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Quantification of natural products in herbal supplements: a combined NMR approach applied on goldenseal

Phuong Mai Le^{a,*}, Clément Milande^b, Estelle Martineau^{b,c}, Patrick Giraudeau^{b,d} and Jonathan Farjon^{b,*}

a Metrology, National Research Council Canada, 1200 Montreal road, Ottawa, ON K1A, 0R6, Canada

b EBSI Team, Chimie et Interdisciplinarité: Synthèse, Analyse, Modélisation (CEISAM), Université de Nantes, CNRS, UMR 6230, BP 92208 – 2, rue de la Houssinière, 44322 Nantes, France

c SpectroMaitrise, CAPACITÉS SAS, 26 Bd Vincent Gâche, 44200 Nantes, France

d Institut Universitaire de France, 1 rue Descartes, 75005 Paris Cedex 05, France

Corresponding authors:

Phuong Mai Le

PhuongMai.Le@nrc-cnrc.gc.ca

Phone 1 613 991 9406

Jonathan Farjon

Jonathan.Farjon@univ-nantes.fr

Phone 33 2 51 12 54 34

Abstract

Authentication of natural products is of major relevance in the context of manufactured drugs or herbal supplements since such active products generate a lucrative market. The analytical method to identify and quantify valuable natural products is critical for quality control and product assignment of herbal supplements. In this framework, we propose to apply a recently developed quantitative 2D NMR approach called Q QUIPU (*Quick QUantItative Perfected and pUre shifted*) in combination with 1D ^1H NMR capable to access the concentration of three major alkaloids, berberine, hydrastine and canadine, in the root extract of goldenseal (*Hydrastis canadensis*), one of the 20 most popular herbal supplements used worldwide. We highlight the complementarity of 1D and 2D quantitative NMR to accurately assess the amount of alkaloids with different range of concentrations and stability within extracts. In particular, unstable natural products having non-overlapped signals like berberine could only be quantified by sensitive and fast 1D ^1H , while overlapped signals of hydrastine and low intense ones of canadine could only be quantified with the recent 2D Q QUIPU HSQC.

Results obtained from this combined approach have led to a good accuracy (<10%) as compared with coupled UPLC-MS/UV techniques. This quantitative NMR approach paves the way to numerous applications where the accurate quantification of targeted compounds in complex mixtures is required, for instance in agricultural, food and pharmaceuticals products.

Keywords: Herbal supplements, Goldenseal (*Hydrastine canadensis*), alkaloids, Quantitative NMR, 1D ^1H and 2D Q QUIPU HSQC

1. Introduction

Counterfeit and/or illegally manufactured drugs as well as herbal medicines have become a worldwide public health issue. Herbal supplements are increasingly used as food or dietary supplements and therefore have become a huge lucrative market. For their perceived benefits, botanical extracts are key ingredients in nutraceuticals and personal care products. Their quality depends on the quantity of active key components and on the absence of undesired materials such as adulterants and residual solvents. The beneficial claims depend on the presence specific molecules in the extracts, which therefore need to be identified and accurately quantified.

Goldenseal (*Hydrastine canadensis*; family Ranunculaceae) is a native North American plant and has long been used in traditional medicine for its pharmacological activities such as antibacterial [1], immunostimulant, antimicrobial [4] and anticancer properties [5]. Approximately 44 alkaloids are described as main phytochemicals in the goldenseal plant [6]. The most abundant alkaloids are berberine, hydrastine, and canadine (Figure 1), which have been recognized as the active constituents for most pharmacological activities [7].

Figure 1 Major alkaloids in goldenseal root extracts.

Today, this plant has emerged as one of the 20 most popular herbal supplements used worldwide. The global sales of goldenseal root as a dietary supplement in pharmaceutical outlets

totaled US\$ 25 million in 2012 [10]. Due to the slow growing nature and the popularity of goldenseal, several other plants containing berberine have been substituted for the plant. The adulterants include Goldthread (*Coptis japonica*), Oregon grape root (*Berberis aquifolium* Pursh), Barberry (*Berberis* spp.), and Yellow root (*Xanthorhiza simplicissima*). Most of these adulterants contain berberine, but hydrastine and canadine are only known from goldenseal [11]. Therefore, berberine, hydrastine and canadine are usually used as the markers and the analysis of these 3 alkaloids for the assessment and quality control of goldenseal products is an important step. Most of the previously published studies have used LC-UV and TLC-MS methods for the identification and quantitative analysis of berberine, canadine and hydrastine in goldenseal [13].

In recent years, quantitative Nuclear Magnetic Resonance (qNMR) has been widely used for the analysis of different compounds in complex mixtures of plant extracts [16], drugs [17] and foods [19]. The implementation of qNMR for identification and quantification of alkaloids in herbal medicines, such as *Rhizoma coptidis* [20], Ephedra species [21], was also documented. qNMR methods provide rapid, accurate, highly repeatable and reproducible quantification of several components together with their unambiguous identification [22, 23]. Therefore, qNMR appears particularly adapted for the quality control of herbal samples [16].

In the quantitative NMR toolbox, 1D ^1H is the most sensitive method to rapidly access concentrations. In particular, 1D ^1H has the advantage that multiple compounds can be quantified in a single experiment relying on a unique reference signal, provided that experiments are carried out under quantitative conditions [25]. However, due to the large number of substances having different numerous signals and the broad associated range of concentrations, peak overlapping issues can be highly challenging. Various approaches have made it possible to improve the separation of overlapped resonances in qNMR, most of them relying on 2D NMR approaches [23],

sometimes associated with fast acquisition methods [26]. While these 2D qNMR methods can reach a good precision and trueness, they require **standard addition or calibration** approaches that rely on commercial standards. Alternative approaches have emerged to make 2D NMR intrinsically quantitative, yielding quantitative results even when a commercial standard is not available [28,30]. Among these methods, we recently developed a faster quantitative approach named Q QUIPU (*Quick QUantItative Perfected and pUre shifted*) HSQC, which combines several time-saving strategies with quantitative pulse sequence elements. In this previous work, complex mixtures at sub-millimolar concentrations could be quantified in 5 hours [31]. This highly dispersed and sensitive method provides an appealing approach to quantify complex mixtures involving a broad range of concentrations. However, when compounds are unstable, the experiment duration could be prohibitive and one may still rely on 1D NMR, provided that the targeted analytes exhibit at least one well-separated signal.

In this context, the main objective of this work is to explore the complementarity of qNMR methods, by combining the sensitivity and rapidity of 1D ^1H with the dispersion power of 2D Q-QUIPU HSQC to identify and quantify 3 natural products in Goldenseal extracts: berberine, canadine and hydrastine. In order to evaluate the accuracy of concentrations derived from the combined NMR strategy, a comparative study using UPLC-MS and UPLC-UV is also performed to evaluate the performances of the combined NMR approach.

2. Materials and Methods

2.1 Plant Material

Dried goldenseal roots used in this study were obtained from a Hamilton (ON, Canada) goldenseal farm in May 2005. The plant material was authenticated by an ethnobotanist (Dr Arnason at Ottawa University).

2.2 Chemicals and reagents

Certified reference materials berberine, hydrastine and canadine (CRM BERB-1, HYDR-1 and CANA-1) with purities of 93.35%; 99.32 and 99.56%, respectively were obtained from National Research Council Canada (Ottawa, Canada). Maleic acid, a reference material, with a purity of 99.35%; was purchased from Fluka. Dimethyl sulfone with a purity of 98%; was purchased from Sigma-Aldrich. All organic solvents and formic acid were of HPLC grade and were purchased from Fisher Scientific (ON, Canada). Ammonium formate was purchased from Sigma-Aldrich (Oakville, ON, Canada). Ultra-pure water (18.2M Ω , cm) was produced in-house with a Thermo Scientific GenPure filtration system. For NMR analysis: Deuterium dimethyl sulfoxide-d₆ (99.9% D) was provided by Cambridge Isotope Laboratories INC (Andover, MA, USA). NMR tubes (5 mm o.d., length of 7", thin wall of 0.38 mm) were from Wilmad-LabGlass Com. (Vineland, NJ, USA).

2.3 Goldenseal extraction

Dried goldenseal roots were milled to a powder (particle size less than 250 μ m), thoroughly homogenized using a rotary mixing drum and thereafter stored at -80°C .

Approximately 40 g of this material sample were extracted by ultrasonic bath, at room temperature for 20 min for 6 x 200 ml of a methanol-water-formic acid mixture (90-9.9-0.1; v/v/v) as an extraction solvent. All extract solutions were pooled, and then filtered. The rest of the supernatant liquid was evaporated down to dry under speed-vac and in the dry vacuum at room temperature for one week. This procedure resulted in 10.6 g of dry crude extract of goldenseal root, which was stored at -20°C for further quantitative analysis by 1D and 2D NMR and also by UV and MS analysis.

2.4 NMR analyses

2.4.1 NMR solvent and signal identification for major alkaloids in goldenseal

DMSO-d₆ was chosen as an NMR solvent because all the three individual pure alkaloids and the goldenseal extract were dissolved well in this solvent.

Approximately 10 mg of berberine, canadine, and hydrastine were individually dissolved in 1 ml DMSO-d₆ for recording 1D and 2D NMR analyses to assign the ¹H and ¹³C NMR chemical shifts of these 3 alkaloids in the NMR spectra of goldenseal extracts.

2D NMR spectra were recorded at 298 K on a Bruker Avance HD 700 spectrometer, at a frequency of 700.13 MHz with a warm TXI probe including z-axis gradients. DMSO-d₆ was used as the internal lock. Different 2D experiments like COSY, HSQC, and HMBC were performed to identify major alkaloids of interest (see [Table S1](#)).

2.4.2 Quantification of major alkaloids in goldenseal by 1D ¹H-NMR

Five samples each containing 123.7; 117.7; 122.8; 123.4 and 115.6 mg, respectively, of dry goldenseal extract were co-dissolved with 15 mM of maleic acid as internal standard in 1 mL DMSO-d₆. The quantification of berberine in the goldenseal extract by ¹H qNMR was performed

on a Bruker Avance III 600 MHz at 20°C. The samples were vortexed, sonicated, then centrifuged and 800 µL of supernatant were loaded into a 5 mm NMR tube, sealed and used for quantitative ¹H qNMR measurements. Five goldenseal samples were analysed by ¹H-NMR and each sample was measured in triplicate.

The ¹H qNMR acquisition was performed with a 90° pulse, a sweep width of 30 ppm (18,000 Hz), 32 scans of 128 K data points preceded by 4 dummy scans, an acquisition time of 3.63 s and a recovery delay of 35 s, requiring 27 min per sample. In order to ensure the full relaxation of ¹H-NMR resonance (including the internal standard) to obtain highly accurate ¹H qNMR spectra, the longitudinal relaxation times (T₁) were measured (prior to the quantitative ¹H qNMR measurements) for a sample containing goldenseal extract and the maleic acid in DMSO-d₆. From this experiment, the longest relevant T₁ was 4.45 s, and the repetition delay was subsequently set to 35 s (*i.e.*, 7 times the longest T₁) to ensure an accuracy of 0.1% with a 90° pulse. Data processing was performed using 64 K data points. The FIDs were Fourier transformed and apodized by an exponential function leading to a line broadening of 0.3 Hz (LB). The resulting spectra were manually phased and a manual polynomial baseline correction encompassing the desired integral regions was carried out prior to integration. The reference frequency was set to the DMSO-d₆ signal at 2.50 ppm. All the spectra were integrated using TopSpin software (version 3.2, Bruker).

2.4.3 Quantification of major alkaloids in goldenseal by 2D HSQC-NMR

Three more samples were prepared with 100, 101 and 201 mg of dried extract respectively dissolved in 1mL of DMSO-d₆ including 2 mM of DMSO₂ as an internal reference.

For all 2D experiments, longitudinal relaxation ¹H times were measured and a recovery time of 22 s was chosen for quantitative HSQCs. Here, 5 times the longest T₁ time was considered as a

compromise between a good accuracy and a reasonable experimental time for a precision of 1% (see below). For the Q QUIPU HSQC, parameters were optimized according to reference [31]. The 2D HSQC maps were recorded in 2 days with 5452 x 160 data points. 2D matrices have been zero filled to 16384 x 1024 and apodized with a cosine in the two dimensions, prior to double Fourier transformation. 176 scans were recorded for each increment in the indirect dimension, to reach a minimum SNR of 50 ensuing a precision in the range of 1%. $^1J_{CH}$ compensation was carried out by varying the INEPT delay throughout the successive scans (8 INEPTs in total: 6 with short delays $\Delta=1.47$ ms and 2 with a longer delay $\Delta=2.96$ ms).

The pure shifting of the acquisition was performed with 10 chunks of 30 ms and BIRD/180° elements were used to ensure the absence J_{HH} evolution during the acquisition time (300 ms). For each HSQC experiment, an echo-antiecho method and an adiabatic heteronuclear decoupling of 28 kHz were used on the ^{13}C channel. Decoupling consisted of 1.5 ms smoothed CHIRP pulses with a total sweep width of 60 kHz and a P5M4 supercycle. Moreover, Q QUIPU is using 50% NUS meaning that 50% of the t_1 increments were actually acquired [32]. A Poisson gap sampling schedule was used since it was shown to yield cleaner spectra after reconstruction. The reconstruction of the 2D spectrum was performed using the compressed sensing algorithm available within the Bruker software [33]. Compressed sensing generates less reconstruction artefacts than recursive multidimensional decomposition (MDD) for 2D spectra [34]. A semi-gaussian/linear function was been used as Variable repetition time (VRT) function [31]. A two-fold aliasing was implemented leading to a 65 ppm apparent spectral width centered on 52.5 ppm along the ^{13}C dimension. Before Fourier transform, 2D maps were weighted with cosine functions over the two dimensions. The resulting spectra were manually phased, an automatic polynomial baseline

correction and referenced to the residual DMSO-d₆ signal at 2.50 ppm. The peak volumes were measured using the 2D integration tool in Topspin 3.2. The integration region limits were systematically optimized for each peak, on the metabolite model sample with the following criteria (i) integration regions centred on the peak of interest and (ii) integration regions size chosen as large as possible to integrate the whole peak but small enough to avoid overlap with neighboring peaks and integration regions.

Three goldenseal samples were analysed by 2D NMR and each sample was measured in triplicate.

In both 1D-¹H [35] and 2D Q QUIPU-HSQC experiments, the area of a signal appearing on a NMR spectrum is directly proportional to the concentration as well as the number of resonant nuclei corresponding to the signal.

2.5 UPLC-UV and UPLC-MS-MS analysis

Stock solutions of berberine, hydrastine and canadine (standard compounds) at a concentration of 1.0 mg/mL in methanol were individually prepared and stored at -20°C for no longer than 1 month. Using these individual alkaloid stock solutions, a mixed standard solution at an appropriate concentration was prepared. The external standard calibration curve was prepared at seven different concentration levels by the serial dilution with MeOH of the mixed standard solution. Finally, the range of the external calibration curve was from 0.1 to 145 µg/mL for UPLC-UV and from 0.002 to 1.45 µg/mL, for UPLC-MS-MS analysis.

The stock solution of goldenseal extract was prepared in MeOH at concentration of 1.20 mg/ml and then diluted 20 and 100 times, respectively, for UPLC-UV and UPLC-MS analysis. 7 samples of goldenseal extract were prepared. The volume of 2 µl of working calibration solution and

goldenseal sample was injected in triplicate into the UV and MS system. All the solutions of samples and standards were gravimetrically prepared.

Analyses were performed with an UPLC (Infinity 1290, Agilent Technologies) system equipped with an Agilent diode array detector (DAD) and/or an ESI-triple quadrupole mass spectrometer (Quantiva TSQ, Thermo Scientific). The samples were separated with an Acquity UPLC Beh C18 column 100 mm x 2.1 mm, 1.7 μ m at 40°C. The mobile phase consisted of A, water containing 15 mM ammonium formate adjusted to pH 3.8 with formic acid, and B, mixture of 15 mM NH₄Formate, pH 3.8 and acetonitrile; 10:90 (v/v). The flow rate was maintained at 0.4 mL/ml and the following gradient program was used: 19–30 % B from 0 to 7 min; 30–95 % B from 7 to 8 min; hold at 95 % B from 8 to 10 min. The total run time for the analysis was 10 min. The standards and goldenseal sample were kept at 10°C during analysis. The 2 μ L of working calibration solution and goldenseal sample were injected in triplicate into the UV and MS system.

The UV chromatograms were recorded at 290, 294 and 344 nm for canadine, hydrastine and berberine, respectively ([Table S2](#)). The DAD spectra from 190 to 600 nm were stored for all peaks. The concentrations of berberine, hydrastine and canadine in goldenseal sample were determined by using the UV detected peak area under the curve to the established calibration curves made from the standard compounds. Data processing was performed using the LAB CDS ChemStation software for UV analysis.

The MS detection was carried out in positive ion mode, using SRM (selected reaction monitoring) mode by monitoring MS/MS transitions m/z 336.1/292.0 for berberine and 384.1/190.1 for hydrastine and 340.1/176.1 for canadine ([Table S2](#)). Experimental conditions for ESI ionization and mass spectrometry detection are summarized in Table 1. Quantitative MS analysis was performed in the SRM mode. The peak area of the SRM ion of berberine, canadine and β -

hydrastine was used to calculate the concentration based on the standard calibration curve. Data processing was performed using the Xcalibur software for MS analysis.

Seven goldenseal samples were analysed by UPLC-UV and UPLC-MS-MS and each sample was measured in triplicate. In addition, the UPLC-MS-MS and UPLC-UV methods were validated in terms of linearity, precision, stability, repeatability and recovery (data not shown). Details on the validation of quantitative UPLC-MS-MS and UPLC-UV methods of alkaloids in goldenseal will be published elsewhere.

2.6 Extract optimization

Various solvent systems were evaluated to obtain the maximum recoveries of 3 major alkaloids (berberine, canadine and hydrastine) in goldenseal root powder, including 100%; 90%; 80% methanol (or acetonitrile ACN); 30% methanol (or acetonitrile) + 0.1% phosphoric acid; 90% MeOH (or acetonitrile) + 0.1% Formic acid; 90% MeOH (or acetonitrile) + 0.1% acetic acid. The recoveries determined by LC-MS of the major alkaloids were found to be higher (>97%) with 90% methanol/water/0.1% Formic acid (v/v/v) than with other solvents. The sonication time was also optimized. 20 and 30 min were tested and no significant increase was observed with the extended treatment times. 20 min was selected as optimum extraction period. There was no analyte observed after the 4th extraction by LC-UV detector and 6th by LC-MS detector. The sixth extraction of samples was considered to be exhaustive.

3 Results and Discussion

As mentioned earlier, the choice of the most appropriate quantitative NMR tool depends on criteria such as 1D peak overlap, concentration, and stability in time. Here, we evaluate both 1D

¹H NMR and 2D Q QUIPU for the quantification of the 3 major alkaloids in goldenseal extract (Figure 1).

3.1 Potential of 1D ¹H NMR for quantification

The easiest way to determine concentrations is the 1D ¹H experiment that is also one of the most sensitive experiments. The representative ¹H-NMR spectra of individual alkaloids and of the goldenseal extract are shown in Figure 2. The assignment of NMR signals for each alkaloid was based on experiments performed on standard solutions (see Table S1).

Signals of the major alkaloids in the extracts are indicated in Figure 2. Accurate quantitative ¹H NMR, is possible when the analyte of interest shows at least one well-separated signal. In the case of goldenseal extract, berberine has two characteristic signals at 9.89 and 8.94 ppm (corresponding to H₆ and H₇ respectively, see Figure 1) that are isolated and easily identified since they do not overlap with other signals in the ¹H spectrum of goldenseal extract. On the contrary, all the peaks of hydrastine were at least partially overlapped by those of minor compounds in the ¹H spectrum, making accurate quantification very difficult by 1D NMR. It is even worse for canadine, which could not be detected due to its low concentration and to the large number of compounds giving rise to overlapping peaks in corresponding spectral region (Figure 2a).

270

271

272 **Figure 2** ^1H NMR spectra of goldenseal extract (a), canadine (b) β -hydrastine (c) and berberine
 273 (d). More details on the assignment can be found in [Table S1](#).

274 There is no need to use calibration curves in ^1H qNMR analysis, since the signal intensity is
 275 directly proportional to the number of resonant nuclei contributing to each signal [22]. The
 276 concentration of the compounds can be calculated by the relative ratio of the peak integral of each
 277 compound to the integral of the peak of a known amount of internal standard (IS), accounting for
 278 the number of protons in each integral. In this study, we chose maleic acid as an IS for the following
 279 reasons: it is not present in goldenseal, it is a reference material with a high purity, it is a non-
 280 hygroscopic and easily weighable solid with a good solubility and stability in DMSO-d_6 and its
 281 unique signal does not overlap with other signals (see Figure S1). As mentioned above, the two
 282 proton signals of berberine (proton H_6 and H_7) were selected to quantify berberine in the goldenseal
 283 extract by ^1H qNMR. As shown in [Table S3](#), the concentrations of berberine (obtained during the
 284 first two hours, before the degradation) in mg per gram of goldenseal extract by ^1H qNMR were in

the range 105.37 mg/g (SD:1.2). The results obtained by ^1H NMR (see Table S3) are consistent from different measurements based on triplicate and from sample to sample.

3.2 2D Q QUIPU capabilities for quantifying goldenseal alkaloids

Due to the limitations of ^1H -qNMR, 2D Q QUIPU HSQC experiments were carried out in order to quantify the two other major alkaloids in the goldenseal root extract, hydrastine and canadine (see Figures S2). As previously shown on breast cancer cell extracts, this ^1H - ^{13}C 2D experiment provides a better sensitivity than standard HSQC: +26 to 92% as compared to the non-pure-shift version (see Figure S3) [28]. The concentrations of β -hydrastine, and canadine were accurately obtained from 2D Q QUIPU maps (see Figure 3) by using DMSO_2 as an internal reference.

The different quantities of the main alkaloids were obtained by averaging all cross peak volumes for each alkaloid of interest (some of them can be seen in Figure 3) deriving from 2D Q QUIPU maps (see Figure S2). In order to evaluate the associated repeatability, 3 spectra were recorded for each sample, leading to a good precision between 1 and 7%. Moreover, 3 different samples were prepared to evaluate the reproducibility (ranging from 0.3 to 1.2%) (see Figure S4 and Table S4).

Figure 3 Parts of 2D Q QUIPU map showing canadine (A), β -hydrastine (B), and DMSO₂ (C) signals with the corresponding integration regions (green squares). DMSO₂ was used as an internal reference.

3.3 Complementarity of 1D ¹H and 2D Q QUIPU

Berberine has shown a tendency to degrade within the first two hours after the sample preparation. Under such conditions, 1D ¹H is the only experiment able to rapidly detect signals of the berberine (see Figure 2) with the best accuracy and repeatability (see Table S3) in only 3 min. In this context the Q QUIPU is not competitive to determine the concentration of berberine. On the contrary, probing the quantity of hydrastine and canadine with 1D ¹H by integration is difficult due to overlaps (see Figure 2). In the case of such overlap, spectral deconvolution of 1D spectra could be suggested as an alternative [36,38]. Thus, a global spectral deconvolution (GSD) using the tool implemented in Mnova 12 was performed. However, the accuracy of the resulting concentrations for hydrastine and canadine is disappointing – between 14 and 55 % as compared to 2D Q QUIPU

(Figure S5B and Table S5 in ESI) – and 2D NMR appears in much better agreement with the results obtained by MS. This shows the limits of the deconvolution approach and highlights the superiority of the quantitative 2D NMR approach in the case of strong overlap.

Although Q QUIPU showed a great ability to access the lower concentration of canadine, 2 days of experiment were required to reach a SNR close to 50 ensuring a precision of 1%. By using a cryogenically cooled probe which was not available at the time of the study, it could be possible to reduce the experimental time by a factor from 9 to 16, leading to a total time from 3 to 5h for the 2D Q QUIPU with the same level of accuracy.

In order to evaluate the quantitative performance of the combined NMR approach, other MS-based analytical techniques were used for comparison.

3.4 UPLC-UV and UPLC-MS-MS analysis

In parallel to the quantification of 3 major alkaloids (berberine, canadine and hydrastine) in goldenseal extract by 1D and 2D-qNMR (as above), we developed the quantification of these three alkaloids by other independent methods such as UPLC-UV and UPLC-MS-MS. The quantification of 3 major alkaloids in goldenseal material has been published in previous studies [13, 14], but not for the same goldenseal material which was used in our study.

The presence of berberine, β -hydrastine and canadine in goldenseal extract was already confirmed in our previous publication [6, 40] by comparison of their retention time, UV wavelength absorbance, MS precursor ion and MS-MS fragmentation ions to those of standard compound.

Here, berberine, β -hydrastine and canadine in goldenseal extract were quantified using the corresponding calibration curves of each compound for UPLC-UV and UPLC-MS-MS method leading to precision from 0 to 1% (see Table 1).

3.5 Comparison of the combined NMR quantification with MS-based methods

For the most concentrated alkaloids (berberine and hydrastine), concentrations derived from NMR are in very good agreement with those obtained by UPLC-UV/MS with trueness in the range of 1 to 3% (Table 1 and Figure 4). Despite the longer acquisition times of the 2D Q QUIPU technique, it was possible to quantify the lowest concentrated canadine in the range of 6 mg/g in 2 days with a good accuracy: 1-3% regarding UPLC-MS, and an acceptable one in the range of 0.3 to 9.6% as compared to UPLC-UV (see Table 1 from the 6th to the 7th columns). The quantities of canadine obtained by UPLC-UV are higher than those obtained by UPLC-MS and 2D-NMR (see Table 1 column 2 versus 3 and 5 columns), which may be explained by the peak overlap between canadine and other compound resulting from the LC-UV method. Using this method, the quantities of canadine was measured based on its UV detected peak area at 290 nm (Table S2) under the standards calibration curve. This method often lacks highly compound specific information, especially for compound with low concentration such as canadine in the complex goldenseal extract, because other compound having a similar retention time on the UV chromatography are also absorbed at around 290 nm. In contrast, the quantities of canadine obtained by the UPLC-MS method using the SRM mode was determined based on both MS precursor ion at m/z 341.1 and MS-MS fragmentation ion at m/z 176.1 (Table S2) and also based on the retention time determined from the calibrations with standard compounds. Therefore, the quantitative conditions of canadine in complex goldenseal extract by UPLC-MS are more selective than those of UPLC-UV, probably leading some discrepancy for canadine amount obtained by UPLC-UV and UPLC-MS. The indices above could be partly explained by reminding that the proposed quantitative NMR approach is more accurate than indirect methods such as UV and MS, because the higher number of steps

required in indirect methods can increase time consumption and introduce sources of bias due to a lower accuracy.

In a general point of view, the quantification by NMR can be made directly on the matrices in the absence of further purifications and does not rely on external calibration because the NMR signal is molar equivalent. With the addition of a known concentration of IS to the matrices (goldenseal extract in this case), the concentration of compound in the matrices can easily be calculated. This is a great advantage when it is difficult to obtain pure standard compounds. An advantage of the qNMR method over UPLC-UV and MS methods is that it requires much less sample preparation time and no calibration curves for quantification are necessary.

Compound	1D ¹ H-NMR	2D Q-QUIPU	UPLC-UV	UPLC-MS-MS	Accuracy (%) NMR/UV	Accuracy (%) NMR/MS
Berberine	105.37 (1.26)	NA	105.08 (0.96)	106.24 (0.76)	0.27	0.82
Hydrastine	NQ	67.01 (2.10)	65.93 (0.81)	65.20 (1.32)	1.61	2.68
Canadine	NQ	5.91 (0.29)	6.48 (0.10)	6.02 (0.27)	9.60	1.86

Table 1 Comparison of the alkaloid concentrations in GDS extract (mg/g $\pm$ SD) determined independently by NMR (n=3), UV and by MS (n=5).

NQ: not quantified due to the peak overlaps issue in 1D ¹H NMR

NA: not applicable due to the long acquisition of 2D Q QUIPU NMR

Figure 4 Concentration of berberine, canadine and β -hydrastine derived from 3 successive 2D Q
QUIPU experiments recorded over 3 samples in comparison with results obtained from triplicate
UPLC-UV and UPLC-MS over 5 samples.

4 Conclusions

2D advanced techniques presented in combination with 1D ^1H NMR have shown their potential for quantifying alkaloids of interest from goldenseal. Despite a longer time requiring 2 days of measurement with a room temperature probe it was possible to access the quantity of the low concentrated canadine at 6 mg/g of extract. Of course under this condition, it was difficult to determine the concentration of berberine due to its degradability within the first hour after sample preparation. The precision rate was very good in the range of 1 to 7 %. Overall, the concentration of berberine, hydrastine and canadine obtained by ^1H and/or 2D qNMR measurement are in acceptable to good agreements with those obtained by MS and UV measurement. The trueness was compared to the data obtained with the coupled UPLC-MS and UPLC-UV leading to variability of 1 to 10 %. Moreover, the ^1H 1D remains the direct method of choice for the quantification of natural

products having moderate overlaps and possible unstable compound in the time. The combination of different NMR techniques is the key to overcome limitations for investigating complex natural mixtures embedding unstable and low concentrated molecules. In this field, 2D ultrafast techniques [26, 27] combined with hyperpolarization methods [42,43] could be useful for enhancing the resolution in seconds to minutes acquisition times with sensitivity enhancement from two to three order of magnitude.

Thus, combined NMR approaches can be used and expanded to quantify other compounds of interest in complex mixtures with a better sensitivity, and resolution, high precision, good accuracy, repeatability and reproducibility and in a less time consuming manner in different areas such as agricultural, food and/or pharmaceutical products.

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References

- [1] F. Scazoccio, M.F. Cometa, L. Tomassini, M. Palmery, Antibacterial activity of *Hydrastis canadensis* extract and its major isolated alkaloids. *Planta Med.* 67 (2001) 561-564.
- [2] K.A. Ettefagh, J.T. Burns, H.A. Junio, G.W. Kaatz, N.B. Cech, Goldenseal (*Hydrastis canadensis* L.) extracts synergistically enhance the antibacterial activity of berberine via efflux pump inhibition. *Planta Med.* 77 (2011) 835-840.

- [3] R. Jalees, J.M. Dillow, S.M. Carter, J. Chou, B. Le, A.S. Maisel, Increased production of antigenspecific immunoglobulins G and M following in vivo treatment with the medicinal plants *Echinacea angustifolia* *Hydrastis canadensis*. *Immunol. Lett.* 68 (1999) 391-395.
- [4] B.Y. Hwang, S.K. Roberts, L.R. Chadwick, C.D. Wu, A.D. Kinghorn, Antimicrobial constituents from goldenseal (the Rhizomes of *Hydrastis canadensis*) against selected oral pathogens. *Planta Med.* 69 (2003) 623-627.
- [5] S.R. Karmakar, S.J. Biswas, A.R. Khuda-Bukhsh, Anti-carcinogenic Potentials of a Plant Extract (*Hydrastis canadensis*): I Evidence from in vivo studies in mice (*Mus musculus*). *Asian Pacific Journal of Cancer Prevention* 11 (2010) 545-551.
- [6] P.M. Le, M. McCooeye, A. Windust, Application of UPLC-QTOF-MS in MSE mode for the rapid and precise identification of alkaloids in goldenseal (*Hydrastis canadensis*). *Anal. Bional. Chem.* 406 (2014) 1739-1749.
- [7] E.J. Gentry, H.B. Jampani, A. Kesharvarz-Shokri, M.D. Morton, D. VanderVelde, H. Telikepalli, L.A. Mitscher, Antitubercular natural products: Berberine from the roots of commercial *Hydrastis canadensis* powder. Isolation of inactive 8-oxotetrahydrothalifendine, canadine, b-hydrastine, and two new quinic acid esters, hydrcandinic acid esters-1 and 2. *J. Nat. Prod.* 61 (1998) 1187-1193.
- [8] P. Bolle, M.F. Cometa, M. Palmery, P. Tucci, Response of rabbit detrusor muscle to total extract and major alkaloids of *Hydrastis canadensis*. *Phytother. Res.* 12 (1998) S86-S88.
- [9] H. Abdel-Haq, M.F. Cometa, M. Palmery, M.G. Leone, B. Silvestrini, L. Saso, relaxant effects of *Hydrastis canadensis* L. and its major alkaloids on guinea pig isolated trachea. *Pharmacol. Toxicol.* 87 (2000) 218-222.
- [10] I.A.F.R.O. Cancer, Goldenseal. In *Some Drugs and Herbal Products*, World Health Organization: France, 2016; Vol. 108, pp 73-86.
- [11] C. Cavaliere, P. Rea, M.E. Lynch, M. Blumenthal, Herbal supplement sales experience - slight rise in 2008. *HerbalGram* (2009) 58-61.
- [12] R. Upton, Goldenseal Root (*Hydrastis canadensis*): Standard of Analysis, Quality Control, and Therapeutics. (2001) 10-18.
- [13] B. Avula, Y.H. Wang, I.A. Khan, Quantitative Determination of Alkaloids from Roots of *Hydrastis canadensis* L. and Dietary Supplements Using Ultra-Performance Liquid Chromatography with UV Detection. *Journal of AOAC Int.* 95 (2012) 1398-1405.

- [14] P.N. Brown, M.C. Roman, Determination of hydrastine and berberine in goldenseal raw materials, extracts, and dietary supplements by high performance liquid chromatography with UV: Collaborative Study. *J. AOAC Int.* 91 (2008) 694-701.
- [15] G.J. Van Berkel, B.A. Tomkins, V. Kerterz, Thin-layer chromatography/desorption electrospray ionization mass spectrometry: investigation of goldenseal alkaloids. *Anal. Chem.* 79 (2007) 2778-2789.
- [16] S.K. Chauthé, R.J. Sharma, F. Aqil, R.C. Gupta, I.P. Singh, Quantitative NMR: An Applicable Method for Quantitative Analysis of Medicinal Plant Extracts and Herbal Products. *Phytochem. Anal.* 23 (2012) 689-696.
- [17] A.A. Salem, H.A. Mossa, Method validation and determinations of levofloxacin, metronidazole and sulfamethoxazole in an aqueous pharmaceutical, urine and blood plasma samples using quantitative nuclear magnetic resonance spectrometry. *Talanta* 88 (2012) 104-114.
- [18] I. Berregi, G.D. Campo, R. Caracena, J.I. Miranda, Quantitative determination of formic acid in apple juices by ¹H NMR spectrometry. *Talanta* 72 (2007) 1049-1053.
- [19] B. Gouilleux, J. Marchand, B. Charrier, G.S. Remaud, P. Giraudeau, High-throughput authentication of edible oils with benchtop Ultrafast 2D NMR. *Food Chemistry* 244 (2018) 153-158.
- [20] G. Fan, M.Y. Zhang, X.D. Zhou, X.R. Lai, Q.H. Yue, C. Tang, W.Z. Luo, Y. Zhang, Quality evaluation and species differentiation of *Rhizoma coptidis* by using proton nuclear magnetic resonance spectroscopy. *Analytica Chimica Acta* 747 (2012) 76-83.
- [21] H.K. Kim, Y.H. Choi, W.T. Chang, R. Verpoorte, Quantitative Analysis of ephedrine analogues from *Ephedra* species using ¹H- NMR. *Chem. Pharm. Bull.* 12 (2003) 1382-1385.
- [22] G.F. Pauli, B.U. Jaki, D.C. Lankin, Quantitative ¹H NMR: Development and Potential of a Method for Natural Products Analysis. *J. Nat. Prod.* 68 (2005) 133-149.
- [23] P. Giraudeau, Quantitative 2D liquid-state NMR. *Magn. Reson. Chem* 52 (2014) 259–272.
- [24] J. Zhao, M. Wang, B. Avula, L. Zhong, Z. Song, Q. Xu, S. Li, I.A. Khan, Effect of Processing on the Traditional Chinese Herbal Medicine *Flos Lonicerae*: An NMR-based Chemometric Approach. *Planta Med.* 81 (2015) 754-764.
- [25] P. Giraudeau, I. Tea, G.S. Remaud, S. Akoka, Reference and normalization methods: Essential tools for the intercomparison of NMR spectra. *J. Pharm. Biomed. Anal.* 93 (2014) 3-16.

- [26] T. Jézéquel, C. Deborde, M. Maucourt, V. Zhendre, A. Moing, P. Giraudeau, Absolute quantification of metabolites in tomato fruit extracts by fast 2D NMR. *Metabolomics* 11 (2015) 1231-1242.
- [27] L. Rouger, B. Gouilleux, M. Pourchet-Gellez, J.N. Dumez, P. Giraudeau, Ultrafast double-quantum NMR spectroscopy with optimized sensitivity for the analysis of mixtures. *Analyst* 141 (2016) 1686-1692.
- [28] C. Mauve, S. Khlifi, F. Gilard, G. Mouille, J. Farjon, Sensitive, highly resolved, and quantitative (1)H-(13)C NMR data in one go for tracking metabolites in vegetal extracts. *Chem. Commun.* 52 (2016) 6142-6145.
- [29] S. Khlifi, C. Mauve, N. Glab, F. Gilard, M. Hodges, G. Mouilled, J. Farjon, An easier analysis of complex mixtures with highly resolved and sensitivity enhanced 2D quantitative NMR: application to tracking sugar phosphates in plants. *Analytical Methods* 9 (2017) 2328-2333.
- [30] K. Hu, W.M. Westler, J.L. Markley, Simultaneous Quantification and Identification of Individual Chemicals in Metabolite Mixtures by Two-Dimensional Extrapolated Time-Zero 1H-13C HSQC (HSQC0). *J. Am. Chem. Soc.* 133 (2011) 1662–1665.
- [31] J. Farjon, C. Milande, E. Martineau, S. Akoka, P. Giraudeau, The FAQUIRE Approach: FAsT, QUantitative, hIghly Resolved and sEnsitivity Enhanced 1H, 13C Data. *Anal. Chem.* 90 (2018) 1845-1851.
- [32] B. Worley, R. Powers, Deterministic multidimensional nonuniform gap sampling. *J. Mag. Reson.* 261 (2015) 19-26.
- [33] A. Stern, J. Hoch, A new approach to compressed sensing for NMR. *Magn. Reson. Chem.* 53 (2015) 908-912.
- [34] V.Y. Orekhov, V.A. Jaravine, Analysis of non-uniformly sampled spectra with multi-dimensional decomposition. *Prog. Nucl. Magn. Reson. Spectrosc.* 59 (2011) 271-292.
- [35] T. Rundlöfa, M. Mathiassona, S. Bekiroglub, B. Hakkarainen, T. Bowden, T. Arvidssona, Survey and qualification of internal standards for quantification by 1H NMR spectroscopy. *J. Pharm. Biomed. Anal.* 52 (2010) 645-651.
- [36] R.S. Phansalkar, C. Simmler, J. Bisson, S.N. Chen, D.C. Lankin, J.B. McAlpine, M. Niemitz, G.F. Pauli, Evolution of Quantitative Measures in NMR: Quantum Mechanical qHNMR Advances Chemical Standardization of a Red Clover (*Trifolium pratense*) Extract. *J. Nat Prod.* 80 (2017) 634-647.

- [37] J.G. Napolitano, T. Gödecke, D.C. Lankin, B.U. Jaki, J.B. McAlpine, S.N. Chen, G.F. Pauli, Orthogonal analytical methods for botanical standardization: Determination of green tea catechins by qNMR and LC–MS/MS. *J. Pharm. Biomed. Anal.* 93 (2014) 59-67.
- [38] T. Schoenberger, S. Menges, M.A. Bernstein, M. Pérez, F. Seoane, S. Sýkora, C. Cobas, Improving the Performance of High-Precision qNMR Measurements by a Double Integration Procedure in Practical Cases. *Anal. Chem.* (2016) 3836-3843.
- [39] M. Tiainen, P. Soininen, R. Laatikainen, J. Magn. Reson. Quantitative Quantum Mechanical Spectral Analysis (qQMSA) of ¹H NMR spectra of complex mixtures and biofluids 242 (2014) 67-78.
- [40] P.M. Le, M. McCooeye, A. Windust, Characterization of the alkaloids in goldenseal (*Hydrastis canadensis*) root by high resolution Orbitrap LC-MS(n). *Anal. Bioanal. Chem.* 405 (2013) 4487-4498.
- [41] A. Bornet, M. Maucourt, C. Deborde, D. Jacob, J. Milani, B. Vuichoud, X. Ji, J.-N. Dumez, A. Moing, G. Bodenhausen, S. Jannin, P. Giraudeau, Highly Repeatable Dissolution Dynamic Nuclear Polarization for Heteronuclear NMR Metabolomics. *Anal. Chem.* 88 (2016) 6179-6183.
- [42] V. Daniele, F. Legrand, P. Berthault, J. Dumez, G. Huber, Single-Scan Multidimensional NMR Analysis of Mixtures at Sub-Millimolar Concentrations by using SABRE Hyperpolarization. *Chemphyschem.* 16 (2015) 3413-3417.
- [43] B. Plainchont, P. Berruyer, J.-N. Dumez, S. Jannin, P. Giraudeau, Dynamic Nuclear Polarization Opens New Perspectives for NMR Spectroscopy in Analytical Chemistry. *Anal. Chem.* 90 (2018) 3639-3650.