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

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1 **Abstract**

2 Authentication of natural products is of major relevance in the context of manufactured drugs or
3 herbal supplements since such active products generate a lucrative market. The analytical method
4 to identify and quantify valuable natural products is critical for quality control and product
5 assignment of herbal supplements. In this framework, we propose to apply a recently developed
6 quantitative 2D NMR approach called Q QUIPU (*Quick QUantitative Perfected and pUre shifted*)
7 in combination with 1D ¹H NMR capable to access the concentration of three major alkaloids,
8 berberine, hydrastine and canadine, in the root extract of goldenseal (*Hydrastis canadensis*), one
9 of the 20 most popular herbal supplements used worldwide. We highlight the complementarity of
10 1D and 2D quantitative NMR to accurately assess the amount of alkaloids with different range of
11 concentrations and stability within extracts. In particular, unstable natural products having non-
12 overlapped signals like berberine could only be quantified by sensitive and fast 1D ¹H, while
13 overlapped signals of hydrastine and low intense ones of canadine could only be quantified with
14 the recent 2D Q QUIPU HSQC.

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

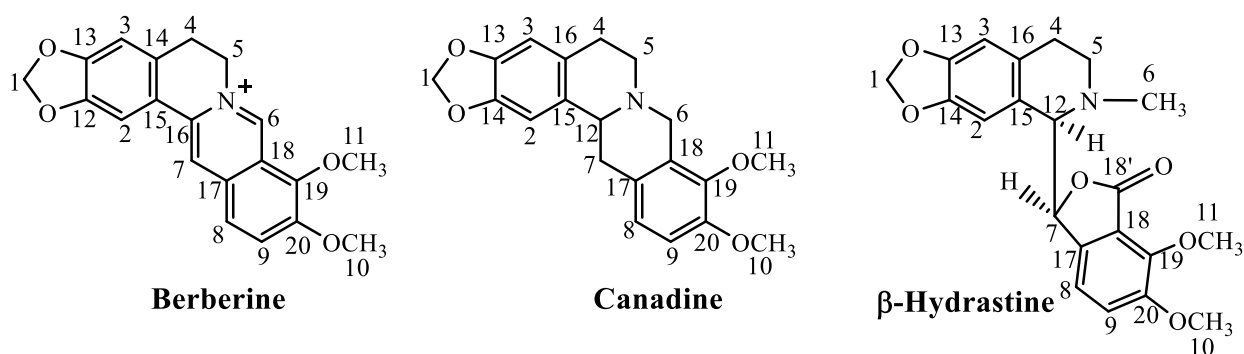
19
20
21 Keywords: Herbal supplements, Goldenseal (*Hydrastine canadensis*), alkaloids, Quantitative
22 NMR, 1D ¹H and 2D Q QUIPU HSQC

23

24 1. Introduction

25
26 Counterfeit and/or illegally manufactured drugs as well as herbal medicines have become a
27 worldwide public health issue. Herbal supplements are increasingly used as food or dietary
28 supplements and therefore have become a huge lucrative market. For their perceived benefits,
29 botanical extracts are key ingredients in nutraceuticals and personal care products. Their quality
30 depends on the quantity of active key components and on the absence of undesired materials such
31 as adulterants and residual solvents. The beneficial claims depend on the presence specific
32 molecules in the extracts, which therefore need to be identified and accurately quantified.

33 Goldenseal (*Hydrastine canadensis*; family Ranunculaceae) is a native North American plant
34 and has long been used in traditional medicine for its pharmacological activities such as
35 antibacterial [1], immunostimulant, antimicrobial [4] and anticancer properties [5]. Approximately
36 44 alkaloids are described as main phytochemicals in the goldenseal plant [6]. The most abundant
37 alkaloids are berberine, hydrastine, and canadine (Figure 1), which have been recognized as the
38 active constituents for most pharmacological activities [7].



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

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

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

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

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

84

85

86

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88

89 2. Materials and Methods

90

91 2.1 Plant Material

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

95

96 2.2 Chemicals and reagents

97 **Certified** reference materials berberine, hydrastine and canadine (CRM BERB-1, HYDR-1 and
98 CANA-1) with purities of 93.35%; 99.32 and 99.56%, respectively were obtained from National
99 Research Council Canada (Ottawa, Canada). Maleic acid, a reference material, with a purity of
100 99.35%; was purchased from Fluka. Dimethyl sulfone with a purity of 98%; was purchased from
101 Sigma-Aldrich. All organic solvents and formic acid were of HPLC grade and were purchased from
102 Fisher Scientific (ON, Canada). Ammonium formate was purchased from Sigma-Aldrich
103 (Oakville, ON, Canada). Ultra-pure water (18.2M Ω , cm) was produced in-house with a Thermo
104 Scientific GenPure filtration system. For NMR analysis: Deuterium dimethyl sulfoxide-d₆ (99.9%
105 D) was provided by Cambridge Isotope Laboratories INC (Andover, MA, USA). NMR tubes (5 mm
106 o.d., length of 7", thin wall of 0.38 mm) were from Wilmad-LabGlass Com. (Vineland, NJ, USA).
107

108 2.3 Goldenseal extraction

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

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

118

119 *2.4 NMR analyses*

120 *2.4.1 NMR solvent and signal identification for major alkaloids in goldenseal*

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

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

126 2D NMR spectra were recorded at 298 K on a Bruker Avance HD 700 spectrometer, at a
127 frequency of 700.13 MHz with a warm TXI probe including z-axis gradients. DMSO-d₆ was used
128 as the internal lock. Different 2D experiments like COSY, HSQC, and HMBC were performed to
129 identify major alkaloids of interest (see [Table S1](#)).

130

131 *2.4.2 Quantification of major alkaloids in goldenseal by 1D ¹H-NMR*

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

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

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

152

153 2.4.3 Quantification of major alkaloids in goldenseal by 2D HSQC-NMR

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

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

158 compromise between a good accuracy and a reasonable experimental time for a precision of 1%
159 (see below). For the Q QUIPU HSQC, parameters were optimized according to reference [31]. The
160 2D HSQC maps were recorded in 2 days with 5452 x 160 data points. **2D matrices have been zero**
161 **filled to 16384 x 1024 and apodized with a cosine in the two dimensions, prior to double Fourier**
162 **transformation.** 176 scans were recorded for each increment in the indirect dimension, to reach a
163 minimum SNR of 50 ensuing a precision in the range of 1%. $^1J_{CH}$ compensation was carried out by
164 varying the INEPT delay throughout the successive scans (8 INEPTs in total: 6 with short delays
165 $\Delta=1.47$ ms and 2 with a longer delay $\Delta=2.96$ ms).

166
167 The pure shifting of the acquisition was performed with 10 chunks of 30 ms and BIRD/180°
168 elements were used to ensure the absence J_{HH} evolution during the acquisition time (300 ms). For
169 each HSQC experiment, an echo-antiecho method and an adiabatic heteronuclear decoupling of
170 28 kHz were used on the ^{13}C channel. Decoupling consisted of 1.5 ms smoothed CHIRP pulses
171 with a total sweep width of 60 kHz and a P5M4 supercycle. Moreover, Q QUIPU is using 50%
172 NUS meaning that 50% of the t_1 increments were actually acquired [32]. A Poisson gap sampling
173 schedule was used since it was shown to yield cleaner spectra after reconstruction. The
174 reconstruction of the 2D spectrum was performed using the compressed sensing algorithm
175 available within the Bruker software [33]. Compressed sensing generates less reconstruction
176 artefacts than recursive multidimensional decomposition (MDD) for 2D spectra [34]. A semi-
177 gaussian/linear function was been used as Variable repetition time (VRT) function [31]. A two-fold
178 aliasing was implemented leading to a 65 ppm apparent spectral width **centered on 52.5 ppm** along
179 the ^{13}C dimension. Before Fourier transform, 2D maps were weighted with cosine functions over
180 the two dimensions. The resulting spectra were manually phased, an automatic polynomial baseline

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

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

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

192

193 *2.5 UPLC-UV and UPLC-MS-MS analysis*

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

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

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

206 Analyses were performed with an UPLC (Infinity 1290, Agilent Technologies) system
207 equipped with an Agilent diode array detector (DAD) and/or an ESI-triple quadrupole mass
208 spectrometer (Quantiva TSQ, Thermo Scientific). The samples were separated with an Acquity
209 UPLC Beh C18 column 100 mm x 2.1 mm, 1.7 μ m at 40°C. The mobile phase consisted of A,
210 water containing 15 mM ammonium formate adjusted to pH 3.8 with formic acid, and B, mixture
211 of 15 mM NH₄Formate, pH 3.8 and acetonitrile; 10:90 (v/v). The flow rate was maintained at 0.4
212 mL/ml and the following gradient program was used: 19–30 % B from 0 to 7 min; 30–95 % B from
213 7 to 8 min; hold at 95 % B from 8 to 10 min. The total run time for the analysis was 10 min. The
214 standards and goldenseal sample were kept at 10°C during analysis. The 2 μ L of working
215 calibration solution and goldenseal sample were injected in triplicate into the UV and MS system.

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

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

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

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

234

235 *2.6 Extract optimization*

236 Various solvent systems were evaluated to obtain the maximum recoveries of 3 major alkaloids
237 (berberine, canadine and hydrastine) in goldenseal root powder, including 100%; 90%; 80%
238 methanol (or acetonitrile ACN); 30% methanol (or acetonitrile) + 0.1% phosphoric acid; 90%
239 MeOH (or acetonitrile) + 0.1% Formic acid; 90% MeOH (or acetonitrile) + 0.1% acetic acid. The
240 recoveries determined by LC-MS of the major alkaloids were found to be higher (>97%) with 90
241 % methanol/water/0.1% Formic acid (v/v/v) than with other solvents. The sonication time was also
242 optimized. 20 and 30 min were tested and no significant increase was observed with the extended
243 treatment times. 20 min was selected as optimum extraction period. There was no analyte observed
244 after the 4th extraction by LC-UV detector and 6th by LC-MS detector. The sixth extraction of
245 samples was considered to be exhaustive.

246

247 **3 Results and Discussion**

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

250 ^1H NMR and 2D Q QUIPU for the quantification of the 3 major alkaloids in goldenseal extract
251 (Figure 1).

252 3.1 Potential of 1D ^1H NMR for quantification

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

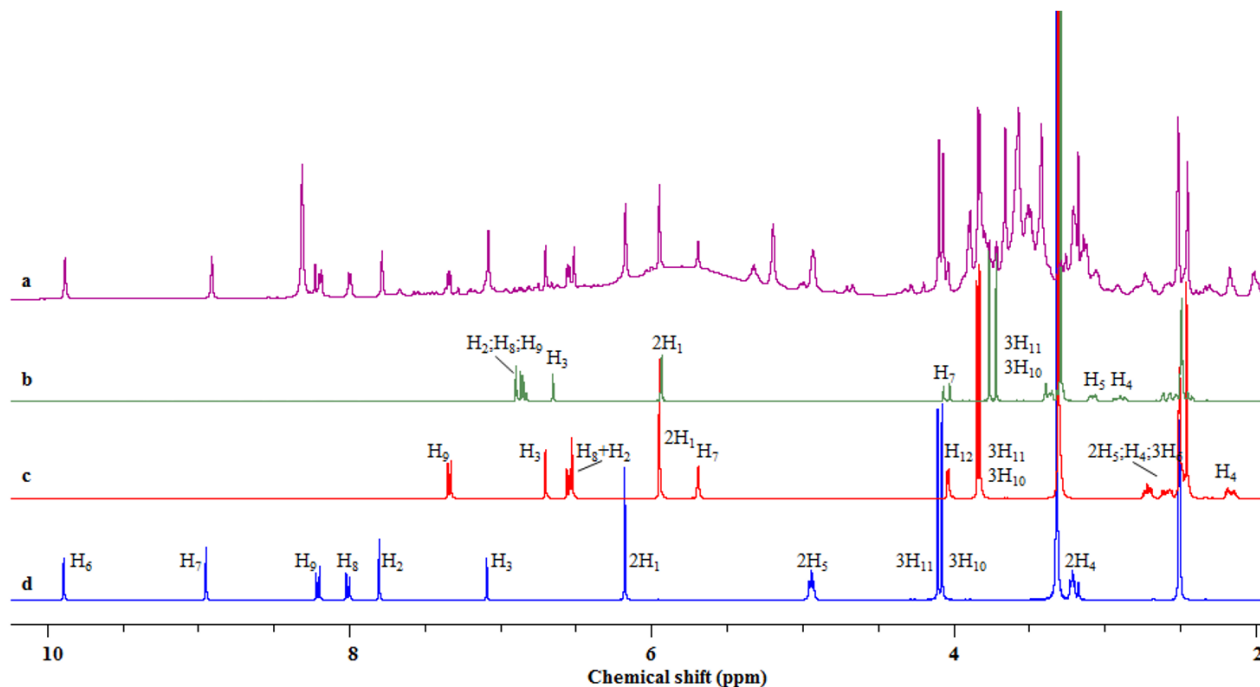
257 **Signals of the major** alkaloids in the extracts are indicated in Figure 2. Accurate quantitative
258 ^1H NMR, is possible when the analyte of interest shows at least one well-separated signal. In the
259 case of goldenseal extract, berberine has two characteristic signals at 9.89 and 8.94 ppm
260 (corresponding to H_6 and H_7 respectively, see Figure 1) that are isolated and easily identified since
261 they do not overlap with other signals in the ^1H spectrum of goldenseal extract. On the contrary,
262 all the peaks of hydrastine were at least partially overlapped by those of minor compounds in the
263 ^1H spectrum, making accurate quantification very difficult by 1D NMR. It is even worse for
264 canadine, which could not be detected due to its low concentration and to the large number of
265 compounds giving rise to overlapping peaks in corresponding spectral region (Figure 2a).

266

267

268

269



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

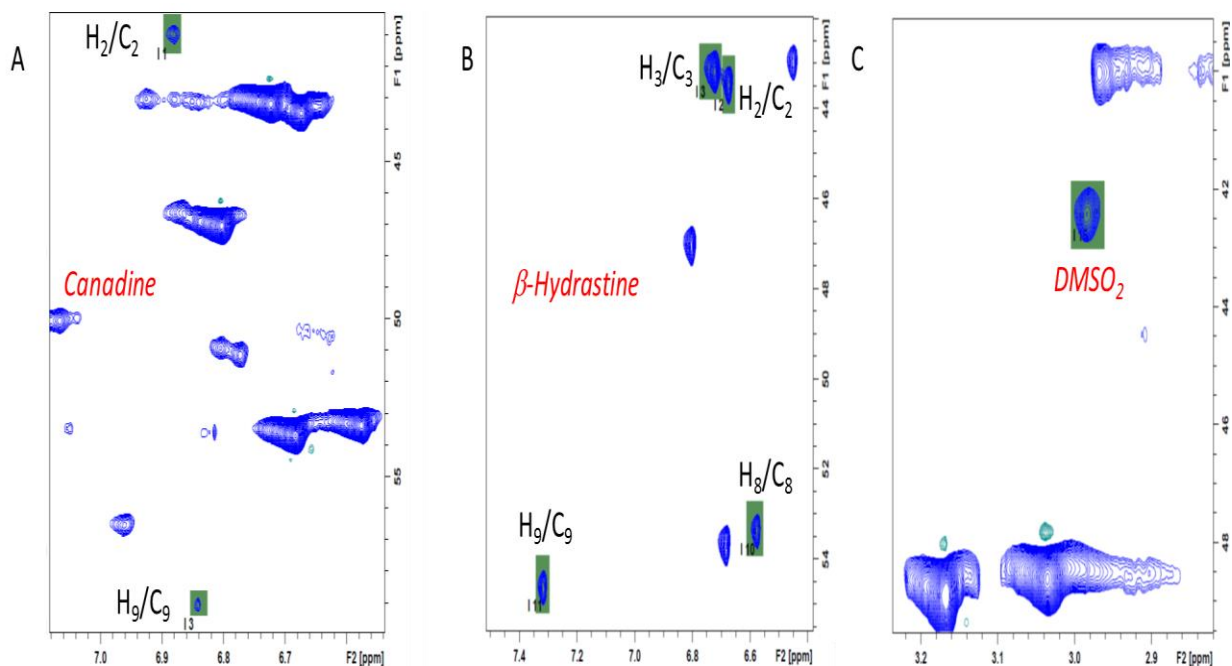
285 the range 105.37 mg/g (SD:1.2). The results obtained by ^1H NMR (see Table S3) are consistent
286 from different measurements based on triplicate and from sample to sample.

287

288 3.2 2D Q QUIPU capabilities for quantifying goldenseal alkaloids

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

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



301
 302 **Figure 3** Parts of 2D Q QUIPU map showing canadine (A), β -hydrastine (B), and DMSO₂ (C)
 303 signals with the corresponding integration regions (green squares). DMSO₂ was used as an internal
 304 reference.

306 3.3 Complementarity of 1D ¹H and 2D Q QUIPU

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

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

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

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

326

327 *3.4 UPLC-UV and UPLC-MS-MS analysis*

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

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

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

339

340 *3.5 Comparison of the combined NMR quantification with MS-based methods*

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

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

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

371

Compound	1D ¹ H-NMR	2D Q-QUIPU	UPLC-UV	UPLC-MS-MS	Accuracy (%)	Accuracy (%)
					NMR/UV	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

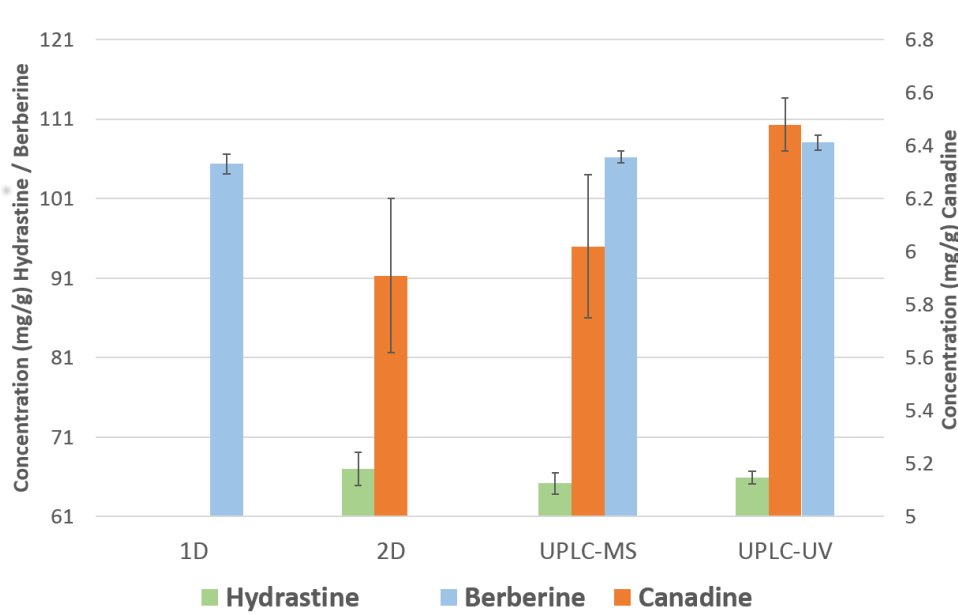
372

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

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

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

377



378

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

382 **4 Conclusions**

383 2D advanced techniques presented in combination with 1D ^1H NMR have shown their potential
 384 for quantifying alkaloids of interest from goldenseal. Despite a longer time requiring 2 days of
 385 measurement with a room temperature probe it was possible to access the quantity of the low
 386 concentrated canadine at 6 mg/g of extract. Of course under this condition, it was difficult to
 387 determine the concentration of berberine due to its degradability within the first hour after sample
 388 preparation. The precision rate was very good in the range of 1 to 7 %. Overall, the concentration
 389 of berberine, hydrastine and canadine obtained by ^1H and/or 2D qNMR measurement are in
 390 acceptable to good agreements with those obtained by MS and UV measurement. The trueness was
 391 compared to the data obtained with the coupled UPLC-MS and UPLC-UV leading to variability of
 392 1 to 10 %. Moreover, the ^1H 1D remains the direct method of choice for the quantification of natural

393 products having moderate overlaps and possible unstable compound in the time. The combination
394 of different NMR techniques is the key to overcome limitations for investigating complex natural
395 mixtures embedding unstable and low concentrated molecules. In this field, 2D ultrafast techniques
396 [26, 27] combined with hyperpolarization methods [42,43] could be useful for enhancing the
397 resolution in seconds to minutes acquisition times with sensitivity enhancement from two to three
398 order of magnitude.

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

403

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409

410 **References**

- 411 [1] F. Scazoccio, M.F. Cometa, L. Tomassini, M. Palmery, Anterbacterial activity of *Hydrastis*
412 *canadensis* extract and its major isolated alkaloids. *Planta Med.* 67 (2001) 561-564.
- 413 [2] K.A. Etefagh, J.T. Burns, H.A. Junio, G.W. Kaatz, N.B. Cech, Goldenseal (*Hydrastis*
414 *canadensis* L.) extracts synergistically enhance the antibacterial activity of berberine vial efflux
415 pump inhibition. *Planta Med.* 77 (2011) 835-840.

- 416 [3] R. Jalees, J.M. Dillow, S.M. Carter, J. Chou, B. Le, A.S. Maisel, Increased production of
417 antigenspecific immunoglobulins G and M following in vivo treatment with the medicinal
418 plants *Echinacea angustifolia* *Hydrastis canadensis*. *Immunol. Lett.* 68 (1999) 391-395.
- 419 [4] B.Y. Hwang, S.K. Roberts, L.R. Chadwick, C.D. Wu, A.D. Kinghorn, Antimicrobial
420 constituents from goldenseal (the Rhizomes of *Hydrastis canadensis*) against selected oral
421 pathogens. *Planta Med.* 69 (2003) 623-627.
- 422 [5] S.R. Karmakar, S.J. Biswas, A.R. Khuda-Bukhsh, Anti-carcinogenic Potentials of a Plant
423 Extract (*Hydrastis canadensis*): I Evidence from in vivo studies in mice (*Musmusculus*). *Asian*
424 *Pacific Journal of Cancer Prevention* 11 (2010) 545-551.
- 425 [6] P.M. Le, M. McCooye, A. Windust, Application of UPLC-QTOF-MS in MSE mode for the
426 rapid and precise identification of alkaloids in goldenseal (*Hydrastis canadensis*). *Anal. Bional.*
427 *Chem.* 406 (2014) 1739-1749.
- 428 [7] E.J. Gentry, H.B. Jampani, A. Kesharvarz-Shokri, M.D. Morton, D. VanderVelde, H.
429 Telikepalli, L.A. Mitscher, Antitubercular natural products: Berberine from the roots of
430 commercial *Hydrastis canadensis* powder. Isolation of inactive 8-oxotetrahydrothalifendine,
431 canadine, b-hydrastine, and two new quinic acid esters,hydcandinic acid esters-1 and2. *J. Nat.*
432 *Prod.* 61 (1998) 1187-1193.
- 433 [8] P. Bolle, M.F. Cometa, M. Palmery, P. Tucci, Response of rabbit detrusor muscle to total extract
434 and major alkaloids of *Hydrastis canadensis*. *Phytother. Res.* 12 (1998) S86-S88.
- 435 [9] H. Abdel-Haq, M.F. Cometa, M. Palmery, M.G. Leone, B. Silvestrini, L. Saso, relaxant effects
436 of *Hydrastis canadensis* L. and its major alkaloids on guinea pig isolated trachea. *Pharmacol.*
437 *Toxicol.* 87 (2000) 218-222.
- 438 [10] I.A.F.R.O. Cancer, Goldenseal. In *Some Drugs and Herbal Products*, World Health
439 Organization: France, 2016; Vol. 108, pp 73-86.
- 440 [11] C. Cavaliere, P. Rea, M.E. Lynch, M. Blumenthal, Herbal supplement sales experience - slight
441 rise in 2008. *HerbalGram* (2009) 58-61.
- 442 [12] R. Upton, Goldenseal Root (*Hydrastis canadensis*): Standard of Analysis, Quality Control, and
443 Therapeutics. (2001) 10-18.
- 444 [13] B. Avula, Y.H. Wang, I.A. Khan, Quantitative Determination of Alkaloids from Roots of
445 *Hydrastis canadensis* L. and Dietary Supplements Using Ultra-Performance Liquid
446 Chromatography with UV Detection. *Journal of AOAC Int.* 95 (2012) 1398-1405.

- 447 [14] P.N. Brown, M.C. Roman, Determination of hydrastine and berberine in goldenseal raw
448 materials, extracts, and dietary supplements by high performance liquid chromatography with
449 UV: Collaborative Study. *J. AOAC Int.* 91 (2008) 694-701.
- 450 [15] G.J. Van Berkel, B.A. Tomkins, V. Kerterz, Thin-layer chromatography/desorption
451 electrospray ionization mass spectrometry: investigation of goldenseal alkaloids. *Anal. Chem.*
452 79 (2007) 2778-2789.
- 453 [16] S.K. Chauthe, R.J. Sharma, F. Aqil, R.C. Gupta, I.P. Singh, Quantitative NMR: An Applicable
454 Method for Quantitative Analysis of Medicinal Plant Extracts and Herbal Products. *Phytochem.*
455 *Anal.* 23 (2012) 689-696.
- 456 [17] A.A. Salem, H.A. Mossa, Method validation and determinations of levofloxacin,
457 metronidazole and sulfamethoxazole in an aqueous pharmaceutical, urine and blood plasma
458 samples using quantitative nuclear magnetic resonance spectrometry. *Talanta* 88 (2012) 104-
459 114.
- 460 [18] I. Berregi, G.D. Campo, R. Caracena, J.I. Miranda, Quantitative determination of formic acid
461 in apple juices by ¹H NMR spectrometry. *Talanta* 72 (2007) 1049-1053.
- 462 [19] B. Gouilleux, J. Marchand, B. Charrier, G.S. Remaud, P. Giraudeau, High-throughput
463 authentication of edible oils with benchtop Ultrafast 2D NMR. *Food Chemistry* 244 (2018)
464 153-158.
- 465 [20] G. Fan, M.Y. Zhang, X.D. Zhou, X.R. Lai, Q.H. Yue, C. Tang, W.Z. Luo, Y. Zhang, Quality
466 evaluation and species differentiation of *Rhizoma coptidis* by using proton nuclear magnetic
467 resonance spectroscopy. *Analytica Chimica Acta* 747 (2012) 76-83.
- 468 [21] H.K. Kim, Y.H. Choi, W.T. Chang, R. Verpoorte, Quantitative Analysis of ephedrine
469 analogues from *Ephedra* species using ¹H- NMR. *Chem. Pharm. Bull.* 12 (2003) 1382-1385.
- 470 [22] G.F. Pauli, B.U. Jaki, D.C. Lankin, Quantitative ¹H NMR: Development and Potential of a
471 Method for Natural Products Analysis. *J. Nat. Prod.* 68 (2005) 133-149.
- 472 [23] P. Giraudeau, Quantitative 2D liquid-state NMR. *Magn. Reson. Chem* 52 (2014) 259-272.
- 473 [24] J. Zhao, M. Wang, B. Avula, L. Zhong, Z. Song, Q. Xu, S. Li, I.A. Khan, Effect of Processing
474 on the Traditional Chinese Herbal Medicine *Flos Lonicerae*: An NMR-based Chemometric
475 Approach. *Planta Med.* 81 (2015) 754-764.
- 476 [25] P. Giraudeau, I. Tea, G.S. Remaud, S. Akoka, Reference and normalization methods: Essential
477 tools for the intercomparison of NMR spectra. *J. Pharm. Biomed. Anal.* 93 (2014) 3-16.

- 478 [26] T. Jézéquel, C. Deborde, M. Maucourt, V. Zhendre, A. Moing, P. Giraudeau, Absolute
479 quantification of metabolites in tomato fruit extracts by fast 2D NMR. *Metabolomics* 11 (2015)
480 1231-1242.
- 481 [27] L. Rouger, B. Gouilleux, M. Pouchet-Gellez, J.N. Dumez, P. Giraudeau, Ultrafast double-
482 quantum NMR spectroscopy with optimized sensitivity for the analysis of mixtures. *Analyst*
483 141 (2016) 1686-1692.
- 484 [28] C. Mauve, S. Khlifi, F. Gilard, G. Mouille, J. Farjon, Sensitive, highly resolved, and
485 quantitative (1)H-(13)C NMR data in one go for tracking metabolites in vegetal extracts. *Chem.*
486 *Commun.* 52 (2016) 6142-6145.
- 487 [29] S. Khlifi, C. Mauve, N. Glab, F. Gilard, M. Hodges, G. Mouilled, J. Farjon, An easier analysis
488 of complex mixtures with highly resolved and sensitivity enhanced 2D quantitative NMR:
489 application to tracking sugar phosphates in plants. *Analytical Methods* 9 (2017) 2328-2333.
- 490 [30] K. Hu, W.M. Westler, J.L. Markley, Simultaneous Quantification and Identification of
491 Individual Chemicals in Metabolite Mixtures by Two-Dimensional Extrapolated Time-Zero
492 1H-13C HSQC (HSQC0). *J. Am. Chem. Soc.* 133 (2011) 1662–1665.
- 493 [31] J. Farjon, C. Milande, E. Martineau, S. Akoka, P. Giraudeau, The FAQUIRE Approach: FAst,
494 QUantitative, hIghly Resolved and sEnsitivity Enhanced 1H, 13C Data. *Anal. Chem.* 90 (2018)
495 1845-1851.
- 496 [32] B. Worley, R. Powers, Deterministic multidimensional nonuniform gap sampling. *J. Mag.*
497 *Reson.* 261 (2015) 19-26.
- 498 [33] A. Stern, J. Hoch, A new approach to compressed sensing for NMR. *Magn. Reson. Chem.* 53
499 (2015) 908-912.
- 500 [34] V.Y. Orekhov, V.A. Jaravine, Analysis of non-uniformly sampled spectra with multi-
501 dimensional decomposition. *Prog. Nucl. Magn. Reson. Spectrosc.* 59 (2011) 271-292.
- 502 [35] T. Rundlöfa, M. Mathiassona, S. Bekiroglub, B. Hakkarainena, T. Bowden, T. Arvidssona,
503 Survey and qualification of internal standards for quantification by 1H NMR spectroscopy. *J.*
504 *Pharm. Biomed. Anal.* 52 (2010) 645-651.
- 505 [36] R.S. Phansalkar, C. Simmler, J. Bisson, S.N. Chen, D.C. Lankin, J.B. McAlpine, M. Niemitz,
506 G.F. Pauli, Evolution of Quantitative Measures in NMR: Quantum Mechanical qHNMR
507 Advances Chemical Standardization of a Red Clover (*Trifolium pratense*) Extract. *J. Nat Prod.*
508 80 (2017) 634-647.

- 509 [37] J.G. Napolitano, T. Gödecke, D.C. Lankin, B.U. Jaki, J.B. McAlpine, S.N. Chen, G.F. Pauli,
510 Orthogonal analytical methods for botanical standardization: Determination of green tea
511 catechins by qNMR and LC-MS/MS. *J. Pharm. Biomed. Anal.* 93 (2014) 59-67.
- 512 [38] T. Schoenberger, S. Menges, M.A. Bernstein, M. Pérez, F. Seoane, S. Sýkora, C. Cobas,
513 Improving the Performance of High-Precision qNMR Measurements by a Double Integration
514 Procedure in Practical Cases. *Anal. Chem.* (2016) 3836-3843.
- 515 [39] M. Tiainen, P. Soinen, R. Laatikainen, J. Magn. Reson. Quantitative Quantum Mechanical
516 Spectral Analysis (qQMSA) of ¹H NMR spectra of complex mixtures and biofluids 242 (2014)
517 67-78.
- 518 [40] P.M. Le, M. McCooeye, A. Windust, Characterization of the alkaloids in goldenseal
519 (*Hydrastis canadensis*) root by high resolution Orbitrap LC-MS(n). *Anal. Bioanal. Chem.* 405
520 (2013) 4487-4498.
- 521 [41] A. Bornet, M. Maucourt, C. Deborde, D. Jacob, J. Milani, B. Vuichoud, X. Ji, J.-N. Dumez,
522 A. Moing, G. Bodenhausen, S. Jannin, P. Giraudeau, Highly Repeatable Dissolution Dynamic
523 Nuclear Polarization for Heteronuclear NMR Metabolomics. *Anal. Chem.* 88 (2016) 6179-
524 6183.
- 525 [42] V. Daniele, F. Legrand, P. Berthault, J. Dumez, G. Huber, Single-Scan Multidimensional
526 NMR Analysis of Mixtures at Sub-Millimolar Concentrations by using SABRE
527 Hyperpolarization. *Chemphyschem.* 16 (2015) 3413-3417.
- 528 [43] B. Plainchont, P. Berruyer, J.-N. Dumez, S. Jannin, P. Giraudeau, Dynamic Nuclear
529 Polarization Opens New Perspectives for NMR Spectroscopy in Analytical Chemistry. *Anal.*
530 *Chem.* 90 (2018) 3639-3650.

531