]^-$ 137	12	119 (41.1)	119 (44.7)	8.4
				14	106 (55.0)	106 (53.7)	4.2

Table 3: ESI-MS/MS monitoring conditions of the target analytes, the relative intensities of the product ions and the relative intensity difference obtained for the standard and sample. EU acceptance criteria are ≤ 10% difference in intensity between standard and sample.

An R. data file containing all constituent samples for each herb was generated. The axes for the UPLC-PDA chromatograms were absorbance (y-axis) and time (x-axis). Similarly, the axes for the ^{1}H NMR spectra were normalized intensity (y-axis) and chemical shift (x-axis). A stack plot was created in order to inspect regions of interest. The baseline of the data set was statistically corrected to remove noise. The data was then normalized to negate small changes in concentration due to sample preparation. The samples were also binned in order to correct narrow peaks that may have shifting retention times. Regions of no interest had to be removed from the dataset before PCA could commence.

This process was fairly straightforward for the UPLC-PDA data, where all regions other than those containing the target peaks were removed. The initial 3 min of the chromatograms containing the solvent and unquantified peaks were removed. The peaks in the wash and equilibration phase were also removed. For the ^{1}H NMR data, the sugar region between 3.0-4.0 ppm was removed as it was not relevant to this study. The region between 4.0-6.0 ppm was also removed since it contained the large methanol-d4 solvent peak. The main regions of PCA relevance was the phenolic region between 6.0-9.0 ppm and the 0.5-3.0 ppm alkyl region.

The classical PCA option that explained the greatest amount of variance for the data set was selected. The Pareto scaling option was chosen for both UPLC-PDA and ^{1}H NMR as it was a compromise between 'no scaling' which heavily weights the largest peaks and 'autoscaling' which weights all the peaks equally. PCA was performed on each data set and the chromatographic PCA and the spectral PCA score plots were compared. The cumulative PC scores (PC1+PC2) range from 66% to 94% for the plots demonstrating that most of the variability is explained by PC1 and PC2 in a 2-dimensional plot.

Anti-inflammatory NO assay

The extract of each herb was dried and stored at -20°C before *in vitro* anti-inflammatory testing. NO release was quantified by the Griess reagent. Briefly the RAW 264.7 macrophages were seeded at 1 × 10^5 cells/well in a 96 well plate for 48 h. The compounds and herbal extracts were added in DMSO (final concentration 0.1% DMSO) 1 h before stimulation with LPS and IFN-γ (50 ng/mL, 50 units/mL). After 18 h the supernatant was removed (180 µL) and reacted with Griess reagent (100 µL) to colorimetrically quantify dissolved nitrates. A MTT solution (60 µL) was used to assess the viability of the remaining cells. The readings were obtained from the plate reader and processed using Graph Pad Prism. A six point (n=9) dose response curve was constructed for each extract and compound tested. The obtained IC_{50} values with 95% confidence intervals are shown in Table 4.

Results and Discussion

Summary of results

The developed analytical method using UPLC-PDA is simple, rapid and precise. A C-18 column is used. The run times range from 7 min to 18 min. This is significantly faster than recent methods that have been reported in recent literature [17-19]. Novel analyte identity confirmation is achieved using tandem ESI-MS/MS and novel structures for breakdown product ions are proposed to be used in bio-active analyte confirmation in the future. The quantitative chemical variability is analysed by comparing the fold-variation in the concentrations of target bio-active analytes. The qualitative chemical variability is studied using PCA. The PCA score plots of the UPLC-PDA chromatograms and the ^{1}H NMR spectra of each corresponding herb are directly compared to look for possible adulteration in samples. Anti-inflammatory activities in the herbal samples were determined using the Griess reagent method. The dose response curve for *A. sinensis* and ferulic acid ranged from 300 to 3.7 µg/mL. The dose response curve for *G. uralensis extracts* and glycyrrhizin ranged from 200 to 0.4 µg/mL. The dose response curve for *R. rosea* and salidroside ranged from 400 to 12.5 µg/mL. Combining information from all these analytical techniques provided extensive information on the phytochemical variability of commercially available medicinal samples of *A. sinensis*, *G. uralensis* and *R. rosea* and exposed the need for better quality control (QC) of these medicinal herbs being sold in the commercial market.

Chromatographic data

The chromatograms are obtained for each sample using the wavelength which gave the maximum absorbance for analyte of interest. Figure 2 shows representative UPLC-PDA chromatograms for the *A. sinensis* sample DG-IV at 325 nm, the *G. uralensis* sample GC-VII at 254 nm and the *R. rosea* sample HJT-I at 268 nm on which the method validations were performed.

Method validation parameters

The recoveries and method validation parameters of the analytes in each herb are shown in Table 5. All the analytes show very good recoveries ranging from 92% to 101%, which were calculated from seven replicates. The %RSD for the recoveries was ≤ 10% which is good for the concentration levels as described by the Horwitz horn [20]. The low values for the limit of detection (LOD) calculated as three times the standard deviation (SD) and limit of quantitation (LOQ) calculated as ten times the SD demonstrate that the analytes are quantified with great confidence using the proposed analytical method as the found concentrations are much higher than the LOD and LOQ. The calibration

***A. sinensis* extracts**												**Analyte**
	DG-I	***DG-II***	***DG-III***	***DG-IV***	***DG-V***	***DG-VI***	***DG-VII***	***DG-VIII***	***DG-IX***	***DG-X***	***DG-XI***	Ferulic acid
NO IC_{50} (μg/mL, n=9)	43	108	118	86	26	86	53	44	45	62	66	128
95% confidence interval at IC50	28 to 65	88 to 132	93 to 151	70 to 107	20 to 32	66 to 112	40 to 71	34 to 58	38 to 53	49 to 78	49 to 90	71 to 232
***G. uralensis* extracts**												**Analyte**
	GC-I	***GC-II***	***GC-III***	***GC-IV***	***GC-V***	***GC-VI***	***GC-VII***					Glycyrrhizin
NO IC_{50} (μg/mL, n=9)	80	152	31	130	39	147	107					N.D.
95% confidence interval at IC50	71 to 91	131 to 175	28 to 35	116 to 146	36 to 43	128 to 169	93 to 123					N.D.
***R. rosea* extracts**												**Analyte**
	HJT-I	***HJT-II***	***HJT-III***	***HJT-IV***	***HJT-V***	***HJT-VI***	***HJT-VII***					Salidroside
NO IC_{50} (μg/mL, n=9)	109	88	102	78	116	141	73					50
95% confidence interval at IC50	86 to 139	80 to 97	85 to 123	64 to 95	94 to 142	117 to 169	65 to 82					44 to 57

Table 4: Griess assay (RAW264.7) results. N.D. (not detected) indicates that the sample had no nitric oxide (NO) scavenging inhibition.

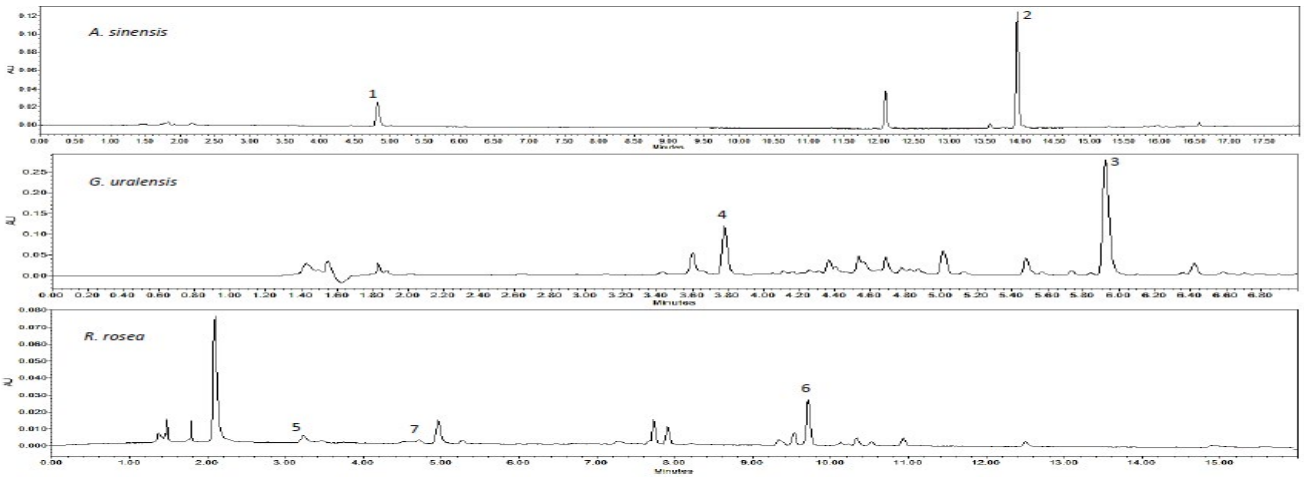

Figure 2: Representative HPLC-PDA chromatographic profiles of *A. sinensis* sample DG-IV at 325nm, *G. uralensis* sample GC-VII at 254 nm and *R. rosea* sample HJT-I at 268 nm with target analytes (1-7) from Table 1 are shown.

Analyte	Retention Time (min)	Average Recovery (%RSD)	LOD (mg/g)	LOQ (mg/g)	Linearity (R^2)	Retention time precision (%)	Peak area precision (%)	Standard stability (h)
Ferulic acid	4.18	101% ± 9%	0.01	0.04	0.9995	0.08	0.63	72
Z-ligustilide	13.98	92% ± 9%	0.11	0.37	0.9994	0.12	0.49	72
Glycyrrhizin	5.93	97% ± 8%	0.75	2.49	0.9995	0.13	0.86	96
Liquiritin	3.80	96% ± 7%	0.45	1.49	0.9993	0.15	0.65	96
Salidroside	3.28	97% ± 9%	0.13	0.41	0.9994	0.10	0.43	120
Rosavin	9.76	96% ± 8%	0.11	0.36	0.9993	0.08	0.46	120
Tyrosol	4.63	96% ± 9%	0.03	0.11	0.9998	0.21	0.62	120

Table 5: The target analyte recoveries and method validation parameters.

curves obtained for the analytes show excellent linearity with R^2 values ≥ 0.999. The peak area and retention time precisions for each analyte are ≤ 1%. The sample extract solution stability was set as the time when the analyte peak decreases by ≥ 2% under specified storage conditions. The *A. sinensis*, *G. uralensis R. rosea* samples were stable for 72 h, 96 h and 120 h respectively when stored at 4°C in a clear glass vial.

Analyte identity confirmation

The UV spectrum of each constituent analyte in both the raw herb and the pure standard in *A. sinensis* (325 nm), *G. uralensis* (254 nm) and *R. rosea* (268 nm) were compared and close overlaps were observed, suggesting good peak purity and providing tentative identity confirmation. Stronger identity confirmation is achieved by monitoring the appropriate *m/z* product ions for the analyte in the standard and sample extract solutions and comparing their relative intensities. The results of this comparison are shown in Table 3. The relative *m/z* intensity differences between the sample and standard for all the fragments are ≤ 10%, so the observed intensity differences are small and well within the guidelines for identity confirmation using MS/MS [14]. The novel proposed structures for each ESI-MS/MS *m/z* product ion are shown in Figure 3. It should be noted that the *m/z* 89 product ion structures are the same for both salidroside and rosavin.

Variability in commercial sources of *A. sinensis, G. uralensis* and *R. rosea*

While fold-variation is useful to determine bio-active compound variability, a larger picture of phytochemical variability is obtained when fold-variation is used in conjunction with PCA, which is a powerful

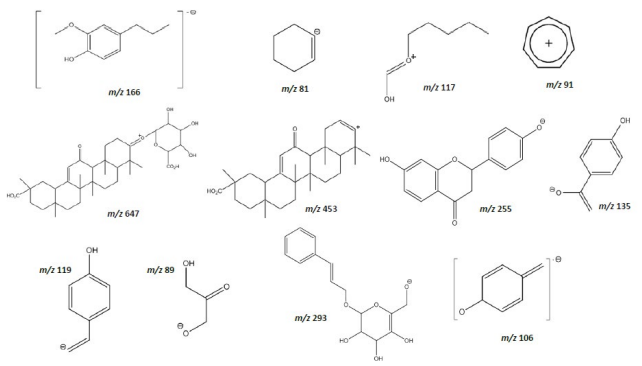

Figure 3: Proposed structures for each *m/z* product ion fragment obtained using ESI-MS/MS.

statistical technique. PCA of UPLC-PDA chromatograms can correlate the variability of multiple target analytes at a specific wavelength where peak areas approximately match up with concentrations of the analytes determined through quantification. PCA of ^{1}H NMR metabolomics is more spectrally rich and therefore more useful to determine the qualitative variability of herbal samples. By comparing the PCA score plots of each technique we can determine whether the UPLC-PDA chromatographic profile correlates with the ^{1}H NMR plot and expose the need for routine chemometrics in future QC. The concentrations of the target analytes of all three herbs in the purchased commercial samples examined are shown in Table 6.

In the *A. sinensis* samples, the concentration fold variation is 3.36 for ferulic acid and 9.39 for Z-ligustilide. Of the three herbs, *A. sinensis* demonstrated the highest quantitative variability of the target bio-actives. The UPLC-PDA PCA plot is not spectrally rich except for the target bio-actives and is not as useful as the spectrally rich ^{1}H NMR PCA plot which shows closer grouping. The PCA groupings are shown in Figure 4. All the samples are shown to be *A. sinensis* as commercially labelled, with sample DG-VIII being a possible outlier. In general, commercially available *A. sinensis* shows poor chemical standardisation, with low concentrations of ferulic acid and great variability of Z-ligustilide across the samples. The qualitative standardisation on the other hand is quite good and the close grouping in the ^{1}H NMR PCA plot demonstrates this. Besides natural biological variation, factors contributing to chemical variability include growth conditions (soil type, climate), age of plant, time of harvest and post-harvest treatment.

In the *G. uralensis* samples, the fold-variation is 1.38 for Glycyrrhizin and 2.30 for liquiritin and the bio-active concentrations are similar to what is reported in literature [21]. *G. uralensis* shows the least chemical variability in concentration of the target bio-actives. The PCA groupings are shown in Figure 5. The UPLC-PDA PCA plot shows that all the samples are grouped very closely. The comparative ^{1}H NMR PCA plot shows a similar close grouping providing proof that the samples are of the *G. uralensis* variety as commercially labelled with only GC-I being a possible outlier. The samples of this herb show high consistency in the concentrations of the target bio-actives across retail distributors which are in stark contrast to the commercial *A. sinensis* samples which show high chemical variability.

In the *R. rosea* samples, the fold-variation is 2.71 for salidroside and 11.7 for tyrosol. Rosavin of the rosavin family of compounds is only present in HJT-I and HJT-V despite being a unique marker compound for *R. rosea* [22]. The *R. rosea* samples show great phytochemical variability in both analyte concentration and possible species identity. The PCA groupings are shown in Figure 6. The UPLC-PDA PCA plot demonstrates little to no grouping among commercial samples due to the presence of three different chromatographic profiles at 268 nm. Three distinct colour coded groups were assigned to distinguish these unique profiles. The ^{1}H NMR PCA plot corroborates this assignment, again indicating the presence of three distinct *Rhodiola* sub-species being marketed as commercial *R. rosea*. HJT-I and HJT-V were identified as *R. rosea* since rosavin of the rosavin family of compounds is a characteristic chemical marker for this sub-species. HJT-III, IV, VI, VII can be identified as *R. kirilowii* from reported literature concentrations for the target bio-actives [23]. HJT-II was an unidentified *Rhodiola* sub-species. The commercial *Rhodiola* samples show very high variability with three different sub-species of the same herb, each with different chemical profiles being sold under the same commercial label. Though the target bio-actives are found in reasonable concentration when they are present, their proportions and presence varies and therefore the efficacy of the commercial product when purchased over-the-counter may be uncertain.

It can be observed in Table 4 that all the extracts tested showed mild NO inhibition. *A. sinensis* was the most potent of the extracts tested with an average IC_{50} of 67 μg/mL and extract DG-V was the most potent with an IC_{50} of 26 μg/mL. Ferulic acid was identified from the Herb MaRS ranking as the most likely anti-inflammatory compound. It however was less active (IC_{50} 128 μg/mL) than the crude extracts which suggest that it is not the most important anti-inflammatory compound in the extract. This is further supported by the lack of correlation (r^2=0.144) between the extracts IC_{50} and ferulic acid concentration.

The G. uralensis extract had a mild NO inhibtion effect with average IC_{50} of 98 μg/mL and extract GC-III showing the most activity with and IC_{50} of 31 μg/mL. Glycyrrhizin was identified as the most likely active compound, however showed no significant inhibition up to 200 μg/mL in the NO assay. Glycyrrhizin concentration and *G. uralensis* extract IC_{50} showed no correlation (r^2=0.0000). Glycyrrhizin is not the compound responsible for *G. uralensis* NO inhibition.

The *R. rosea* extract had the lowest potency in terms of NO inhibition. The average IC_{50} was 101 μg/ml and the most potent sample was sample HJT-II with and IC_{50} of 73 μg/mL. The selected marker compound, salidroside, showed greater activity than the extracts with an IC_{50} of 50 μg/mL. Despite the promising activity it is unlikely that salidroside is the main component that is responsible for the NO inhibition based on the average quantity of salidroside in the extract 4.25 mg/g calculated form Table 6. At the average IC_{50} of the extract (73 μg/mL) the concentration of the salidroside is calculated to be 0.31 μg/mL. This is significantly below the IC_{50} valμe of salidroside (50 μg/mL). This is further supported by the lack of correlation (r^2=0.0261) between the extracts IC_{50} and salidroside concentration.

The NO inhibition results suggest that despite the reported activity in the literature and Herb MaRS ranking the marker compounds ferulic acid for *A. sinensis,* glycyrrhizin for *G. uralensis* and salidroside for *R. rosea* were not correlated with the extracts activity. It is likely that besides the compounds, the anti-inflammatory activity may also be due to other components that have not been quantified such as polysaccharides or there may be synergy occurring between phytochemicals present in the extract. It is also possible that as NO is only one measure of anti-inflammatory activity the markers may be acting by a different anti-inflammatory mechanism not detected in this assay. For example if the target compounds act directly on the COX enzyme like common NSAIDs this would not be reflected in the current assay. Therefore the pharmacological QC for these commercial products cannot be performed using these two corresponding bio-actives for each herb

Analyte in (mg/g)	Commercial Sample										
	DG-I	***DG-II***	***DG-III***	***DG-IV***	***DG-V***	***DG-VI***	***DG-VII***	***DG-VIII***	***DG-IX***	***DG-X***	***DG-XI***
Ferulic acid	0.25	0.21	0.15	0.39	0.48	0.49	0.46	0.24	0.32	0.23	0.28
***Z*-ligustilide**	2.98	10.40	1.27	7.24	11.93	7.11	7.16	2.82	4.52	11.72	2.96
(mg/g)	***GC-I***		***GC-II***	***GC-III***	***GC-IV***		***GC-V***	***GC-VI***		***GC-VII***	
Glycyrrhizin	40.98		35.03	41.27	41.26		31.60	38.45		30.01	
Liquiritin	12.14		10.09	17.43	10.03		7.67	10.06		10.40	
(mg/g)	***HJT-I***		***HJT-II***	***HJT-III***	***HJT-IV***		***HJT-V***	***HJT-VI***		***HJT-VII***	
Salidroside	2.09		4.74	5.28	4.25		2.13	5.66		5.59	
Rosavin	2.62		N.D.	N.D.	N.D.		2.76	N.D.		N.D.	
Tyrosol	0.57		2.26	5.37	5.20		0.55	5.98		6.44	

Table 6: The present concentrations in mg/g of each target analyte in all commercially obtained samples of the three herbs. N.D. (not detected) indicates that the analyte was not detected in the sample.

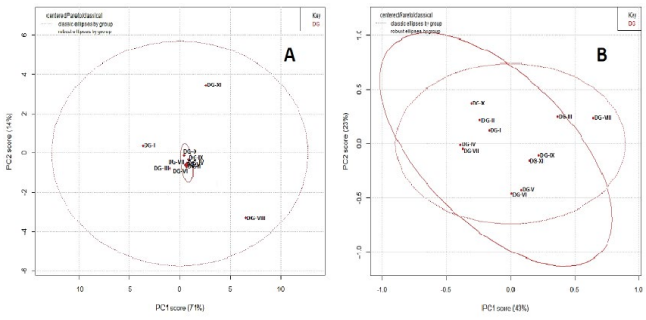

Figure 4: The PCA score plots for A. sinensis samples using LC-PDA (A) and 1H NMR (B).

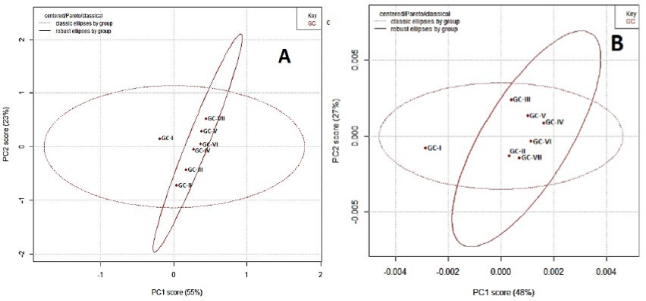

Figure 5: The PCA score plots for *G. uralensis* samples using LC-PDA (A) and ^{1}H NMR (B).

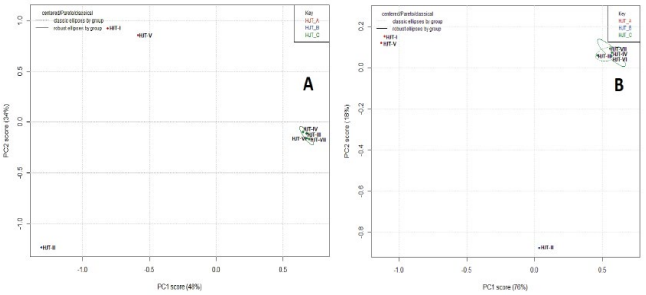

Figure 6: The PCA score plots for *R. rosea* samples using LC-PDA (A) and ^{1}H NMR (B).

alone. It can be concluded that specific assays are needed for QC before specific claims can be made on a labelled commercial product.

Conclusions

Three rapid, simple and precise UPLC-PDA methods were developed for the quantitation of major bio-actives in *A. sinensis*, *G. Uralensis* and *R. Rosea*. These methods used a common C-18 column and ranged from 7 to 18 min which represents a significant reduction in run time compared to methods reported in literature. The major bio-actives were identified using ESI-MS/MS and new novel fragmentations were achieved for identity confirmation. The three method validations are comprehensive, detailed and were used to study the quantitative chemical variability of commercial herbal extracts. The PCA analysis performed on ^{1}H NMR spectra and UPLC-PDA chromatograms provided information on the qualitative chemical variability of the samples. The *in vitro* anti-inflammatory tests were a useful additional tool to demonstrate the need for better pharmacological QC standardisation of commercial herbal products. The combined techniques used in this study demonstrate how to ensure the efficacy of the selected commercial herbal products in the future.

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Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication.

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