Quantification by LC–MS/MS of astragaloside IV and isoflavones in Astragali radix can be more accurate by using standard addition

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Abstract
Introduction: Astragali radix (AR), the root of Astragalus, is an important medical herb widely used in traditional Chinese medicine. Bioactive components include isoflavones and a unique class of triterpenoid saponins (named astragalosides).

Objectives: Accurate measurement of bioactive components, especially astragaloside IV, is necessary for confirming AR authenticity, quality control and future medical research.

Methodology: Liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) is a suitable technique but suffers from ion suppression effects due to sample matrix. This can be corrected by using isotopic labelled internal standards, but these are not available for many phytochemicals. We explored the use of standard addition to circumvent this issue.

Results: LC–MS/MS and liquid chromatography coupled with ultraviolet (LC-UV) detection provided linear calibration curves ($R^2 > 0.99$). LC–MS/MS provided superior selectivity and detection limits below 10 ng/mL, which was 2–3 magnitudes lower than LC-UV detection. Precision and accuracy were overall improved by using LC–MS/MS with diluted sample extracts, resulting in an inter series coefficient of variation (CV) of 12% or less and mean recovery estimates in the 85–115% range. LC–MS/MS quantification by standard addition resulted in significantly higher concentrations of astragaloside IV measured in the samples. Concentrations calculated by standard addition were unaffected by large variation in signal response caused by matrix effects, independent of variation in slope of the standard addition curves.

Conclusion: Sample dilution was helpful but not sufficient for reducing effects of ion suppression. We have shown that LC–MS/MS quantification by standard addition can be a powerful approach for accurate measurement of phytochemicals in the absence of isotopic labelled internal standards.

Keywords
astragaloside, Astragalus, isoflavone, LC–MS/MS, standard addition

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Astragalus mongholicus Bunge (English: Mongolian milkvetch, Chinese: huáng qí) and Astragalus propinquus Schischkin are important medicinal plants in traditional Chinese medicine (TCM). The root of these plants is known as Astragali radix (AR) and is often clinically applied as a medical herb in TCM and Western phytomedicine. AR preparations are classified according to TCM, as tonifiers of vital energy (Qi). AR is the second strongest TCM medicine in this category, after the much more expensive and rare Ginseng. Meta-studies and reviews of randomised controlled trials (RCTs) have claimed positive effects of AR in breast cancer, acute myocardial infarction, diabetic nephropathy and many more diseases.

AR is known to be a rich source of astragalosides (AG) which are triterpenoid saponins unique to the Astragalus species. There are at least seven AG components present in different plant tissues, with astragaloside IV (AG-IV) being the most well-known because of its high bioactivity. This component is a 9,19-cycloartane type major active triterpene glycoside, which has been reported to increase T and B lymphocyte proliferation. Other effects of AG-IV include cardioprotective, neuroprotective, immune stimulating and anti-inflammatory properties. Upon ingestion, AG-IV is metabolised to cycloastragenol (CAG), which is its corresponding aglycon (glycoside-free) sapogenin. CAG extend T-cell proliferation by increasing the telomerase activity, a vital process to delay cellular ageing. CAG has been marketed for human applications by T.A. Sciences (New York, NY, USA) as a dietary supplement for anti-ageing under the brand name TA-65. Herbal medicinal products, including TCM herbs, are regulated in the European Union (EU) by the EU medicine directive 2001/83/EC amended by the 2004/24/EC directive. TCM products need approval by the European Medicines Agency (EMA). Both for quality control and to correlate pharmaceutical effects with consumption of TCM herbs, it is essential to know their chemical composition, accurately measure concentrations of their active components and study the pharmacokinetics in humans. Most pharmacopeias state that AG-IV should be above 0.04%. The European pharmacopeia suggest using an ammonia solution during sample preparation for increasing the level of AG-IV in the extract. This occurs by hydrolysis of several other AGs into AG-IV. However, by adding ammonia we might not measure the true concentration of AG-IV naturally present in samples intended for intake. Consequently, it is questionable to perform pharmacokinetic studies based upon ammonia-based results.

Other components in AR include isoflavones such as calycosin, ononin and formononetin (Figure 1). Their effects include boosting of the immune system, as well as having impact on glucose homeostasis and lipid metabolism. Isoflavone concentrations in AR can be assessed by liquid chromatography coupled with ultraviolet (LC-UV) detection. However, AG-IV and CAG do not contain chromophores, and UV detection is therefore not possible. Instead, their quantification can be done by LC coupled with evaporative light scattering detector (ELSD) or with mass spectrometry (MS) detection. For quality control of AR, it was recently shown that LC–MS provides a more sensitive and convenient quantification of AG-IV than with LC-MS/MS performed with atmospheric pressure chemical ionisation (APCI) can quantify AGs in the negative mode it is more common to increase sensitivity by applying electrospray ionisation (ESI) in the positive
mode. However, ESI is known to suffer from ion suppression due to various effects of the sample matrix. This is typically seen as a variation in the signal response caused by sample composition, making accurate quantification more difficult. Isotope labelled internal standards are applied to correct for ion suppression in the LC–MS/MS analysis, but no such internal standards exist for AG-IV and CAG.

Most studies on the analysis of AR samples have used UV detectors, ELSDs and some have used MS detectors. Reports indicate extensive use of MS/MS detectors for AR samples and even for bioavailability of compounds during animal studies. None of these have utilised standard addition to compensate for ion suppression effects in the LC–MS/MS analysis of AR, although standard addition was successfully reported for quantification of AGs by LC–ELSD.468–471 In the absence of an isotope labelled internal standard, we explored the use of standard addition to improve the accuracy of quantitative assessment of bioactive components extracted from EU-approved AR. By compensating for various matrix effects of the samples, standard addition is expected to provide concentration measurements that are closer to the true levels in samples.

2 EXPERIMENTAL

2.1 Chemicals and reagents

Chemical standards, AG-IV (98%, Lot no: PRF90922502), ononin (98%, Lot no: PRF9060501) and calycosin 7-O-β-D-glucoside (98%, Lot no: PRF8071905) were purchased from Chengdu Biopurify Phytochemicals Limited (Sichuan, China). Formononetin (≥ 98%, Lot no: BCBZ9069) and cycloastragenol (≥ 98%, Lot no: SLM2014V) were obtained from Sigma-Aldrich (St Louis, MO, USA). Acetonitrile, methanol and formic acid were of high-performance liquid chromatography grade (HPLC)-LC MS grades and were obtained from VWR International (Radnor, PA, USA). All other chemicals used were analytical grades. Purified water was obtained from Elga LabWater (High Wycombe, United Kingdom). All the standards were diluted to different concentrations for HPLC-UV detection as follows: 2.5, 5, 10 and 20 μg/mL. Similarly, for LC–MS/MS the stock solution with mixture of all components with final concentration of 0.1 mg/mL in methanol was prepared and the standard dilutions for all components were: 0.1565, 0.3125, 0.625, 1.25, 2.5, 5, 10 and 20 μg/mL. For standard addition, diluted samples were spiked to concentrations of 0, 0.5, 1 and 2 μg/mL.

2.2 Sample extracts

The analysed AR samples were dried herb, granulate or hydrophilic concentrate of Astragalus mongholicus Bunge, all purchased from Naturapotheek (Pijnacker, the Netherlands). Sample extracts (Table 1) were prepared from solid samples (5 g) either by boiling in water for 60 min or by ultrasonication for 60 min in 70% methanol at 40 °C. Sample extracts were centrifuged at 4000 rpm for 10 mins twice to remove impurities (Eppendorf Centrifuge 5702), followed by drying in a rotary evaporator (IKA HB 10, VWR International), and reconstitution in methanol to a final volume of 20 mL. The hydrophilic concentrate liquid was analysed directly without extraction.

2.3 Standard solutions

Pure analytical standard chemicals were weighed and dissolved in methanol to a concentration of 1 mg/mL. Addition of 4 to 5% of aceton in methanol, as well as heating to 40 °C, was used to dissolve ononin and formononetin. All the standards were diluted to different concentrations for HPLC-UV detection as follows: 2.5, 5, 10 and 20 μg/mL. Similarly, for LC–MS/MS the stock solution with mixture of all components with final concentration of 0.1 mg/mL in methanol was prepared and the standard dilutions for all components were: 0.1565, 0.3125, 0.625, 1.25, 2.5, 5, 10 and 20 μg/mL. For standard addition, diluted samples were spiked to concentrations of 0, 0.5, 1 and 2 μg/mL.

2.4 LC-UV detection

LC-UV analysis was performed by using an Alliance 2795 coupled with a PDA 966 diode array detector (Waters 2795, Waters Corporation, Milford, MA, USA). Components were separated on a 50 mm long and 2.1 mm inner diameter (ID) reversed-phase C18 column with 3 μm particle size and 90–100 Å pore size (ACE AQ, Advanced Chromatography Technology Ltd, Aberdeen, UK) and by using a 4 mm long and 2.1 mm ID Gemini C18 guard column (Phenomenex, Torrance, CA, USA). Column temperature was 40 °C. The mobile phase delivered at 0.4 mL/min was a mixture of (A) 0.2% formic acid and (B) acetonitrile, using the following gradient elution: 0–1 min (5–25% B), 1–4 min (25–50% B), 4–5 min (50–90% B), 5–7 min (90% B). The injection volume was 8 μL of both non-diluted samples and calibration standards with the following concentrations: 2.5, 5, 10 and 20 μg/mL. Absorbance was monitored in the 190–400 nm wavelength range and chromatograms at 254 nm were used for quantification. Samples were analysed directly without dilution by LC-UV detection, and quantification was by external calibration.

| Sample extract | Sample type | Manufacturer | Lot number | Extraction conditions | Extraction solvent |
|----------------|-------------|--------------|------------|----------------------|-------------------|
| A              | Granulates  | Kaiser pharmaceutical, Taiwan | GR-77/3/19 | Ultrasonication | 70% Methanol      |
| B              | Dried roots | Pharmaceutical wholesalers | HB-01350146 | Ultrasonication | 70% Methanol      |
| B1             | Dried roots | Pharmaceutical wholesalers | HB-01350146 | Boiling water | Water             |
| C              | Hydrophilic concentrate | Conforma NV, Belgium | HC-17 J10/V90291 | No extraction | —                |
2.5 | LC–MS/MS

LC–MS/MS analysis was performed by using an Acquity UPLC coupled with a Quattro Premier XE mass spectrometer (Waters). Components were separated on a 100 mm long and 2.1 mm ID reversed-phase BEH C18 column with 1.7 μm particle size and 130 Å pore size (Waters). Column temperature was 50 °C. The mobile phase delivered at 0.5 mL/min was a mixture of (A) 0.2% formic acid and (B) methanol, using the following gradient elution: 0–5 mins (5–95% B), 5–6 mins (95% B). Parent ion adducts were created by positive ESI with a capillary voltage of 3 kV and detected by multiple reaction monitoring (MRM) with the transitions shown in Table 2. Detection in the negative mode resulted in nearly 10 times lower signal response for AG-IV and was therefore not applied. Samples were analysed by LC–MS/MS, both non-diluted and after 10 times dilution, and were quantified by external calibration. For quantification by standard addition, all samples were diluted 10 times.

2.6 | Method validation

The methods were validated by following the guidelines provided by Eurachem Guide\textsuperscript{25} and CPMP/ICH/381/95 Validation of Analytical Procedures guideline available from EMA (https://www.ema.europa.eu). For LC-UV detection, identification of isoflavones was by retention time only, and selectivity was assessed by observation of co-eluting matrix components. For LC–MS/MS, component identification was by peak retention time in two MRM channels, as well as ion ratio matching with calibration standards. Information on linearity was obtained from the external calibration curves and from the standard addition curves, both using linear regression fit. The accuracy and precision for both LC-UV and LC–MS/MS methods were determined. Limit of detection (LOD) and limit of quantification (LOQ) were established from the calibration curves, by using a signal-to-noise of 3 and 10, respectively. For quantification by external calibration, reproducibility (coefficient of variation percentage, CV %) was obtained by analysis of same sample at different days (n = 6). For quantification by the standard addition method, within day repeatability (CV %) was obtained by a total of six curve replicates. Standard addition resembles a recovery testing experiment, with exact amounts of analyte added at increased concentrations to samples, so the analyte recovery can be incorporated within the standard addition calibration curve. Sample concentration is obtained at the curve intercept on the x-axis, typically analysing 3–6 spiked samples and one non-spiked sample. However, recovery percentage is calculated by difference between one spiked and one non-spiked sample, relative to the exact spike concentration. In general, 85–115% recovery is considered acceptable during method validation, and sample concentrations are usually not corrected.

3 | RESULTS AND DISCUSSION

3.1 | LC-UV and LC–MS/MS methods characteristics

Example chromatograms of standards and sample extracts are provided in Supporting Information Figures S1 and S2. Table 3 shows the analyte detectability with the two different chromatographic methods. Calibration curves were linear (R\textsuperscript{2} > 0.99) from the LOQs up to the highest calibrator concentrations, which were either 200 or 20 μg/mL respectively for LC-UV detection and LC–MS/MS. The

| Component name | Molecular formula | Parent ion adduct | Parent ion m/z | Daughter ion m/z | Cone voltage (V) | Collision voltage (V) |
|----------------|-------------------|-------------------|----------------|------------------|------------------|----------------------|
| Formononetin   | C\textsubscript{16}H\textsubscript{12}O\textsubscript{4} | [M + H]\textsuperscript{+} | 269.2          | 118.1\textsuperscript{b} | 50               | 28                   |
|                |                   |                   |                | 213.3            | 50               | 25                   |
|                |                   |                   |                | 237.3            | 50               | 25                   |
|                |                   |                   |                | 237.3            | 28               | 46                   |
|                |                   |                   |                | 269.2\textsuperscript{b} | 28               | 22                   |
| Ononin         | C\textsubscript{22}H\textsubscript{22}O\textsubscript{9} | [M + H]\textsuperscript{+} | 431.25         | 118.1            | 28               | 46                   |
|                |                   |                   |                | 237.3            | 28               | 44                   |
|                |                   |                   |                | 269.2\textsuperscript{b} | 28               | 22                   |
| Calycosin 7-O-β-D glucoside | C\textsubscript{22}H\textsubscript{22}O\textsubscript{10} | [M + H]\textsuperscript{+} | 447.25         | 270.3            | 28               | 38                   |
|                |                   |                   |                | 285.35\textsuperscript{b} | 28               | 20                   |
| Cycloastragenol | C\textsubscript{30}H\textsubscript{50}O\textsubscript{5} | [M + H]\textsuperscript{+} | 491.5          | 143              | 45               | 10                   |
|                |                   | [M + Na]\textsuperscript{+} | 513.4          | 513.4\textsuperscript{a} | 80               | 2                    |
| Astragaloside IV | C\textsubscript{41}H\textsubscript{66}O\textsubscript{14} | [M + H]\textsuperscript{+} | 785.45         | 143\textsuperscript{a} | 70               | 20                   |
|                |                   | [M + Na]\textsuperscript{+} | 807.4          | 203.1            | 120              | 55                   |
|                |                   |                   |                | 627.5\textsuperscript{b} | 120              | 50                   |

\textsuperscript{a}Daughter ion used in the MRM transition for quantification
\textsuperscript{b}Parent ion used in MRM transition for quantification (no viable fragment ion).
selectivity of compounds was based upon the retention time and MRM channels of respective standards. As expected, AG-IV and CAG were not detected by the LC-UV method, even when monitoring absorbance at a very low wavelength (203 nm). LC–MS/MS provided LODs and LOQs less than 10 ng/mL that were 2–3 orders of magnitude lower than LC-UV method for the isoflavones. The intraday (n = 3) and interday (n = 6) precision and accuracy for measurement of the three isoflavones with spiked concentration of 5 to 20 μg/mL by LC-UV detection (Table 4) and all five analytes by LC–MS/MS were assessed by analysis of samples spiked to concentrations in the 2.5–10 μg/mL range (Table 5). The LC–MS/MS method was found to be more precise and accurate, with mean recovery for compounds found to be 102 ± 6% for AG-IV, 97 ± 9% for cycloastragenol, 101 ± 8% for formononetin, 97 ± 8% for ononin, and 95 ± 7% for calycosin 7-O-β-D-glucoside. However, to achieve such high recoveries, it was necessary to quantify by applying the slope obtained from the standard addition calibration curves described later.

### TABLE 3 Analyte detectability with liquid chromatography coupled with ultraviolet (LC-UV) detection and liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS)

| Compound name | LC-UV LOD (ng/mL) | LC-UV LOQ (ng/mL) | LC–MS/MS LOD (ng/mL) | LC–MS/MS LOQ (ng/mL) |
|---------------|-------------------|-------------------|----------------------|----------------------|
| Formononetin  | 643               | 1949              | 3.8                  | 9.9                  |
| Ononin        | 997               | 3,021             | 0.4                  | 1.1                  |
| Calycosin 7-O-β-D glucoside | 188               | 571               | 0.7                  | 1.7                  |
| Astragaloside IV (AG-IV) | NP               | NP                | 0.6                  | 1.5                  |
| Cycloastragenol (CAG) | NP               | NP                | 0.4                  | 0.9                  |

LOD, limit of detection; LOQ, limit of quantification; NP, not possible to detect

### TABLE 4 Method repeatability and reproducibility for liquid chromatography coupled with ultraviolet (LC-UV) detection

| Compounds (standard concentration, μg/mL) | Intraday (n = 3) Precision (%) | Intraday (n = 3) Accuracy (%) | Interday (n = 6) Precision (%) | Interday (n = 6) Accuracy (%) |
|------------------------------------------|--------------------------------|------------------------------|--------------------------------|------------------------------|
| Formononetin                             |                                |                              |                                |                              |
| 5                                        | 11                             | 75                           | 23                             | 74                           |
| 10                                       | 12                             | 81                           | 21                             | 97                           |
| 20                                       | 2.8                            | 99                           | 11                             | 95                           |
| Ononin                                   |                                |                              |                                |                              |
| 5                                        | 5.5                            | 66                           | 16                             | 63                           |
| 10                                       | 6.1                            | 88                           | 16                             | 87                           |
| 20                                       | 0.7                            | 97                           | 1.2                            | 97                           |
| Calycosin 7-O-β-D glucoside              |                                |                              |                                |                              |
| 5                                        | 28                             | 19                           | 33                             | 37                           |
| 10                                       | 9.2                            | 75                           | 12                             | 81                           |
| 20                                       | 2.7                            | 93                           | 3.5                            | 95                           |

### TABLE 5 Method repeatability and reproducibility for liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS)

| Compounds (standard concentration, μg/mL) | Intraday (n = 3) Precision (%) | Intraday (n = 3) Accuracy (%) | Interday (n = 6) Precision (%) | Interday (n = 6) Accuracy (%) |
|------------------------------------------|--------------------------------|------------------------------|--------------------------------|------------------------------|
| Formononetin                             |                                |                              |                                |                              |
| 2.5                                      | 3.5                            | 96                           | 12                             | 86                           |
| 5                                        | 5.1                            | 87                           | 8.9                            | 90                           |
| 10                                       | 1.5                            | 96                           | 5.7                            | 99                           |
| Ononin                                   |                                |                              |                                |                              |
| 2.5                                      | 5.2                            | 93                           | 3.4                            | 93                           |
| 5                                        | 7.8                            | 87                           | 6.2                            | 90                           |
| 10                                       | 6.4                            | 99                           | 4.5                            | 99                           |
| Calycosin 7-O-β-D glucoside              |                                |                              |                                |                              |
| 2.5                                      | 1.5                            | 90                           | 3.9                            | 86                           |
| 5                                        | 2.7                            | 82                           | 5.4                            | 86                           |
| 10                                       | 1.0                            | 93                           | 4.8                            | 97                           |
| Astragaloside IV                         |                                |                              |                                |                              |
| 2.5                                      | 2.1                            | 94                           | 5.7                            | 98                           |
| 5                                        | 1.4                            | 84                           | 5.2                            | 88                           |
| 10                                       | 0.9                            | 97                           | 1.2                            | 97                           |
| Cycloastragenol                          |                                |                              |                                |                              |
| 2.5                                      | 4.0                            | 80                           | 5.3                            | 84                           |
| 5                                        | 4.3                            | 93                           | 3.7                            | 92                           |
| 10                                       | 4.6                            | 99                           | 3.9                            | 100                          |

3.2 | Standard addition

For quantification by standard addition, 100 μL of the sample extract was added to four autosampler vials together with 100 μL of standard mixture containing 0, 5, 10 or 20 μg/mL of each analyte, followed by dilution to 1 mL with methanol. Figure 2 shows the quantification results for AG-IV in two different diluted sample extracts. The most extraordinary observation when applying standard addition was the large variation in the slopes of the curves, which did not affect the concentrations calculated by standard addition. This large variation in signal response, despite similar composition, would normally be attributed to ion suppression caused by sample matrix components. However, as the diluted samples contained the same amount and composition of matrix, this explanation could be less likely. Ion suppression is typically causing significant variation in signal between samples and not within samples. An alternative more probable explanation for this observation, is a variation in ionisation efficiency (ion enhancement) due to small variation of the mobile phase.
Quantification of AG-IV was done by monitoring the sodium adduct parent ion [M + Na]⁺. Ionisation efficiency therefore depends on trace amounts of sodium in the mobile phase. In the present work and in previous works the trace amount of sodium is most probably derived from the glass bottles of the solvent. However, the level of sodium can vary in these bottles, even for the same solvent product. It is therefore expected that signal response of sodium cluster parent ions may vary too. In our experience, trace levels of sodium can originate from the injection of biological samples. Of note, sodium chloride is in fact rather soluble in methanol (14.9 g/L). Regardless of this variation in signal response, quantification by standard addition resulted in mean concentrations with an acceptable precision. Most important however, is that standard addition provides a result with an embedded recovery correction and the results are expected to be closer to the true concentration levels of the samples. This can be useful in future work towards creating a Certified Reference Material (CRM) for AR. Availability of CRMs is a necessity to ensure traceability and for bias assessment in any type of laboratory analysis.

Standard addition is slightly more time consuming than other quantification methods, as it requires more samples to be analysed by LC–MS/MS. However, there are no commercial isotopic labelled internal standard available for AG-IV, making it difficult to fully compensate for ion suppression or ion enhancement using internal standard calibration. In general, the availability of isotopic labelled internal standards for phytochemicals is limited since manufacturing of isotopic labelled internal standards requires an organic synthesis. For many phytochemicals, such a synthesis does not yet exist. Many studies have therefore used different components as internal standards, some of them are ginsenoside 1, 6,14 digoxin and mycophenolic acid. In future improvements of the method by inclusion of internal standards, the validation should be carried out by comparing results with a standard addition quantification. It is simply not sufficient to assess method trueness, by investigation of analyte recovery in a limited number of samples.

3.3 Sample concentrations

Isoflavones and AG-IV concentrations (weight of dry sample) in extracts from the original solid herb samples, by LC-UV detection (isoflavones only) or LC–MS/MS with two different calibration methods are shown in (Table 6). The concentrations for sample C (hydrophilic concentrate liquid) were determined directly and by using a liquid density of 0.83 g/mL. Cycloastragenol was not detected in
any of the samples. Measurements by LC-UV detection indicated a fairly good agreement with the LC–MS/MS method, except for large overestimation by LC-UV detection of calycosin 7-O-β-D glucoside in sample A. Co-elution of interfering matrix components were observed in the LC-UV chromatogram for this sample. The LC–MS/MS analysis of diluted samples resulted in higher concentration estimates than the non-diluted samples. This clearly indicated more ion suppression for the non-diluted samples, caused by higher levels of interfering matrix components. For AG-IV, quantification by standard addition revealed higher concentration levels than by external calibration. Standard addition thus reduced the negative contribution of ion suppression even more than dilution only.

The AR samples analysed were selected from various formula-tions and raw herbs from one specific vendor. The samples were extracted by ultrasonication or boiling in water, instead of traditional Soxhlet extraction, which requires large quantity of samples and solvents. We hypothesised that the extraction technique would affect the measured concentration levels. Indeed, this was observed for the dried root herb where more AG-IV was extracted into boiling water (sample B1) than into 70% methanol by sonication (sample B). Hot water seemed to be the better solvent for this rather polar substance.

Accurate identification and quantification of AR samples is needed, because of the presence of complex and multiple bioactive components and their therapeutic mechanisms. These can be helpful for optimising the dosage regimens, as well as pharmacological and pharmacokinetic profiling of the components. As shown in the present work, the superior analyte detectability of MS/MS over UV detection, illustrates the advantages of using LC–MS/MS, not only for AR quality control, but potentially also in future pharmacokinetic studies that require low LODs.

Quantification by standard addition provides an embedded correction of ion suppression or ion enhancement, that would otherwise affect the measured concentration levels of bioactive components in AR by LC–MS/MS. This method provides better accuracy and can be used in the absence of an isotopic labelled internal standard. It ensures that measured concentrations are closer to the true levels in the sample extracts. This approach can be beneficial in future herb quality control measurements, and for validation of methods depending on unlabelled internal standards.

### Table 6

Concentrations measured in samples (μg/g) of isoflavones determined by liquid chromatography coupled with ultraviolet (LC-UV) detection and of isoflavones and astragaloside IV determined by liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS).

| Method          | LC-UV   | LC–MS/MS |
|-----------------|---------|----------|
|                 | Non-diluted sample extract | Non-diluted sample extract | 10 × diluted sample extract | 10 × diluted sample extract |
|                 | (n = 4) | (n = 3)  | (n = 3) | (n = 3) |
| Calibration method | External | External | External | Standard addition |
| Sample A        |         |         |         |         |
| Formononetin    | 23 ± 1  | 20 ± 2  | 28 ± 3  | 21 ± 2  |
| Ononin          | 86 ± 6  | 16 ± 2  | 35 ± 2  | 35 ± 3  |
| Calycosin 7-O-β-D glucoside | 525 ± 33 | 39 ± 5  | 125 ± 2 | 173 ± 41 |
| Astragaloside IV | NP      | 6 ± 1   | 74 ± 2  | 203 ± 5 |
| Sample B        |         |         |         |         |
| Formononetin    | 59 ± 3  | 40 ± 7  | 69 ± 4  | 65 ± 11 |
| Ononin          | 46 ± 4  | 33 ± 12 | 59 ± 7  | 63 ± 12 |
| Calycosin 7-O-β-D glucoside | 188 ± 10 | 57 ± 16 | 163 ± 11 | 177 ± 34 |
| Astragaloside IV | NP      | 1.1 ± 0.2 | 21 ± 2 | 37 ± 7 |
| Sample B1       |         |         |         |         |
| Formononetin    | 7.4 ± 0.3 | 13 ± 5  | 28 ± 1  | 24 ± 5  |
| Ononin          | 34 ± 2  | 19 ± 7  | 64 ± 3  | 76 ± 37 |
| Calycosin 7-O-β-D glucoside | 170 ± 7  | 38 ± 11 | 209 ± 7 | 65 ± 17 |
| Astragaloside IV | NP      | 4 ± 1   | 47 ± 4  | 145 ± 8 |
| Sample C        |         |         |         |         |
| Formononetin    | ND      | 8.1 ± 0.4 | 13 ± 1  | 13 ± 1  |
| Ononin          | 27 ± 1  | 10 ± 1  | 23 ± 1  | 21 ± 4  |
| Calycosin 7-O-β-D glucoside | 94 ± 3  | 18 ± 1  | 72 ± 3  | 61 ± 40 |
| Astragaloside IV | NP      | 1.7 ± 0.1 | 17 ± 2  | 23 ± 5  |

ND, not detected; NP, not possible to detect.
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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.