Validated Quantitative $^1$H NMR Method for Simultaneous Quantification of Indole Alkaloids in Uncaria rhynchophylla

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ABSTRACT: Uncariae Ramulus Cum Uncis, known as “Gou-Teng” in Chinese, is derived mainly from the dried hook-bearing stems of Uncaria rhynchophylla. Quantitative determination of monoterpenoid indole alkaloids is critical for controlling its quality. In the present study, a rapid, accurate, and precise method was developed for the simultaneous quantitation of four characteristic components, namely, rhynchophylline (1), isorhynchophylline (2), corynoxeine (3), and isocorynoxeine (4), through $^1$H NMR spectrometry techniques. This method was performed on a 600 MHz NMR spectrometer with optimized acquisition parameters for performing quantitative experiments within 14 min. The highly deshielded signal of NH was at $\delta$9 10−11 in the aprotic solvent DMSO-$d_6$, which enables satisfactory separation of the signals to be integrated. Validation of the quantitative method was also performed in terms of specificity, linearity, sensitivity, accuracy, and precision. The method is linear in the concentration range of 25−400 $\mu$g/mL. The lower limit of quantification is 25 $\mu$g/mL. The intra- and interday relative standard deviation across three validation runs over the entire concentration range is less than 2.51%. The accuracy determined at three concentrations was within ±4.4% in terms of relative error. The proposed qNMR method was demonstrated to be a powerful tool for quantifying the alkaloids in traditional Chinese medicines (TCMs) due to its unique advantages of high precision, high reproducibility, and nonrequirement of standard compounds for calibration curve preparation. Moreover, qNMR represents a feasible alternative to high-performance liquid chromatography-based methods for the quality control of TCMs.

1. INTRODUCTION

Uncariae Ramulus Cum Uncis, known as “Gou-Teng” in Chinese, is a commonly used herbal medicine that has been widely used in China and other Asian countries, such as Japan, for thousands of years. Currently, Gou-Teng is officially documented in the Chinese Pharmacopoeia (2020 edition) for the treatment of hyperpyrexia, epilepsy, pre-eclampsia, and hypertension. This herbal medicine is mainly derived from the dried hook-bearing stems of five Uncaria species (Rubiaceae), including Uncaria rhynchophylla (Miq.) Miq. ex Havil., Uncaria hirsuta Havil., Uncaria sinensis (Oliv.) Havil., Uncaria macrophylla Wall., and Uncaria sessilifructus Roxb. Among them, U. rhynchophylla is the most widely used. Monoterpenoid indole alkaloids have been acknowledged as the characteristic and active components of Uncaria plants due to their various pharmacological activities, such as antiproliferative, antihypertensive, and agonistic activities on the melatonin receptor. Therefore, rhynchophylline, isorhynchophylline, and their analogues, usually serve as markers for the quality control of Gou-Teng.

High-performance liquid chromatography (HPLC) with different detectors, including UV (ultraviolet), diode array detector, and MS (mass spectrometry) detectors, is the most common method for the quantitative determination of indole alkaloids in Gou-Teng samples. However, HPLC methods are time-consuming and cannot always provide satisfactory results due to the highly similar structures of these alkaloids. Moreover, HPLC methods usually require a strict mobile phase containing the buffer salt to control a proper pH, which will potentially result in the column damage. In addition, the standard chemicals used to prepare the calibration curves in HPLC-based methods are expensive and difficult to obtain. The absence of such standards represents a major challenge to investigate their concentration in the HPLC method. Quantitative $^1$H nuclear magnetic resonance (qNMR) is an ideal strategy to address these problems, as it has the advantages of short measuring times, nonrequirement of reference markers for standard curve preparation, the simplicity of the method, the ease of sample preparation, and lower solvent usage.
In the present study, a rapid, accurate, and precise method for the simultaneous quantification of four characteristic components, namely, rhynchophylline (1), isorhynchophylline (2), corynoxeine (3), and isocorynoxeine (4) (Figure 1), in Gou-Teng samples (U. rhynchophylla) using $^1$H NMR spectroscopy was developed. The method is based on the highly deshielded $^1$H NMR signal of NH at $\delta_{NH}$ 10–11 in the aprotic solvent DMSO-d$_6$. The studied compounds were assigned with the aid of one-dimensional and two-dimensional (2D) NMR spectral data. Validation of the quantitative method was performed in terms of specificity, linearity, sensitivity, accuracy, and precision. This work implied that qNMR represents a feasible alternative to HPLC-based methods for the quantitation of the active compounds in TCMs.

2. RESULTS

2.1. Signal Identification and Selection for Quantification. Generally, the quantitative signals used in qNMR must be well resolved from any other resonances in the tested sample. As the signal separation of the $^1$H NMR spectrum is largely influenced by the solvent, the total alkaloids from the Gou-Teng sample were first dissolved in several common deuterated solvents, including CDCl$_3$, CD$_3$OD, pyridine-d$_5$, and DMSO-d$_6$, for $^1$H NMR experiments to select the optimal solvent for the present analysis. It was found that almost all of the signals ($\delta_{NH}$ 0.5–8) that belong to these conventional protons of the tested compounds were severely overlapped in the above deuterated solvents, including the aromatic ($\delta_{NH}$ 6.5–7.5, for H-8 to H-12), olefinic ($\delta_{NH}$ 5.5–6.0, for H-17, H-18, and H-19), and saturated hydrocarbon protons ($\delta_{NH}$ 0.5–4.0). This could be mainly attributed to their highly similar chemical structures. Additionally, the spin–spin coupling and splitting that widely exist in these structures increase the complexity of the $^1$H NMR spectrum. Exceptionally, the highly deshielded signals at $\delta_{NH}$ 10–11 in DMSO-d$_6$ which belong to the NH group of indole alkaloids, displayed a relatively higher resolution (Figure 2E). Previous studies have reported that low-field signals from reactive hydrogen protons, such as OH in flavonoids, could also be applied for the direct quantification of the corresponding compounds in plant extracts. Moreover, the low-field signals are rather sensitive to substituent effects, which are beneficial to be implemented in the qNMR analysis of highly similar analogues. Thus, the low-field signals of NH present in the aprotic solvent DMSO-d$_6$ might also be used in the qNMR analysis of indole alkaloids in the Gou-Teng samples.

Considering that the spectral data of alkaloids to be measured were all reported in CDCl$_3$ or CD$_3$OD in previous studies, their full spectral data in DMSO-d$_6$ were assigned completely by using 2D NMR, including HMBC (heteronuclear multiple bond correlation), HSQC (heteronuclear single quantum coherence), and $^1$H–$^1$H COSY ($^1$H–$^1$H correlation) experiments for the first time (Table S1). Subsequently, four singlets at $\delta_{NH}$ 10.11, 10.32, 10.13, and 10.33 s could be easily assigned to be the NH groups of rhynchophylline (1), isorhynchophylline (2), corynoxeine (3), and isocorynoxeine (4), respectively. In addition, it should be noted that the integral area of these NH resonances is sufficiently compared with other protons, which means that they can be used as quantitative signals. To ensure the specificity of the selected signals, 2D NMR including HMBC and HSQC experiments for the total alkaloids of the Gou-Teng sample was performed. As shown in Figure 3, the HMBC spectrum of the total alkaloids showed only four cross-peaks under the signal at $\delta_{NH}$ 10.11 s, which could be unambiguously assigned to the HMBC corrections from NH to C-7 ($\delta_{CH}$ 55.2 s), C-8 ($\delta_{CH}$ 133.8 s), C-13 ($\delta_{CH}$ 141.9 s), and C-2 ($\delta_{CH}$ 179.6 s) in rhynchophylline (1). The same HMBC correlation patterns were observed for alkaloids 2–4. Another selective HMBC experiment ($\delta_{NH}$ 9–12, $\delta_{CH}$ 40–180) with improved resolution also showed no exessent correlation under the four selected signals. The HSQC spectrum showed no cross-peak under these four signals, which further demonstrated the specificity of these NH signals. In summary, these results indicated that the selected resonances do not overlap with other signals from the sample and could be used for integration in qNMR analysis.

2.2. Selection of Internal Standard. In qNMR analysis, the internal standards usually have the characteristics of high purity, low proton number, relative stability, and no interference, overlap, and interaction with the quantitative peak of the test samples. Based on the above principles, 3,4,5-trimethoxybenzaldehyde (5) was selected as an internal standard for quantitation. The selected internal standard exerts a signal at $\delta_{CH}$ 9.88 s (CHO) in DMSO-d$_6$ which is close and well separated from the quantitative NH signals, thus reducing the deviation from the data-processing procedure. The qNMR analyses require some modifications or optimizations of certain acquisition and processing parameters to ensure that the obtained data meet the requirements of analytical methods. For example, the relaxation delay (d1) should be long enough to ensure complete relaxation for all of the protons involved. In the present study, the longitudinal relaxation time (T1) of the selected resonance peaks in alkaloids to be measured along with the internat standard was determined by using the inversion recovery pulse sequence (t1ir), acquiring 18 recovery delays between 0.001 and 15 s. The T1 values of the selected $^1$H NMR signals were measured to be 0.99 s for rhynchophylline (1), 0.99 s for isorhynchophylline (2), 1.40 s for corynoxeine (3), 1.11 s for isocorynoxeine (4), and 2.33 s for the internal standard (I.S.). Generally, a high-precision qNMR spectrum can be achieved when d1 is greater than...
As the longest $T_1$ was 2.33 s, $d_1$ should be set to at least 11.65 s. However, this would result in a longer measurement time and hence lose the competitive advantage when compared with other analysis techniques. It has also been reported that in the case of a 30° pulse angle, a relatively shorter $d_1$ ($\geq 7/3$ of the longest $T_1$) is considered to be sufficient to completely relax the protons to be analyzed. Therefore, $d_1$ of 10 s at a 30° pulse angle was used in the present study to ensure accuracy and to reduce the measurement time as much as possible.

The number of scans ($n$) is a key factor that influences the sensitivity of qNMR analyses. Although increasing $n$ is helpful to improve the signal-to-noise ratio (S/N), it would inevitably increase the measurement time. Generally, a S/N ratio greater than 150-fold could be satisfied with quantitative NMR. In the present study, $^1$H NMR experiments using different numbers of scans ($n = 8, 16, 32, 64$, and $128$) were carried out. The results indicated that the S/N ratios of the selected five signals of analytes were all more than 150-fold after 64 scans in the $^1$H NMR spectra, which means that the set of $n = 64$ will be sufficient for the present qNMR analyses.

Figure 2. $^1$H NMR spectra of alkaloids I–4 (A: rhynchophylline (1); B: isorhynchophylline (2); C: corynoxeine (3); and D: isocorynoxeine (4)), and the total alkaloids of Gou-Teng (E) in DMSO-d$_6$.

Considering that automatic phase and baseline corrections might give inconsistent results depending on the tested samples, manual phase and baseline corrections were also performed for all of the acquired $^1$H NMR spectra in the present study. Finally, a careful manual integration of quantitative peaks was performed to ensure accuracy. Each selected signal was integrated three times to obtain an average value to minimize the error of manual integration.

2.4. Validation of the Quantitative Method. The specificity of the analytical method represents its ability to measure a particular substance rather than another. In qNMR, it means that the selected quantitative peaks could be unambiguously assigned and are not overlapped by any other resonance. As described in Section 2.1, the assignments of quantitative peaks were achieved by comparing the chemical shifts of the tested compounds in Gou-Teng with those from reference standards. These selected peaks were proven to not overlap with other resonances by HMBC and HSQC experiments, indicating good specificity for the current qNMR method.
To determine the linearity of the developed qNMR method, six solutions of four test alkaloids with a concentration range of 25–400 μg/mL per 50 μg of internal standard in 0.5 mL of DMSO-$d_6$ were prepared and analyzed in triplicate. The calibration curve was obtained by plotting the ratio between the peak areas of alkaloids to be measured ($S_x$) and the internal standard ($S_{I.S}$) versus concentration ($C_x$). As shown in Table 1, the correlation coefficients ($R^2$) for alkaloids 1–4 were >0.999, indicating that the method had satisfying linearity over this concentration range (25–400 μg/mL).

A S/N ratio of threefold was used to determine the limit of detection (LOD). The standard solution with known concentrations was diluted and measured, and the LOD values obtained were 10.2 μg/mL for 1, 4.2 μg/mL for 2, 5.1 μg/mL for 3, and 5.4 μg/mL for 4. The LOD of 1 is higher than the others, which may be attributed to the fact that the NH group in 1 is broader than the others due to the higher exchange rate. The limit of quantitation (LOQ) was 25 μg/mL for the alkaloids 1–4. When compared with previous studies,29,30 the relatively high sensitivity of the current method could be attributed to the use of cryoprobes and higher magnetic fields.

The measurement of intra- and interday precisions was performed by analyzing the standard solutions at three concentration levels (50, 100, and 300 μg/mL). The intraday precision was obtained from one test within 1 day, while the interday precision was obtained using the same method as the intraday precision for 3 consecutive days. The precision was expressed as the percentage of the relative standard deviation (RSD %). The RSD values of intraday and interday precisions for the four alkaloids were found to be in the range of 0.40–2.51% and 0.41–1.73%, respectively.

Recovery tests were performed to determine the accuracy of the qNMR method, which was done by the standard addition method. Three different concentrations of mixed standard solutions (80, 100, and 120%) were spiked into a known amount of sample. The results are shown in Table 1. The average recoveries were between 97.1 and 104.4%.

![Partial HMBC spectrum of the total alkaloids of Gou-Teng.](image)

**Table 1. Linearity, Sensitivity, Precision, and Accuracy for Alkaloids 1–4**

|          | 1       | 2       | 3       | 4       |
|----------|---------|---------|---------|---------|
| Linear range (μg/mL) | 25–400  | 25–400  | 25–400  | 25–400  |
| Correlation coefficient ($R^2$) | 0.9995  | 0.9996  | 0.9994  | 0.9995  |
| LOD (μg/mL) | 10.2    | 4.2     | 5.1     | 5.4     |
| LOQ (μg/mL) | 25.0    | 25.0    | 25.0    | 25.0    |
| Precision (RSD %) |         |         |         |         |
| Intraday | 0.40–1.38 | 0.85–2.51 | 0.19–0.86 | 1.22–2.19 |
| Interday | 0.85–1.73 | 1.01–1.63 | 0.41–1.53 | 0.99–1.49 |
| Recovery (%) | 98.3–103.6 | 99.5–104.4 | 97.9–103.5 | 97.1–104.0 |
Table 2. Content of Alkaloids 1–4 in the Total Alkaloids of Gou-Teng Samples Determined by qNMR and LC−MS

| nos. | qNMR | LC−MS | qNMR | LC−MS | qNMR | LC−MS | qNMR | LC−MS |
|------|------|-------|------|-------|------|-------|------|-------|
| S1   | 9.22 ± 0.16 | 8.85 ± 0.20 | 8.41 ± 0.11 | 8.25 ± 0.04 | 6.12 ± 0.13 | 6.04 ± 0.09 | 6.15 ± 0.15 | 6.13 ± 0.24 |
| S2   | 2.61 ± 0.08  | 2.55 ± 0.08  | 5.11 ± 0.06  | 5.14 ± 0.07  | 3.50 ± 0.03  | 3.66 ± 0.07  | 2.28 ± 0.03  | 2.12 ± 0.02  |
| S3   | 2.72 ± 0.03  | 2.60 ± 0.07  | 5.41 ± 0.05  | 5.54 ± 0.11  | 3.70 ± 0.11  | 3.87 ± 0.05  | 2.37 ± 0.02  | 2.19 ± 0.08  |

Note: Data presented as means ± standard deviations (n = 3).

2.5. Quantitative Determination of Four Alkaloids in Gou-Teng. Using the developed qNMR method, four alkaloids were determined simultaneously in the total alkaloids from three Gou-Teng samples (Table 2). The content of alkaloids 1–4 in the total alkaloids was in the range of 2.61−9.22%, 5.11−8.41%, 3.50−6.12%, and 2.28−6.15%. The observed discrepancies in the quantitative composition with previous studies might result from the differences in the geographical origin, the age, and the time (vegetation period) of collection of the official parts. The quantification of all compounds in the total alkaloids of Gou-Teng samples was additionally validated by HPLC−MS analysis, and no significant differences were found between the results obtained by either methodology.

3. DISCUSSION

A quantitative 1H NMR method with good linearity, precision, repeatability, and accuracy was successfully developed for the simultaneous determination of four indole alkaloids in Gou-Teng. Compared with the conventional HPLC method, the established qNMR method is rapid, requiring approximately 14 min per sample, which is only one-third of the HPLC analysis time (approximately 40−50 min). The testing procedure of the method is simple without sample pretreatment or equilibration of the instrument. It is also worth noting that no standard compounds are required to prepare calibration curves for quantitative determination because qNMR is a primary ratio method. Moreover, this is a great advantage when it is difficult to obtain pure standard compounds. The proposed methodology has the potential as a fast and reliable method for the quality control of TCMs and may be extended to other herbal medicines, especially alkaloid-containing medicines.

Active hydrogen protons are commonly the most important and frequently encountered functionalities in natural products, for example, OH groups in phenolics and NH groups in alkaloids. In 1H NMR spectra, active hydrogen protons usually appear as broad signals, and their linewidth is inversely proportional to the intermolecular proton exchange rate, which depends mainly on the concentration of the solute molecule and of the residual H2O. Linewidth broadening of active hydrogen protons might result in an insufficient integral area, which hinders their application in qNMR analysis. Although several studies have reported that OH group protons from phenolic acids and flavonoids could serve as quantitative signals by the use of the aprotic solvent DMSO-d6, there has been no report that involves the utilization of NH in the qNMR analysis of alkaloids. The present study demonstrated the practicability of NH from oxindole alkaloids in qNMR analysis for the first time, which can be applied in other analogues such as corynantheine, hirsutine, geissoschizine methyl ether, and so forth within this herbal medicine after careful attribution of their corresponding NH signals. In addition, it could be further applied in TCMs rich in alkaloids, thus extending the applied scope of active hydrogen protons in qNMR. On the other hand, the application of qNMR analysis is always hampered by resonance overlapping. As extracts from TCMs possess various compounds with complex molecular structures, the signals in 1H NMR spectra easily overlap in such a limit range of spectral width. In most cases, it is difficult to find a practicable quantitative signal from these overcrowded regions (δH 1−8). The highly deshielding region (δH 9−13) from active hydrogen protons is much simpler than these conventional overcrowded regions and is beneficial for use in the analysis of complex extracts from TCMs. Moreover, active hydrogen protons are rather sensitive to substituent effects. It has been reported that OH shielding occurs in at least 11 bonds. In the present study, NH of indole alkaloids exhibited distinguished chemical shifts despite their highly similar structures and thus could be utilized as a quantitative signal. The current study offers an example for the qNMR analysis of TCMs containing highly similar compounds.

4. CONCLUSIONS

A rapid, accurate, and precise method for the simultaneous quantitation of four marker components in the total alkaloids of U. rhynchophylla using 1H NMR was developed. The use of DMSO-d6 as the solvent enables the utilization of a highly deshielded 1H NMR signal of NH of these alkaloids at δH 10−11. The current method is time-saving and does not require any preliminary treatment. This work implied that qNMR represents a feasible alternative to HPLC-based methods for the quantitation of the active compounds in TCMs.

5. MATERIALS AND METHODS

5.1. Materials and Chemicals. Gou-Teng was purchased from different herbal markets in Zhusai City, China. Deuterated solvents, including chloroform-d (CDCl3, 99.8%, containing 0.03% TMS), methanol-d4 (CD3OD, 99.8%, containing 0.03% TMS), pyridine-d5 (CD5N, 99.5%, containing 0.05% TMS), and DMSO-d6 (99.9% atom % D, containing 0.03% TMS), were purchased from Adamas Reagent Ltd. (Shanghai, China). I.S. 3,4,5-trimethoxybenzaldehyde (98%) and the standard alkaloids to be measured, including rhynchophylline (99%), isorhynchophylline (99%), corynoxine (99%), and isocorynoxine (99%), were purchased from Sigma-Aldrich Co., LLC. (MO, USA). Ultrapure water was obtained from a Milli-Q Gradient Water System (Millipore Corp., Bedford, MA, USA). All other chemicals used were of analytical grade.

5.2. Sample Preparation. The dried Gou-Teng samples were ground to 60 mesh. The powders (10.0 g) were immersed in 100 mL of HCl (1%) for ultrasonic extraction three times (30 min each). The extracting solutions were filtered, combined, and basified with ammonia (10%) to pH 9−10, which were then extracted with ethyl acetate three
times. The ethyl acetate solutions were concentrated in a rotary evaporator, giving the total alkaloids as a yellowish powder. After drying using a nitrogen blow, the total alkaloids (3.0 mg, each) dissolved in DMSO-<i>d</i><sub>6</sub> (500 μL) containing 50 μg of I.S. were used for <sup>1</sup>H NMR assay.

5.3. <sup>1</sup>H NMR Spectrometric Parameters. All <sup>1</sup>H NMR spectra were recorded on a Bruker AVANCE III 600 MHz NMR spectrometer equipped with a 5 mm Cryoprobe (CP DCH6000S 3/C-HD-05 Z) (Bruker, Karlsruhe, Germany). All samples were dissolved in DMSO-<i>d</i><sub>6</sub> at the same concentration (6.0 mg/mL for total alkaloids). <sup>1</sup>H NMR spectra were acquired with a 5 mm ST-500-7 NMR tube (NORELL, Landisville, USA) using the standard Bruker pulse program zg30 with the following settings: flip angle of 90°, relaxation delay (1s), size of fid (TD) = 65536, number of scans (NS) = 64, spectral width (SW) = 16.0221 ppm, acquisition time (AQ) = 3.408 s, and requested probe temperature (TE) = 300 K. The test time for each sample was 14 min. Data acquisition and processing were performed with TopSpin 4.1.0 (Bruker) or MestReNova 12.0 (Mestrelab Research S.L., Santiago de Compostela, Spain).

5.4. Quantitative <sup>1</sup>H NMR Method Validation. The proposed qNMR method was validated using calibration curves, LOD and LOQ precision, and accuracy assays. The <sup>1</sup>H NMR spectra of the different concentrations of alkaloids 1–4 (25–400 μg/mL) in DMSO-<i>d</i><sub>6</sub> containing 50 μg of I.S. were recorded. Calibration curves were constructed by plotting the ratios of the peak areas of each standard alkaloid detected by qNMR to that of I.S. LOD is defined as the lowest concentration of an analyte that can be detected. Typically, it is the concentration of a threefold S/N ratio, while LOQ is defined as the lowest concentration of an analyte that can be accurately and precisely quantified. Intra- and interday precisions were measured by analyzing the standard solutions at three concentration levels (50, 100, and 300 μg/mL). The results of precision experiments are expressed as RSD %. The accuracy was determined using recovery tests by adding the mixed standard solutions with three different concentrations (80, 100, and 120%) to the known amounts of analytes and calculated as follows: recovery (%) = (m<sub>found</sub> − m<sub>original</sub>) / m<sub>addition</sub> × 100%.

5.5. Determination of the Content of Indole Alkaloids by <sup>1</sup>H NMR. From the obtained total alkaloids, 3.0 mg was weighed and dissolved in 0.5 mL of DMSO-<i>d</i><sub>6</sub> containing 50 μg I.S. The solution was then used for quantitative <sup>1</sup>H NMR assay. The content (C<sub>x</sub>) of a certain alkaloid relative to total alkaloids was calculated by the following equation: C<sub>x</sub> = 98% × (S<sub>X</sub> × m<sub>T,A</sub> × M<sub>x</sub>) / (S<sub>I,S</sub> × M<sub>I,S</sub> × m<sub>T,A</sub>), where S<sub>X</sub> and S<sub>I,S</sub> are the integrals of the selected signal of the tested alkaloid and I.S., respectively; M<sub>X</sub> and M<sub>I,S</sub> are the molecular weights (g/mol) of the tested alkaloid and I.S., respectively; and m<sub>T,A</sub> and m<sub>TA</sub> are the masses (μg) of the tested alkaloid, total alkaloids, and I.S. in NMR tubes, respectively.

5.6. LC–MS/MS Analysis. LC–MS/MS analysis was performed by using electrospray ionization (ESI) on a TSQ Quantum Access MAX triple-stage quadrupole mass spectrometer (Thermo Fisher, San Jose, USA) in positive ion mode. The mass spectrometer was coupled with a Thermo Accela HPLC system consisting of a binary gradient pump and an autosampler. Chromatographic separation was performed on an Agilent ZORBAX SB C<sub>18</sub> column (2.1 mm × 150 mm, 3.5 μm) which was maintained at 40 °C. The total running time was 7 min. The mobile phase was composed of acetonitrile and 0.1% formic acid in water (v/v, 25/75) at a flow rate of 0.3 mL/min. After chromatographic separation, the mobile phase was directly introduced into the MS system via an ESI source operating in the positive mode. MS conditions were as follows: vaporizer temperature, 280 °C; capillary temperature, 320 °C; sheath gas pressure, 40 psi; auxiliary gas pressure, 20 psi; and spray voltage, 3000 V. Quantification was accomplished in multiple reaction monitoring (MRM) by monitoring the transition of m/z 385.2 → 160.3 for both rhynchophylline (1) and isorhynchophylline (2) and IRN and m/z 383.5 → 160.3 for corynoxeine (3) and isocorynoxeine (4). Standard stock solutions of rhynchophylline (1), isorhynchophylline (2), corynoxeine (3), and isocorynoxeine (4) were separately prepared in acetonitrile at 500 μg/mL. The stock solutions were serially diluted with acetonitrile to prepare the working solutions. The calibration standards were prepared at 7.8125–250 ng/mL. The standard calibration curves were generated by plotting the peak area ratios of the concentrations of analytes, with the 1/C<sup>2</sup> weighted least-squares linear regression model. The concentrations of ingredients in the samples were all calculated based on the regression parameters derived from the standard calibration curves prepared on the same day. All system control, data acquisition, mass spectral data evaluation, and data processing were performed using XCalibur software version 2.0.7 (Thermo Fischer, San Jose, CA, USA) and Thermo LCQuan software version 2.5.6 (Thermo Fischer).
Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.1c04464

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Notes
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REFERENCES
(1) Heitzman, M. E.; Neto, C. C.; Winiarz, E.; Vaisberg, A. J.; Hammond, G. B. Ethnobotany, phytochemistry and pharmacology of Uncaria (Rubiaceae). Phytochemistry 2005, 66, 5−29.
(2) Zhang, Q.; Zhao, J. J.; Xu, J.; Feng, F.; Qu, W. Medicinal uses, phytochemistry and pharmacology of the genus Uncaria. J. Ethnopharmacol. 2015, 173, 48−80.
(3) Commission, C. P. Chinese Pharmacopoeia: Sectin I; China Medical Science and Technology Press: Beijing, 2020.
(4) Chen, T.; Charlotte, M. T. Flora of China 2001, 19, 348−353.
(5) Ndagijimana, A.; Wang, X.; Pan, G.; Zhang, F.; Feng, H.; Olaley, O. A review on indole alkaloids isolated from Uncaria rhynchophylla and their pharmacological studies. Fitoterapia 2013, 86, 35−47.
(6) Geng, C.-A.; Huang, X.-Y.; Ma, Y.-B.; Hou, B.; Li, T.-Z.; Zhang, X.-M.; Chen, J.-J. (+−)−Ucralins A and B, dimeric isoechinulin-type alkaloids from Uncaria rhynchophylla. J. Nat. Prod. 2017, 80, 959−964.
(7) Chen, Z.; Tian, Z.; Zhang, Y.; Feng, X.; Li, Y.; Jiang, H. Monoterpenoid indole alkaloids in Uncaria rhynchophylli (Mic.) Jacks chinensis and their chemotaxonomic significance. Biochem. Syst. Ecol. 2020, 91, 104057.
(8) Guo, Q.; Yang, H.; Liu, X.; Si, X.; Liang, H.; Tu, P.; Zhang, Q. New zwitieronic monoterpenoid indole alkaloids from Uncaria rhynchophylla. Fitoterapia 2018, 127, 47−55.
(9) Zhang, J.-G.; Chen, J.-J.; Geng, C.-A. Advances in indole alkaloids from traditional Chinese medicine of Uncariae Ramulus Cum Uncis documented in Chinese Pharmacopoeia. J. Chin. Mater. Med. 2019, 44, 685−695.
(10) Lu, W.; Yang, F.; Wang, S. Development of an HPLC fingerprint for quality control and species differentiation of Uncaria rhynchophylla (Mic.) ex Havi. Acta Chromatogr. 2012, 24, 643−651.
(11) Xie, S.; Shi, Y.; Wang, Y.; Wu, C.; Liu, W.; Feng, F.; Xie, N. Systematic identification and quantification of tetracyclic monoterpenoid oxindole alkaloids in Uncaria rhynchophylla and their fragmentations in Q-TOF-MS spectra. J. Pharm. Biomed. Anal. 2013, 81−82, 56−64.
(12) Bertol, G.; Franco, L.; de Oliveira, B. H. HPLC analysis of oxindole alkaloids in Uncaria tomentosa: sample preparation and analysis optimisation by factorial design. Phytochem. Anal. 2012, 23, 143−151.
(13) Qu, J.; Gong, T.; Ma, B.; Zhang, L.; Kano, Y.; Yuan, D. Comparative study of fourteen alkaloids from Uncaria rhynchophylla hooks and leaves using HPLC-diode array detection-atmospheric pressure chemical ionization/MS method. Chem. Pharm. Bull. 2012, 60, 23−30.
(14) Owczarek, A.; Klys, A.; Olszewska, M. A. A validated 1H nQMR method for direct and simultaneous quantification of euscilin, fraxin and (-)-epicatechin in Hippocastani cortex. Talanta 2019, 192, 263−269.
(15) Saito, N.; Kitamaki, Y.; Otsuka, S.; Yamanaka, N.; Nishizaki, Y.; Sugimoto, N.; Imura, H.; Ibara, T. Extended internal standard method for quantitative 1H NMR assisted by chromatography (EIC) for analyte overlapping impurity on 1H NMR spectra. Talanta 2018, 184, 484−490.
(16) Kemprai, P.; Protim Mahanta, B.; Kumar Bora, P.; Jyoti Das, D.; Lakshmi Hati Boruah, J.; Proteem Saikia, S.; Haldar, S. A 1H NMR spectroscopic method for the quantification of propenylbenzenes in the essential oils: Evaluation of key odorsants, antioxidants and post-harvest drying techniques for Piper betle L. Food Chem. 2020, 331, 127278.
(17) Charsides, P.; Primikry, A.; Eckehou, V.; Tzakos, A.; Gerothanassias, I. P. Unprecedented ultra-high-resolution hydroxy group 1H NMR spectroscopic analysis of plant extracts. J. Nat. Prod. 2011, 74, 2462−2466.
(18) Zhang, J.; Yang, C. J.; Wu, D. G. Chemical constituents of Uncaria rhynchophylla (III). Chin. Tradit. Herb. Drugs 1999, 30, 12−14.
(19) Yuan, D.; Ma, B.; Wu, C.; Yang, J.; Zhang, L.; Liu, S.; Wu, L.; Kano, Y. Alkaloids from the leaves of Uncaria rhynchophylla and their inhibitory activity on NO production in lipopolysaccharide-activated microglia. J. Nat. Prod. 2008, 71, 1271−1274.
(20) Ma, X.-L.; Zou, P.-P.; Lei, W.; Wu, L.; Liu, W.; Feng, F.; Li, C. Y.; Lin, C. H.; Wu, T. S. Quantitative analysis of flavonoid metabolomes via data mining and quantification of hydroxyl NMR signals. Anal. Chem. 2020, 92, 4954−4962.
(21) Lu, W.; Zhang, J.; Yuan, D.; Wu, C.; Ma, B.; Wu, L.; Liu, S.; Wu, L.; Kano, Y. Alkaloids from the leaves of Uncaria rhynchophylla and their inhibitory activity on NO production in lipopolysaccharide-activated microglia. J. Nat. Prod. 2008, 71, 1271−1274.
(22) Charisiadis, P.; Primikyri, A.; Exarchou, V.; Tzakos, A.; Gerothanassias, I. P. Unprecedented ultra-high-resolution hydroxy group 1H NMR spectroscopic analysis of plant extracts. J. Nat. Prod. 2011, 74, 2462−2466.
(23) Zhang, J.; Yang, C. J.; Wu, D. G. Chemical constituents of Uncaria rhynchophylla (III). Chin. Tradit. Herb. Drugs 1999, 30, 12−14.
(24) Yuan, D.; Ma, B.; Wu, C.; Yang, J.; Zhang, L.; Liu, S.; Wu, L.; Kano, Y. Alkaloids from the leaves of Uncaria rhynchophylla and their inhibitory activity on NO production in lipopolysaccharide-activated microglia. J. Nat. Prod. 2008, 71, 1271−1274.
differentiation of Rhizoma coptidis by using proton nuclear magnetic resonance spectroscopy. *Anal. Chim. Acta* **2012**, *747*, 76–83.

(31) Yan, Y. M.; Lei, X. L.; Fang, L. H.; Mo, Z. X. *Chin. Med. Mater* **2011**, *34*, 1558–1562.

(32) Yin, T. P.; Cai, L.; Ding, Z. T. An overview of the chemical constituents from the genus Delphinium reported in the last four decades. *RSC Adv.* **2020**, *10*, 13669–13686.

(33) Gonzalez, B.; Fagundez, C.; Peixoto de Abreu Lima, A.; Suescún, L.; Sellanes, D.; Seoane, G. A.; Carrera, I. Efficient access to the Iboga skeleton: Optimized procedure to obtain voacangine from *Voacanga africana* root bark. *ACS Omega* **2021**, *6*, 16755–16762.

(34) Ning, Y. C. *Structural Identification of Organic Compounds and Organic Spectroscopy*; Scincen Press: Beijing, 2018.

(35) Nerantzaki, A. A.; Tsiafoulis, C. G.; Charisiadis, P.; Kontogianni, V. G.; Gerothanassis, I. P. Novel determination of the total phenolic content in crude plant extracts by the use of $^1$H NMR of the -OH spectral region. *Anal. Chim. Acta* **2011**, *688*, 54–60.

(36) Charisiadis, P.; Kontogianni, V. G.; Tsiafoulis, C. G.; Trakos, A. G.; Gerothanassis, I. P. Determination of polyphenolic phytochemicals using highly deshielded -OH $^1$H-NMR Signals. *Phytochem. Anal.* **2017**, *28*, 159–170.

(37) Charisiadis, P.; Exarchou, V.; Troganis, A. N.; Gerothanassis, I. P. Exploring the “forgotten”-OH NMR spectral region in natural products. *Chem. Commun.* **2010**, *46*, 3589–3591.