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Evaluation of low-field versus high-field proton NMR spectroscopy for quality control of cinnamon samples

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1 **Abstract**

2 The use of cinnamon as a spice for cooking or as a dietary supplement for its nutraceutical
3 properties is widespread around the world. In this study, 28 samples, 14 cooking spices and
4 14 dietary supplements, were analysed using both high-field (400 MHz) and low-field (60
5 MHz) NMR. High-field NMR analysis was performed for profiling, quantitative analysis and
6 classification of the samples between Ceylon cinnamon and cassia cinnamon. Then, the
7 capabilities and limitations of low-field NMR for quality control of cinnamon were studied
8 and discussed. At last, the chemometric treatment of low-field NMR spectra has proved to be
9 an interesting way of predicting quantification of their contents in coumarin and (E)-
10 cinnamaldehyde. For dietary supplements, this approach allowed to identify samples
11 containing a higher quantity of hepatotoxic coumarin and thus presenting a potential risk to
12 the health of consumers.

13

14 **Keywords: cinnamon, NMR, compact NMR, flavor, dietary supplement, nutraceuticals**

15

16

17 **1. Introduction**

18 Cinnamon is a well-known spice widely used for cooking in many countries around
19 the world. Recent studies reviewed the use of cinnamon in human health with its beneficial as
20 well as its adverse effects (Silva, Bernardo, Singh, & Mesquita, 2019; Hajimonfarednejad,
21 Ostovar, Raei, Hashempur, Mayer, & Heydari, 2019). Medicinal properties against diabetes,
22 inflammations, gastrointestinal disorders, urinary infections and as a neuroprotective or
23 antimicrobial agent were reported (Nabavi, Di Lorenzo, Izadi, Sobarzo-Sanchez, Daglia, &
24 Nabavi, 2015; Silva, Bernardo, Singh, & Mesquita, 2019). Because of its actual or perceived
25 medicinal properties, many health and cosmetic products take nowadays advantage of
26 cinnamon.

27 Two main varieties of cinnamon are commonly available, "true cinnamon" or Ceylon
28 cinnamon which corresponds to the *Cinnamomum verum* species, and cassia cinnamon which
29 has several origins. The most common is Chinese cassia (*Cinnamomum cassia* Blume, syn.
30 *Cinnamomum aromaticum* Nees) but there are also Saigon cinnamon (*Cinnamomum loureirii*
31 Nees) from Vietnam and Indonesian cassia (*Cinnamomum burmanini* Blume), mainly from
32 Indonesia and the Philippines (Avula, Smillie, Wang, Zweigenbaum, & Khan, 2015;
33 Woehrlin, Fry, Abraham, & Preiss-Weigert, 2010).

34 "True cinnamon" has a characteristic taste different from cassia one because of a
35 different chemical composition. Ceylon cinnamon has low levels of coumarin (0.004% w/w)
36 by comparison to cassia cinnamon that contains until 1% (Avula, Smillie, Wang,
37 Zweigenbaum, & Khan, 2015). Coumarin is known to be hepatotoxic and a tolerable daily
38 intake of 0.1 mg/kg body weight has been defined in Europe to insure safety of consumers
39 (EFSA, 2004). As cassia cinnamon is cheaper, it is more and more widely available in the
40 European market; it is thus relevant to explore new quality control methods for the analysis of
41 cinnamon samples.

42 The aim of this study was to evaluate the ability of low-field (LF) NMR as a method
43 for quality control of cinnamon samples. With the recent introduction of compact NMR
44 spectrometers that use permanent magnets giving rise to proton NMR frequencies between 40
45 and 80 MHz, new areas of applications have been opened (Blumich, 2019; Blumich & Singh,
46 2018). In the field of food chemistry, analysis of meat (Jakes, Gerdova, Defernez, Watson,
47 McCallum, Limer, et al., 2015), authenticity of Arabica coffee (Gunning, Defernez, Watson,
48 Beadman, Colquhoun, Le Gall, et al., 2018), analysis of edible oils (McDowell, Defernez,
49 Kemsley, Elliott, & Koidis, 2019; Parker, Limer, Watson, Defernez, Williamson, & Kemsley,

50 2014) and adulteration of dietary supplements with added active pharmaceutical ingredients
51 (Pages, Gerdova, Williamson, Gilard, Martino, & Malet-Martino, 2014) were described.

52 The present work is the first application of LF NMR to cinnamon analysis for both
53 food spices and dietary supplements. The analyses were performed using high-field (HF)
54 NMR as the reference method and the LF capabilities and limits were assessed and discussed.
55 At last, a statistical treatment of LF NMR data was explored to model LF data and predict
56 quantification.

57

58 **2. Experimental**

59 **2.1. Materials**

60 (E)-cinnamaldehyde (Acros Organic, 99%), coumarin (Acros Organic, 99%),
61 cinnamyl alcohol (Acros Organic, 99%), dimethyl maleate (Sigma-Aldrich, 99%), o-methoxy-
62 cinnamaldehyde (Sigma-Aldrich, 98%), (E)-cinnamic acid (Acros Organic, 98%), eugenol
63 (Sigma-Aldrich, 99%), 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid sodium salt (TSP, Acros
64 Organic, 99.8% D), tetramethylsilane (TMS, Acros Organic, 99% D), Eu(fod)₃ (Sigma-
65 Aldrich, 99%), EuCl₃ (Sigma-Aldrich, 99.9%), Eu(NO₃)₃.5H₂O (Sigma-Aldrich, 99.9%),
66 dimethyl sulfone (Sigma-Aldrich, 98%) were used as received without any further
67 purification unless otherwise described. All deuterated solvents (99.80% D) were supplied
68 from Eurisotop. An authentic sample of *Cinnamomum verum* was obtained for Extrasynthese
69 (Lyon, France).

70 The fourteen samples of cinnamon for cooking were bought from French malls or
71 markets, and the fourteen dietary supplements were purchased online or in French specialized
72 shops like organic groceries or dietetic stores (Table 1). All samples were analysed before
73 their expiry date.

74

75 **2.2. Preparation of samples for NMR analysis**

76 **2.2.1. Extraction methodology**

77 All the preliminary extraction experiments described in this section were analysed
78 with the HF NMR spectrometer. In order to choose the best solvent to extract commercial
79 samples, solid-liquid extractions with deuterated methanol (MeOH-d₄), deuterated
80 chloroform (CDCl₃), heavy water (D₂O) and mixtures of solvents MeOH-d₄:D₂O (50%:50%;
81 80%:20%) were investigated. A liquid-liquid extraction of an aqueous suspension of
82 cinnamon powder with CDCl₃ was also tested.

83 Briefly, around 100 mg of the powdered commercial cinnamon sample **25** were
84 weighed and mixed with 1 mL of the chosen solvent, shaken for 20 s with a vortexer,
85 sonicated for 10 min in a stoppered tube and centrifuged at 5000 g for 5 min. For the liquid-
86 liquid extraction, 0.5 mL D₂O were added to the powdered sample followed by 1 mL CDCl₃.
87 The sample was then mixed, sonicated and centrifuged as described above. After
88 centrifugation, the CDCl₃ phase was submitted to NMR analysis. In the final step, 700 μL of
89 supernatant were put into a 5 mm NMR tube and 60 μL of TSP were added (10 mM in
90 MeOH-d₄) for NMR analysis. For the samples extracted with CDCl₃, 10 μL of a solution
91 (120 mM) of dimethylsulfone as internal reference were mixed with 500 μL of supernatant.
92 Each extraction condition was tested in duplicate.

93 Experiments with successive extractions of the powdered samples were also
94 implemented. For the first step, the extraction protocol was the same as previously described,
95 i.e. around 100 mg of samples **10** (n=2) and **25** (n=2) were mixed with 1 mL MeOH-d₄,
96 shaken for 20 s with a vortexer, sonicated for 10 min and centrifuged. The supernatant was
97 removed and 700 μL were mixed with 60 μL TSP (10 mM, MeOH-d₄) for NMR analysis.
98 The residual powder was dried on a filter paper to absorb the residual liquid and then re-
99 extracted leading to the second extraction sample. This step was repeated once to obtain the
100 third extraction sample. The supernatants from the second and third extractions were then
101 analysed by mixing 700 μL of supernatant with 60 μL TSP (10 mM, MeOH-d₄). The mean
102 results of extraction provided a recovery of $88.2 \pm 0.9\%$ and $86.1 \pm 1.8\%$ for (E)-
103 cinnamaldehyde and coumarin respectively in the first extraction. Residual active compounds
104 were measured at $10.6 \pm 0.8\%$ and $12.4 \pm 1.6\%$ in the second extraction and $1.2 \pm 0.1\%$ and
105 $1.5 \pm 0.2\%$ in the third extraction. The same procedure of three successive extractions was
106 repeated for the two samples **10** and **25** with only 15 mg of powder. The recoveries were 96.8
107 $\pm 0.9\%$ and $97.5 \pm 0.7\%$ for (E)-cinnamaldehyde and coumarin respectively in the first
108 extraction, $3.2 \pm 0.9\%$ and $2.5 \pm 0.7\%$ in the second extraction and nothing in the third one.

109

110 **2.2.2. Preparation of commercial samples for NMR analysis**

111 One mL of MeOH-d₄ was added to around 15 or 100 mg exactly weighed of each
112 powdered commercial cinnamon sample placed in a stoppered tube. After 20 s of vortex
113 mixing and 10 min of sonication, the mixture was centrifuged at 5000 g for 5 min. 700 μL of
114 supernatant were then collected, mixed with 60 μL of TSP (10 mM) and transferred into a 5
115 mm NMR tube. The extracts from 100 mg of cinnamon powder were analysed with both HF
116 and LF NMR and those from 15 mg only with HF NMR for the absolute quantification of (E)-

117 cinnamaldehyde and coumarin. All samples were prepared and analysed in duplicate for both
118 HF and LF NMR.

119

120 ***2.2.3. Experiments with chemical shift reagents***

121 100 mg of sample **25** were extracted with MeOD-d4 as described in section 2.2.2. The
122 supernatant (700 μ L) was mixed with 20 μ L of a solution of Eu(fod)₃, EuCl₃, or Eu(NO₃)₃ in
123 MeOH-d4 at a concentration of 750 mM. TSP (60 μ L, 10 mM) was added as an internal
124 reference. The final concentrations for europium salts were 20 mM. For the blank sample, 20
125 μ L of MeOD-d4 were used instead of the europium salt solution.

126

127 ***2.2.4. Standard samples for statistical analysis***

128 Solutions of standard (E)-cinnamaldehyde and coumarin in MeOH-d4 at different
129 concentrations were prepared as follows. Stock solutions (5 mg/mL) of each standard were
130 diluted to obtain concentrations corresponding to real amounts in commercial samples, i.e. 3,
131 5, 8, 10, 12, 15, 17, 20, 25 and 30 mg/g for (E)-cinnamaldehyde and 2, 4 and 6 mg/g for
132 coumarin. These values take into account a correction factor linked to the extraction yield of
133 real samples (see section 3.2.1.) A blank MeOH-d4 solution was also analysed. A solution of
134 TSP as internal reference was added for NMR analysis as previously described.

135

136 2.3. NMR analysis

137 2.3.1. HF NMR analysis

138 HF ^1H NMR experiments were performed on a Bruker Avance 400 spectrometer
139 (Bruker Biospin AG, Fallanden, Switzerland) equipped with a TXO probe at 298 K. For
140 qualitative experiments, HF ^1H NMR spectra were recorded with the following parameters:
141 pulse width 13.5 μs (flip angle 90°), acquisition time 2.56 s, spectral width 16 ppm, 32K data
142 points and relaxation delay 1.0 s. The number of scans was 32 or 128, corresponding to a
143 recording time of *ca.* 2 or 8 min. Typical acquisition parameters for quantitative experiments
144 were as follows: pulse width 4.53 μs (flip angle 30°), acquisition time 5.12 s, spectral width
145 16 ppm, 64K data points, and a relaxation delay of 10 s; the number of scans was 256 or 512
146 giving a recording time of *ca.* 65 or 130 min. A 30° pulse angle was selected instead of 90° to
147 both ensure relaxation and save recording time because the relaxation delay required is then
148 lower. The signal of TSP set at 0 ppm was used as an internal reference for chemical shift
149 measurement and quantification.

150 Before quantitative analyses, the longitudinal relaxation times T1 of protons of (E)-
151 cinnamaldehyde, coumarin, and TSP in standard solutions and in the commercial samples **10**
152 and **25** were measured by the inversion-recovery pulse sequence method with recovery delays
153 from 0.001 to 50 s. The T1s found were less than 6 s (i.e. H1 (E)-cinnamaldehyde 5.0 s, H4
154 coumarin 5.8 s), whereas the TSP protons have a relaxation time of 3.7 s. All ^1H resonances
155 were thus considered as fully relaxed since 99% of the signal intensity of the proton with the
156 longest T1 (5.8 s) were recovered with a repetition time of 15.1 s.

157 NMR assignments of (E)-cinnamaldehyde, coumarin, (E)-cinnamic acid, o-methoxy-
158 cinnamaldehyde, (E)-cinnamyl alcohol and eugenol were achieved by comparison with
159 spectra of standard compounds and with ^{13}C and 2D (gCOSY, gHSQC, gHMBC) experiments
160 to clear up ambiguities. Data are given in the supplementary section (Table S1).

161 1D ^1H NMR data were processed using the TOPSPIN 3.1 software with one level of
162 zero-filling and Fourier transformation after multiplying FIDs by an exponential line-
163 broadening function of 0.3 Hz, then phasing and baseline correction were applied. T1 values
164 were calculated with the T1/T2 relaxation module included in the TOPSPIN software.

165

166 2.3.2. LF NMR analysis

167 Spectra were acquired on a PulsarTM benchtop NMR spectrometer (Oxford
168 Instruments, Abingdon, UK) operating at a frequency of 59.7 MHz for ^1H . The temperature

169 inside the spectrometer was 310 K. The acquisition was performed with the SpinFlow 1.2.0.1
170 software (Oxford Instruments) and the processing with MNova 11.0 (Mestrelab Research,
171 Santiago de Compostela, Spain). FIDs were recorded with a flip angle of 90° (12.0 μs), a
172 spectral width of 5000 Hz, and 8K complex points (acquisition time of 1.64 s). The relaxation
173 delay was set at 2 s, and 2000 transients were recorded leading to a total acquisition time of 2
174 h. For data processing, the FIDs with one level of zero-filling were apodized with an
175 exponential (0.3 Hz) filter and automatic Whittaker smoother baseline correction was applied.

176 The T1s of standard (E)-cinnamaldehyde (H1), coumarin (left part of the H4 doublet)
177 and TSP were measured (n=4) in MeOH-d4 by the inversion recovery pulse sequence method
178 with 20 recovery delays from 0.001 to 40 s. Values were 3.8 ± 0.1 s, 4.3 ± 0.2 s and 3.1 ± 0.1
179 s, respectively. T1 measurements were also performed on the commercial samples **10** and **25**
180 in duplicate. Values obtained were 3.5 ± 0.2 s, 3.9 ± 0.4 s and 3.1 ± 0.1 s for T1 of (E)-
181 cinnamaldehyde (H1), coumarin (H4) and TSP, respectively. From these T1 measurements, it
182 was possible to calculate the recovery of the signals compared to TSP in our recording
183 conditions. It was 90% for (E)-cinnamaldehyde and 82% for coumarin with RSD <2% for
184 standard samples but reaching 5% for commercial samples for which variability is greater.

185 At last, the LOD and the apparent LOQ were determined by analyzing the diluted
186 standard solutions of known concentration for signal-to-noise ratios (SNR) of 3 and 10,
187 respectively. We mention here an "apparent" LOQ insofar as we will show later that the
188 experimental conditions used were not fully quantitative. The SNR was estimated using the
189 SNR peak calculator tool included in the MNova software. The LOD of the left signal of H4
190 of coumarin was 0.096 mg/mL and the apparent LOQ was 0.29 mg/mL for the same signal.
191 For the H1 of (E)-cinnamaldehyde, the LOD and apparent LOQ values were 0.048 mg/mL
192 and 0.19 mg/mL respectively. The limits of apparent quantification thus corresponded to 1.9
193 mg/g and 2.9 mg/g for (E)-cinnamaldehyde and coumarin, respectively, in real samples.

194

195 **2.4. HF NMR quantification**

196 Concentrations were measured by comparing the signal areas of targeted protons [i.e.
197 H1 (δ 9.66 ppm, doublet (d)) of (E)-cinnamaldehyde, H4 (δ 7.96 ppm, d) of coumarin, H2 (δ
198 6.47 ppm, d) of (E)-cinnamic acid, right part of the H1 d at 9.62 ppm of o-methoxy-
199 cinnamaldehyde, H2 (δ 6.36 ppm, doublet triplet) of (E)-cinnamyl alcohol, and -O-CH₃ (δ
200 3.82 ppm, singlet) of eugenol (Table S1)] to the area of the reference signal, the area of each
201 NMR peak being directly proportional to the number of protons giving rise to it.

202 The amount of each compound expressed in mg/g of sample was determined using the
203 following general equation:

$$204 \quad Amount \ (mg/g) = [REF] \times \frac{A_x}{A_{REF}} \times \frac{N_{REF}}{N_x} \times V \times \frac{MW_x}{W_{sample}} \quad (1)$$

205 [REF] is the concentration of the internal reference (TSP),

206 A_x and A_{REF} the integrated signal areas of the targeted compound and TSP,
207 respectively,

208 N_x and N_{REF} the number of protons giving rise to the signal considered,

209 V the volume of solution,

210 MW_x the molecular weight of each compound,

211 W_{sample} the mass of cinnamon sample used for the NMR assay.

212 The standard deviations (SD) (Table 1) were obtained from the amounts calculated
213 from $n=2$ or $n=3$ experiments depending on samples.

214

215 **2.5. Chemometric analysis of LF spectra**

216 Fourier transform, phasing and manual multipoint baseline correction were performed
217 on each spectrum. Then the data matrix was obtained by an automatic binning approach (bin
218 width of 0.01 ppm) and buckets were normalized by dividing their areas by that of the internal
219 reference TSP for standard solutions and also by the correction factor linked to the extraction
220 yield (see section 3.2.1.) for commercial samples. Principal Component Analyses (PCA) with
221 Pareto scaling were performed on the LF NMR spectra of all commercial samples (56
222 spectra). Partial least squares regressions (PLS1) were built with the areas of specific LF
223 NMR signals of standard (E)-cinnamaldehyde (22 spectra) and coumarin (8 spectra) solutions
224 as the set of independent variables (X) and their known concentrations as the set of dependent
225 variables (Y). These PLS1 models were validated using cross-validation method (Q^2) and CV-
226 ANOVA. The quantification of (E)-cinnamaldehyde and coumarin in commercial samples
227 was then predicted according to their respective PLS1 models. PCAs and PLS1s were
228 performed with the SIMCA-P+ 13.0 software (Umetrics, Umea, Sweden).

229

230 **3. Results and discussion**

231 **3.1. HF and LF NMR assignment of signals**

232 Figure 1 compares the spectra of the same NMR tube containing a solution of standard
233 (E)-cinnamaldehyde (A and B) or coumarin (C and D) recorded at 400 (A and C) and 60 (B
234 and D) MHz. As the coupling constants are independent of the magnetic field, the signals

235 appear obviously much more spread with more overlaps in the spectrum at LF. For instance
236 the H2 doublet of doublet of (E)-cinnamaldehyde becomes a second order system with a
237 slight "roof" effect at LF. Likewise, H3 and H4 of coumarin give two doublets at HF ($\Delta\nu/J$
238 >60) but tend to an AB system at LF with $\Delta\nu/J=9.4$. Besides, some signals overlap e.g. H7, 8,
239 9 and 10 of coumarin co-resonate as a broad multiplet that spreads from 7.1 to 7.7 ppm at LF.
240 NMR assignments of spectra recorded at 400 MHz are reported in Table S1.

241 Figure 2 illustrates the ^1H NMR profiles of the cinnamon food sample **2**. The HF
242 spectrum shows, in addition to signals of coumarin and (E)-cinnamaldehyde, some typical
243 resonances of o-methoxy-cinnamaldehyde, cinnamyl alcohol and (E)-cinnamic acid that were
244 assigned by comparison to previous work (Farag, Labib, Noletto, Porzel, & Wessjohann,
245 2018) and by addition of standard compounds in the medium. NMR assignments of spectra of
246 standard compounds recorded at 400 MHz are reported in Table S1. Another flavouring
247 compound, eugenol, was detected in HF spectra of some samples (not present in the spectrum
248 shown Figure 2A) thanks to its characteristic singlet at 3.82 ppm (O-CH₃) (Farag, Labib,
249 Noletto, Porzel, & Wessjohann, 2018) and other signals from its aromatic ring (δ (ppm) 6.72, d
250 2 Hz; 6.69, d 8.0 Hz; 6.58, dd 8.0, 2.0 Hz). Only (E)-cinnamaldehyde (H1, δ 9.66 ppm and
251 H2, δ 6.73 ppm) and coumarin (H3, δ 6.42 ppm and H4, δ 7.94 ppm) signals are readily
252 observed in the LF spectrum of the cinnamon food sample **2** (Figure 2B). To complete the
253 samples screening, the HF ^1H NMR spectrum of an additional sample of an authentic standard
254 of *Cinnamomum verum* is reported in Figure S1 and compared with sample **2**. In this
255 spectrum, signals of (E)-cinnamaldehyde, o-methoxy-cinnamaldehyde, cinnamyl alcohol, (E)-
256 cinnamic acid and eugenol were observed whereas those of coumarin could not be detected.

257

258 **3.2. Implementation of the experimental conditions**

259 **3.2.1. Extraction tests and solvent choice**

260 To select the best extraction procedure, different extraction tests were performed on
261 the commercial dietary supplement **25** which contains significant amounts of (E)-
262 cinnamaldehyde and coumarin as shown later.

263 In the literature different solvents have been proposed for the quantification of active
264 compounds of cinnamon. The most common are methanol (Archer, 1988; Farag, Labib,
265 Noletto, Porzel, & Wessjohann, 2018; Wang, Avula, Nanayakkara, Zhao, & Khan, 2013) and
266 methanol/water or ethanol/water mixtures (Ballin & Sørensen, 2014; Blahova & Svobodova,
267 2012; Ding, Wu, Liang, Chen, Tran, Hong, et al., 2011; Lungarini, Aureli, & Coni, 2008;

268 Sproll, Ruge, Andlauer, Godelmann, & Lachenmeier, 2008). Only one study carried out by
269 NMR used CDCl₃ as extracting solvent (Killday, Markus, Fischer, & Colson, 2014).

270 A first set of experiments was thus dedicated to the screening of different solvents.
271 MeOH-d₄, mixtures MeOH-d₄:D₂O (80:20 and 50:50) and CDCl₃ were tested with a direct
272 extraction of 100 mg of powder. Moreover, a liquid-liquid extraction of a D₂O suspension of
273 powder by CDCl₃ was also explored. The extraction yields were evaluated by integration of
274 the H1 signal of (E)-cinnamaldehyde and H3 and H4 signals of coumarin on the HF ¹H NMR
275 spectra. Results are reported in Figure S2. The solvents that extracted the highest quantities of
276 (E)-cinnamaldehyde and coumarin were MeOH-d₄ and the mixture MeOH-d₄:D₂O in the
277 80:20 ratio. This better extraction yield with methanol is in agreement with most published
278 studies (Ballin & Sørensen, 2014; Blahova & Svobodova, 2012; Farag, Labib, Noletto, Porzel,
279 & Wessjohann, 2018). The extraction yields found in CDCl₃ relatively to MeOH-d₄ were
280 poor (10.6 and 9.5% for (E)-cinnamaldehyde and coumarin, respectively) demonstrating that
281 it was not possible to implement a quantitative analysis of cinnamon samples in this solvent
282 unlike previously reported (Killday, Markus, Fischer, & Colson, 2014). For the next steps of
283 the study, since our objective was to propose a method on a LF NMR spectrometer, we chose
284 to use MeOH-d₄ and not a MeOH-d₄:D₂O mixture as extraction solvent. Indeed a single
285 solvent facilitates the implementation of the LF NMR experiments because the lock procedure
286 is more convenient.

287 In order to establish the extraction procedure, three successive extractions were
288 performed on 100 mg or 15 mg of samples **10** and **25**. From these preliminary experiments
289 (see results in section 2.2.1), we chose to analyse all the commercial samples after a single
290 extraction in MeOH-d₄. HF NMR analyses were done from both 15 and 100 mg of powder.
291 The 15 mg samples were considered for the assay of (E)-cinnamaldehyde and coumarin as we
292 showed that a lower amount of powder ensures a better extraction yield, and the 100 mg
293 samples for the assay of the other chemicals (eugenol, o-methoxy-cinnamaldehyde, (E)-
294 cinnamyl alcohol, (E)-cinnamic acid) whose concentrations are much lower. The LF
295 capabilities were assessed with 100 mg of powder only (discussed in 3.2.3. section).

296

297 **3.2.2. LF NMR exploratory experiments with Chemical Shift Reagents (CSR)**

298 CSR have been explored extensively in the years 1960-70 when NMR spectrometers
299 operated with ¹H frequencies around or under 100 MHz. Recently, the use of CSR was
300 reported for the new generation of compact LF NMR spectrometers to increase the frequency
301 dispersion of the targeted NMR signals (Singh & Blumich, 2016). In most experiments with

302 CSR, the best solvent for improving spectral resolution is CDCl_3 . In the context of cinnamon
303 analysis, we showed above that CDCl_3 did not provide a good extraction yield and that
304 MeOH-d_4 was the best solvent. Although the interaction of the paramagnetic ion with the
305 oxygen atom of MeOH-d_4 reduces its availability for interacting with other species, we
306 nevertheless decided to test CSR in our experimental conditions. Our aim was to spread the
307 signals and particularly to downfield slightly (≈ 20 Hz) the H4 signal of coumarin thus making
308 its area easier to measure. Three CSR ($\text{Eu}(\text{fod})_3$, $\text{Eu}(\text{NO}_3)_3$ and EuCl_3) were tested (Figure
309 S3). For a TSP calibrated at 0 ppm, the chemical shift of the H4 signal did not shift
310 significantly. Indeed, the left part of the doublet moves from 8.02 ppm without CSR to 8.03
311 ($\Delta\delta = 0.5$ Hz), 8.09 ($\Delta\delta = 4.4$ Hz) and 8.18 ppm ($\Delta\delta = 9.7$ Hz) with addition of $\text{Eu}(\text{fod})_3$,
312 $\text{Eu}(\text{NO}_3)_3$ and EuCl_3 respectively. Moreover, for EuCl_3 which induces the highest chemical
313 shift modification of the coumarin resonance, a broadening of the signals, as well as a sharp
314 decrease of the (E)-cinnamaldehyde signal, were observed because EuCl_3 induced strongest
315 interaction with the chemicals. From these experiments, it appears that the use of CSR for the
316 analysis of cinnamon extracts under our experimental conditions did not constitute an
317 interesting tool for better discrimination of signals. Experiments with CSR were not thus
318 deeply investigated due to the restricted solvent choice imposed by the extraction step.

319

320 ***3.2.3. LF quantitative analysis: attempt to implement and limitations***

321 Quantitative HF NMR is nowadays a recognized method for the analysis of complex
322 samples (Bharti & Roy, 2012; Monakhova & Diehl, 2018; Simmler, Napolitano, McAlpine,
323 Chen, & Pauli, 2014) and was taken as the standard reference method in this study. However,
324 the implementation of LF quantitative analysis remains a challenging task (Matviychuk, Yeo,
325 & Holland, 2019). In this section, we discuss the attempt to set up and encountered limitations
326 for cinnamon analysis.

327 The first option was to quantify the samples prepared with a correct extraction yield,
328 i.e. using the same solution for both HF and LF NMR quantifications. For HF ^1H NMR
329 quantitative analysis, 15 mg of commercial sample were sufficient to measure accurate
330 concentrations in a reasonable recording time of 1 h with a LOQ of 0.09 mg/g and 0.15 mg/g
331 for (E)-cinnamaldehyde and coumarin respectively. With the same parameters and recording
332 time on the LF spectrometer, the LOQ was estimated at 6 mg/g and 10 mg/g due to the
333 decrease of the SNR per unit time by a factor of ≈ 60 . This means that if we wanted to obtain
334 a correct SNR ratio for coumarin quantification, the experimental time would have been
335 superior to 100 h for each analysis of commercial samples. Even with extracts from 100 mg of

336 commercial powder, at least 17 h were necessary to obtain a SNR of ≈ 10 for samples
337 containing ≈ 3 mg/g of coumarin (Figure S4). It thus appeared that it was not possible to
338 implement correct quantitative measurements of coumarin with both a full relaxation of the
339 signals and under a reasonable recording time compatible with routine analyses.

340 Another way to proceed was to run LF NMR analysis using concentrated samples, i.e.
341 samples extracted from 100 mg, recorded in a reasonable experiment time of 2 h in non-fully
342 relaxed conditions, and to consider several correction factors: (i) a correction factor linked to
343 the extraction yield, (ii) a second integrating the fact that signals were not fully relaxed in 2 h
344 of recording (Bharti, Sinha, Joshi, Mandal, Roy, & Khetrpal, 2008), (iii) a third applied to
345 coumarin only, linked to the roof effect of the second order system of H4 and H3 protons.
346 Moreover, the LF quantification was also altered by the automatic baseline correction
347 procedure that introduces experimental errors related to the SNR of the considered signal.
348 Taking into account all the correction factors described above, the global resulting RSD for
349 quantification would be $>15\%$. We thus concluded that cinnamon samples could not be
350 accurately quantified at LF in our experimental conditions. Nevertheless, we will further
351 investigate in section 3.3.2. a statistical treatment of LF NMR spectra obtained from
352 commercial samples recorded for 2 h after extraction of 100 mg samples.

353

354 **3.3. Analysis of commercial samples**

355 **3.3.1. HF NMR analysis: from profiling to quantitative analysis**

356 Amounts of (E)-cinnamaldehyde, coumarin, eugenol, o-methoxy-cinnamaldehyde,
357 cinnamyl alcohol and (E)-cinnamic acid in cinnamon commercial samples measured from HF
358 ^1H NMR spectra are reported in Table 1. These data allow distinguishing several categories of
359 samples. In four dietary supplements (**16**, **19**, **21** and **24**), neither (E)-cinnamaldehyde nor
360 coumarin was detected but only signals of fatty acids and/or sugars. None of the typical
361 chemicals of cinnamon being detected, not even (E)-cinnamaldehyde, we assess that these
362 dietary supplements did not contain cinnamon powder (or at trace level) contrary to what was
363 announced on the label. Eugenol which was previously reported as a marker of *Cinnamomum*
364 *verum* (Frag, Labib, Noleto, Porzel, & Wessjohann, 2018; Wang, Avula, Nanayakkara,
365 Zhao, & Khan, 2013) was observed and quantified in samples **5**, **6**, **9**, **17** and **18**. Moreover,
366 coumarin was not detected in these samples which could be thus classified as "true cinnamon"
367 (*Cinnamomum verum*) samples. Indeed, the amount of coumarin in *Cinnamomum verum* (0.04
368 mg/g) is under the LOQ of HF NMR for this compound (0.15 mg/g). Two samples of food
369 cinnamon, **2** and **11**, contained the highest amounts of both (E)-cinnamaldehyde and coumarin

370 in agreement with the Vietnamese origin claimed by the producers and previously reported
371 data on Vietnamese cinnamon (*Cinnamomum loureirii*) (Wang, Avula, Nanayakkara, Zhao, &
372 Khan, 2013). Samples containing detectable levels of coumarin could be classified as cassia
373 cinnamon (samples **1-4**, **8**, **10-15**, **20**, **25-28**) without a more precise classification according
374 to geographical origin or species (*burmanii*, *zeylanicum*...). Classification of samples **7**, **22**
375 and **23** could not be assessed from NMR data insofar as only (E)-cinnamaldehyde was
376 detected. The proposed classification of samples from HF NMR data is reported in Table 1.
377 Moreover, we can observe that the two samples **22** and **23** contain high levels of (E)-cinnamic
378 acid with 4.1 and 3.7 mg/g respectively, which can be due to a longer storage of these samples
379 that leads to the oxidation of (E)-cinnamaldehyde (Woehrlin, Fry, Abraham, & Preiss-
380 Weigert, 2010).

381 The toxicity of cinnamon samples is related to their coumarin content and the
382 hepatotoxic level of 0.1 mg/kg body weight must be considered. Consumption of cinnamon in
383 food is very variable and linked to dietary habits of each country. For instance, it was reported
384 that in Germany the consumption of cinnamon present in various desserts can reach 17 g over
385 the ten days of the Christmas period, so a mean value of 1.7 g/day (Abraham, Wohrlin,
386 Lindtner, Heinemeyer, & Lampen, 2010). In food cinnamon samples reported in Table 1, the
387 highest concentration of coumarin found was 6.1 mg/g. This kind of spice sample may thus
388 present a risk for the health of a big consumer of cinnamon cookies or other Christmas
389 desserts.

390 The issue is different for dietary supplements for which a dosage is recommended by
391 the manufacturer. Indeed, the coumarin content of dietary supplements can be discussed
392 considering the daily amount ingested according to the recommended dosage indicated on the
393 packaging. For samples **15**, **20**, **25-28**, it was calculated, from HF NMR data, between 0.65
394 and 8.43 mg (mean 4.6 mg). For the two dietary supplements **27** and **28**, the daily coumarin
395 intake, 7.72 and 8.43 mg respectively, is over the hepatotoxic level of 0.1 mg/kg considering
396 an average body weight of 70 kg. Therefore, their daily ingestion can be at risk for consumers.
397

398 **3.3.2. LF NMR analysis: from chemometric approach to quality control**

399 As shown above (see section 3.2.3.), the LF quantification of cinnamon samples could
400 not be done correctly. We nevertheless chose to extract 100 mg of powder from commercial
401 samples, to record the LF NMR spectra in non-fully relaxed conditions and then to apply a
402 statistical treatment.

403 First, an unsupervised PCA of all samples was performed as it provides an informative
404 first look at the dataset structure and indicates trends of clustering. The PCA was built from
405 the LF NMR spectra limited to the region of olefinic and aromatic protons (5.7-11 ppm) of all
406 samples (Figure 3A). Other parts of NMR spectra were excluded in order to remove (i) areas
407 affected by the residual signals of water and methanol at 3.8-5.7 ppm and 2.8-3.8 ppm
408 respectively, (ii) signals of fatty acids (0.5-2.5 ppm) and (iii) noise (2.5-2.8 ppm) (Figure 2B).
409 Based on the quantitative HF analysis, three classes of samples were colored on the PCA
410 score plot : samples which contain neither (E)-cinnamaldehyde nor coumarin (**16, 19, 21, 24**)
411 in red, samples in agreement with Ceylan cinnamon i.e. without coumarin but containing
412 eugenol (**5, 6, 9, 17, 18**) in blue, and all the remaining samples in green. Two compact clusters
413 were identified: the first one corresponds to the dietary supplements which contain no
414 cinnamon (in red) and the second to samples containing true cinnamon (*Cinnamomum verum*)
415 (in blue). The wide dispersion of the last group (in green) is related to the NMR signals at 6.4-
416 7.0 ppm and 7.3-7.7 ppm corresponding to all the protons (except H1) of (E)-cinnamaldehyde
417 (Figure 1) which is present in varying amounts (0.2 to 36 mg/g) (Table 1). The effect of (E)-
418 cinnamaldehyde in the samples dispersion is shown by the loading plot illustrated in Figure
419 S5.-For coumarin, in order to check if the whole doublet can be considered for the next step, a
420 PCA score plot based on parts of the H3 (right component) and H4 (left component) was
421 performed. It showed a clear grouping of samples where coumarin was not detected (red and
422 blue in Figure 3B). Samples **2** and **11** with the highest levels of coumarin (6.1 and 5.4 mg/g
423 respectively, Table 1) are located in the lower right part of the score plot. The two samples **22**
424 and **23** seem atypical because they contain significant amounts of (E)-cinnamic acid (Table 1)
425 whose H2 has the same chemical shift than the right resonance of the H3 of coumarin at LF
426 (Figure S6). In the same way, the broad signal around 8 ppm is only detected in sample **22**
427 and to a lesser extent in sample **23**. For the next steps, the two formulations **22** and **23** were no
428 longer considered and the analysis was restricted to the left resonance of the H4 of coumarin.

429 Figure 4 shows that a quantification based on the LF NMR data focused on typical
430 signals of (E)-cinnamaldehyde (H1) or coumarin (left component of the H4 doublet) can be
431 predicted. For this approach, spectra of standard and commercial samples were acquired and
432 processed under the same conditions. Only the correction factor linked to the extraction yields
433 of commercial samples was included in the normalization of the data. PLS1 models were first
434 built with the LF NMR spectra of solutions of standard (E)-cinnamaldehyde and coumarin of
435 known concentrations corresponding to real amounts in commercial samples. Each model was
436 validated ($Q^2 > 0.99$ and $CV\text{-ANOVA} < 10^{-8}$) and used to predict concentrations of these

437 compounds in commercial samples. The first scatter plot (Figure 4A) shows the good
438 correlation ($R^2 = 0.98$) between the predicted concentrations of (E)-cinnamaldehyde and the
439 real concentrations measured with HF NMR for the three classes of samples (red, blue and
440 green) from 0 to ≈ 40 mg/g for the more concentrated samples **2** and **11**. The same result was
441 obtained for coumarin in food samples (Figure 4B). Figure 4C shows the bar plot of the
442 approximate concentrations of coumarin predicted by the same PLS1 but restricted to dietary
443 supplements (without samples **22** and **23**). Here, the maximal recommended daily dosage was
444 considered for data treatment. This graph highlights that the two samples **27** and **28**
445 containing ≈ 6 -8 mg of coumarin may lead to the ingestion of daily high levels of coumarin by
446 the consumer. They can thus pose a health risk because they are located in the hepatotoxic
447 range for a body weight of about 70 kg.

448 This chemometric approach demonstrates that key information on the quality of
449 commercial cinnamon samples can be reached from LF NMR data obtained with a quick and
450 easy sample preparation step and 2 h of NMR spectra recording. Moreover, it allows
451 predicting quantification of (E)-cinnamaldehyde and coumarin levels in commercial samples.

452

453 **Conclusion**

454 The aim of the present study was to evaluate the pros and cons of LF NMR, a
455 technique reported here for the first time for quality control of commercial cinnamon samples.
456 Although LF NMR spectra of cinnamon samples are crowded and assignments tricky due to
457 signal overlaps, characteristic resonances of (E)-cinnamaldehyde and coumarin can
458 nevertheless be detected. LF absolute quantification cannot be proposed as a routine control
459 method for cinnamon analysis but the treatment of LF NMR data with a chemometric
460 approach is a promising way to enhance the power of LF NMR and thus to consider it as a full
461 analytical technique. These encouraging results must lead to continuing the evaluation of
462 benchtop LF NMR spectrometers for quality control applications in food analysis.

463

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466 Chantal Galaup for helpful discussions on CSR reagents.

467

468 **Captions for figures**

469 **Figure 1.** ^1H NMR spectra of solutions of standard (E)-cinnamaldehyde (A and B) and
470 coumarin (C and D) in MeOH- d_4 . (A) and (C) spectra are recorded at HF and (B) and (D) at
471 LF. Recording times were 2 min at HF and 2 h at LF. See Figure 2 for chemical structures and
472 proton numbering of (E)-cinnamaldehyde and coumarin.

473
474 **Figure 2.** (A) HF and (B) LF ^1H NMR spectra of cinnamon food (sample **2**) highlighting
475 typical signals of the main components. Recording times were 8 min at HF and 2 h at LF.

476
477 **Figure 3.** (A) Three-dimensional score plot of a PCA built from the LF NMR data of all the
478 samples using signals in the range 5.7 to 11 ppm. (B) Score plot of a PCA built from the LF
479 NMR data focusing on the signals of H3 (right component at 6.34 ppm) and H4 (left
480 component at 8.03 ppm) of coumarin. Samples without cinnamon (**16, 19, 21, 24**) are colored
481 in red, samples with true cinnamon (**5, 6, 9, 17, 18**) in blue and other samples in green.

482
483 **Figure 4.** Chemometric analysis of LF NMR data. Representation of predicted values
484 obtained from PLS1 models built with LF NMR data versus HF NMR quantitative data for
485 (A) (E)-cinnamaldehyde in all samples and (B) coumarin in food samples. (C) Bar plot of
486 predicted contents of coumarin in dietary supplement samples according to their
487 recommended dosage.

488
489

490 **Declaration of Competing Interest**

491 The authors declare that they have no known competing financial interests or personal
492 relationships that could have appeared to influence the work reported in this paper.

493

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Figure 1

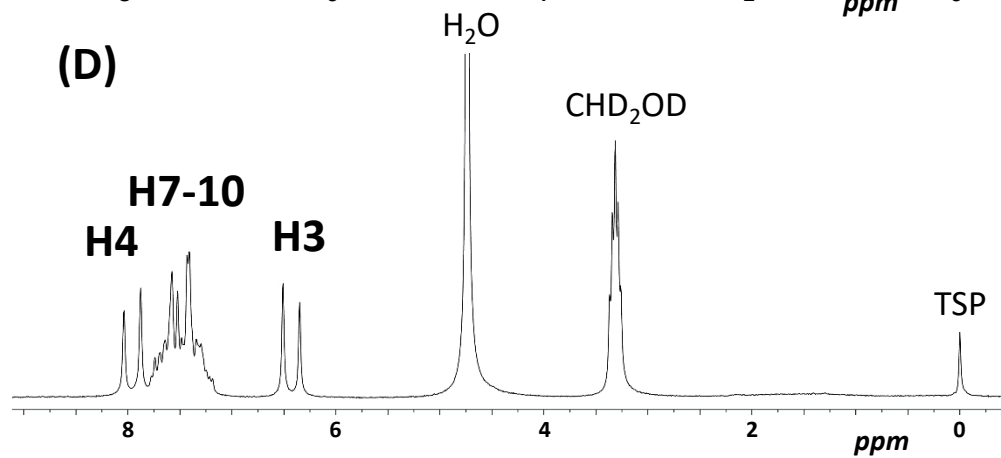
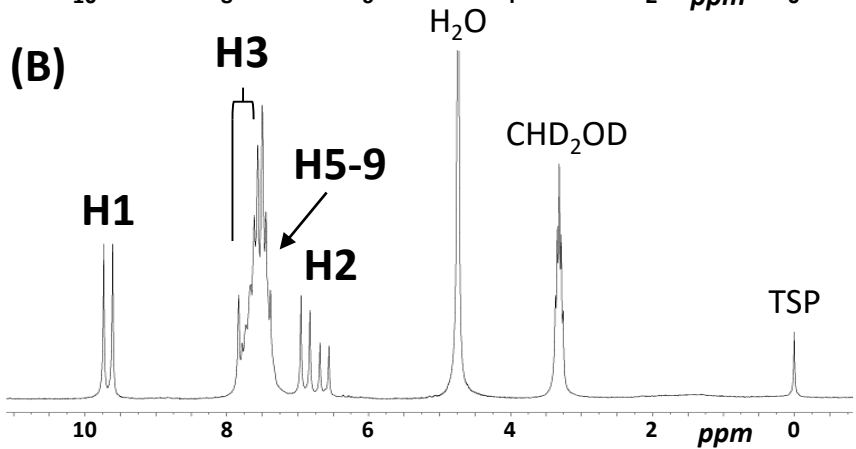
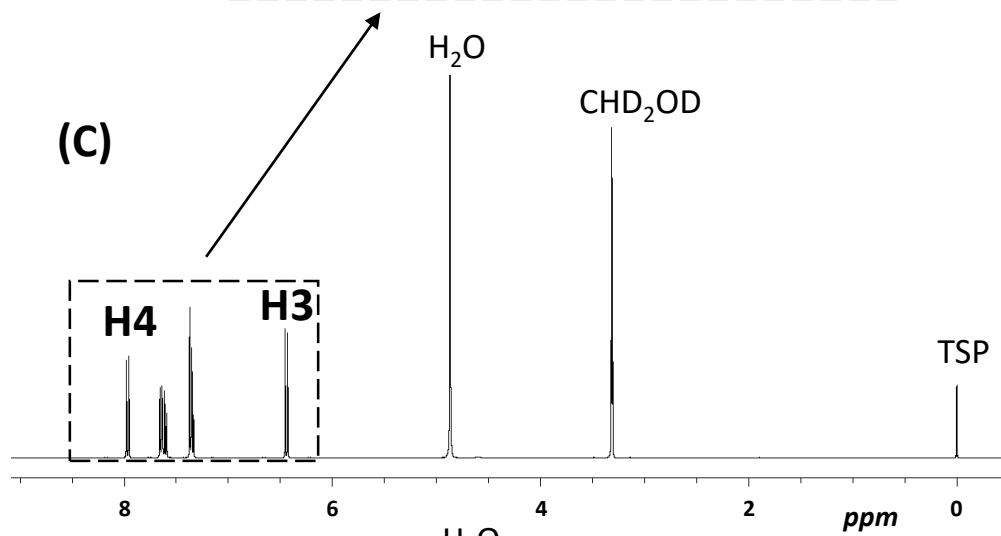
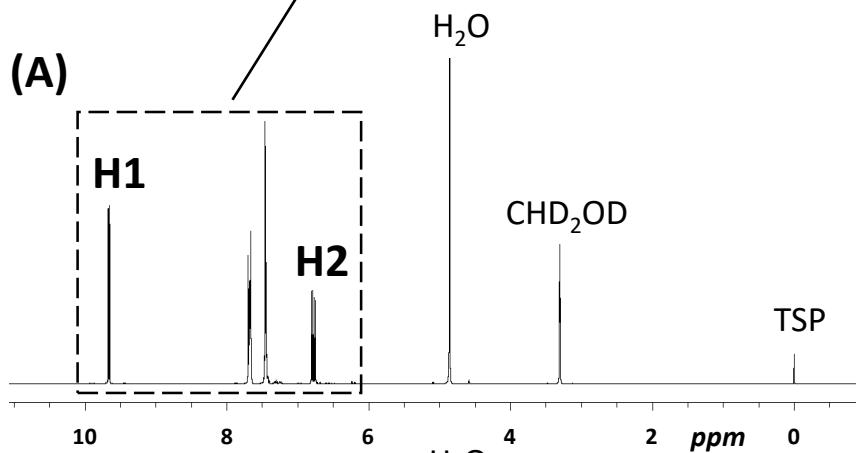
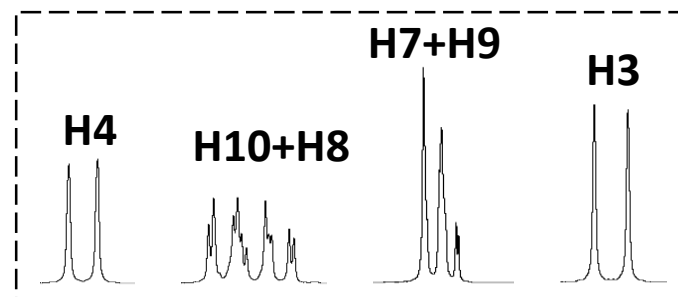
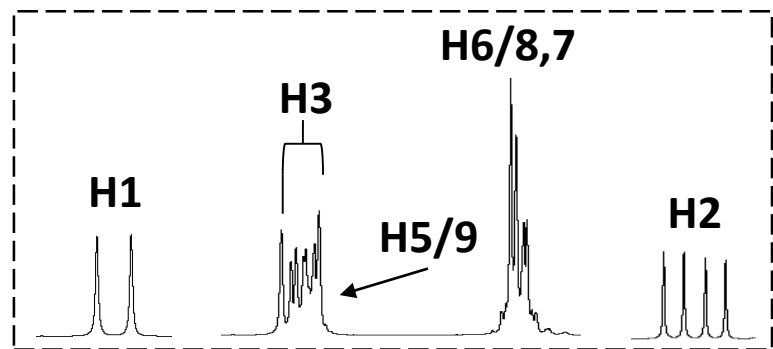


Figure 2

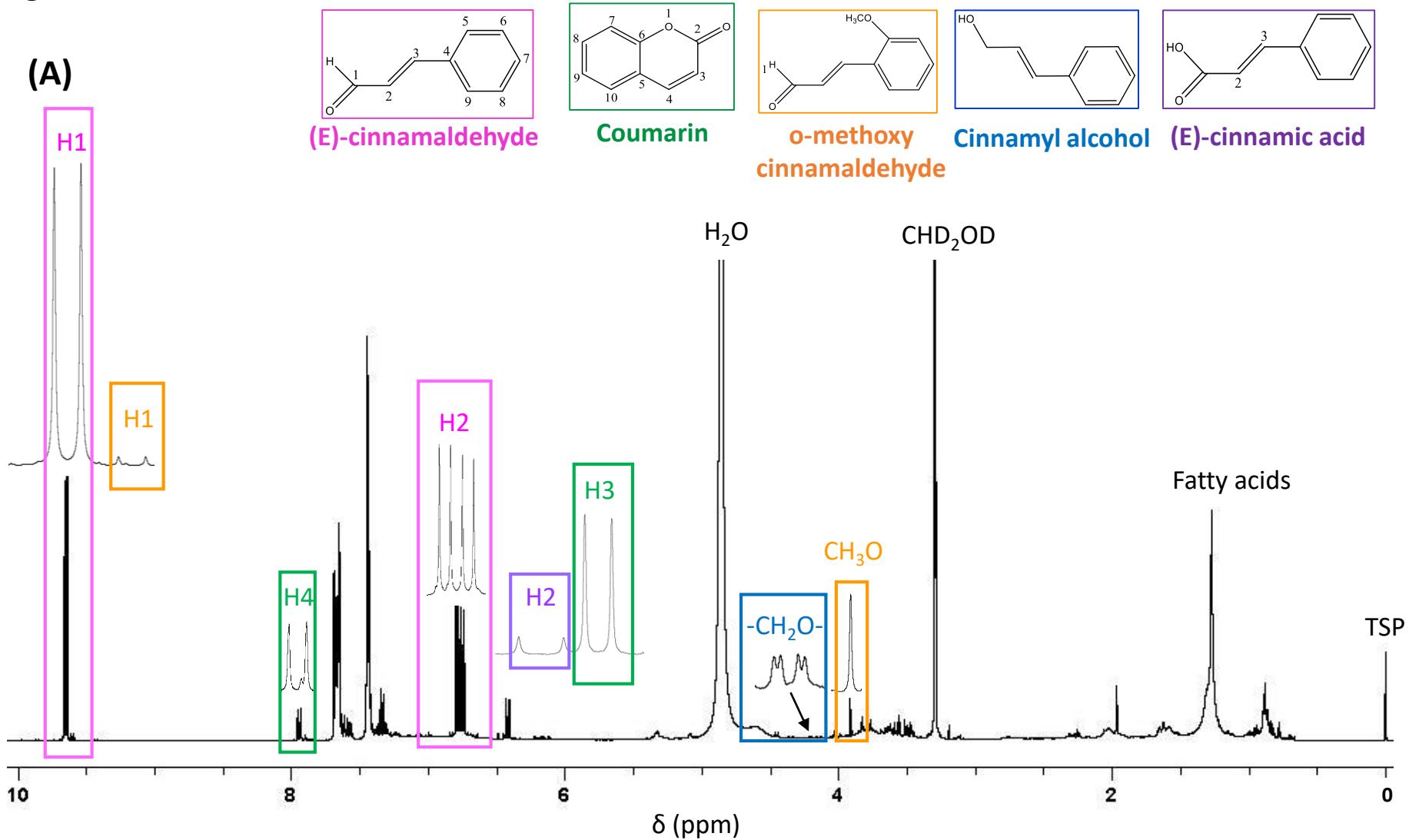


Figure 2

(B)

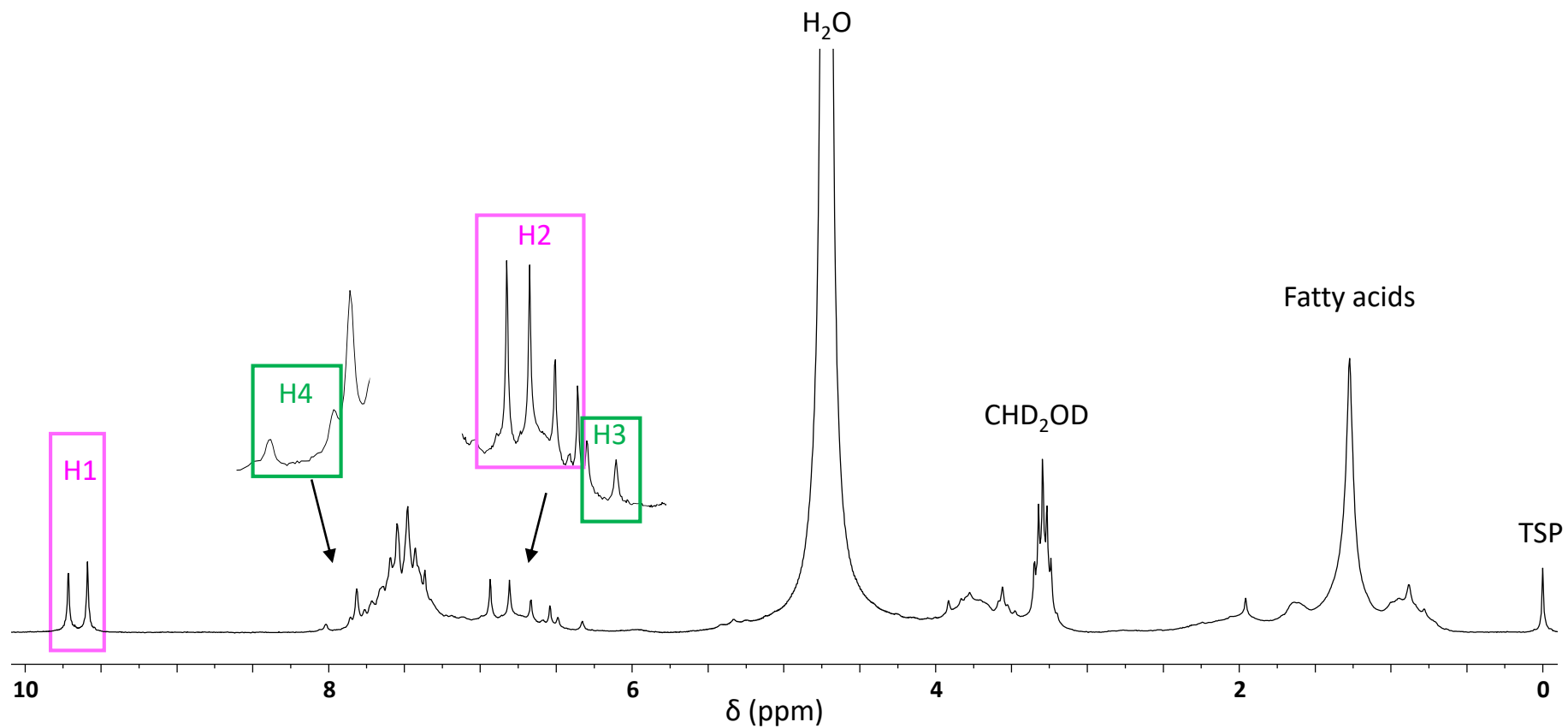


Figure 3

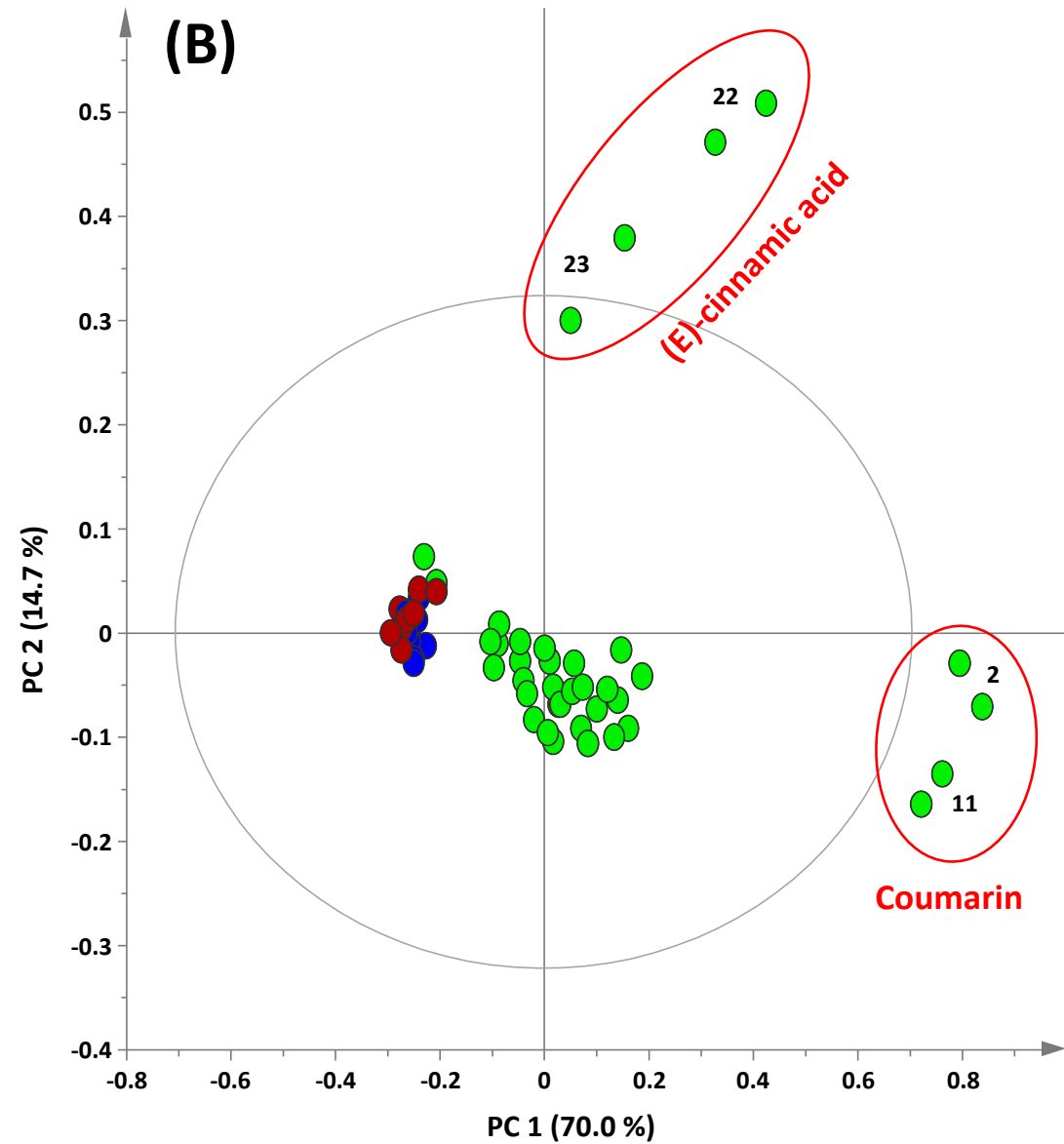
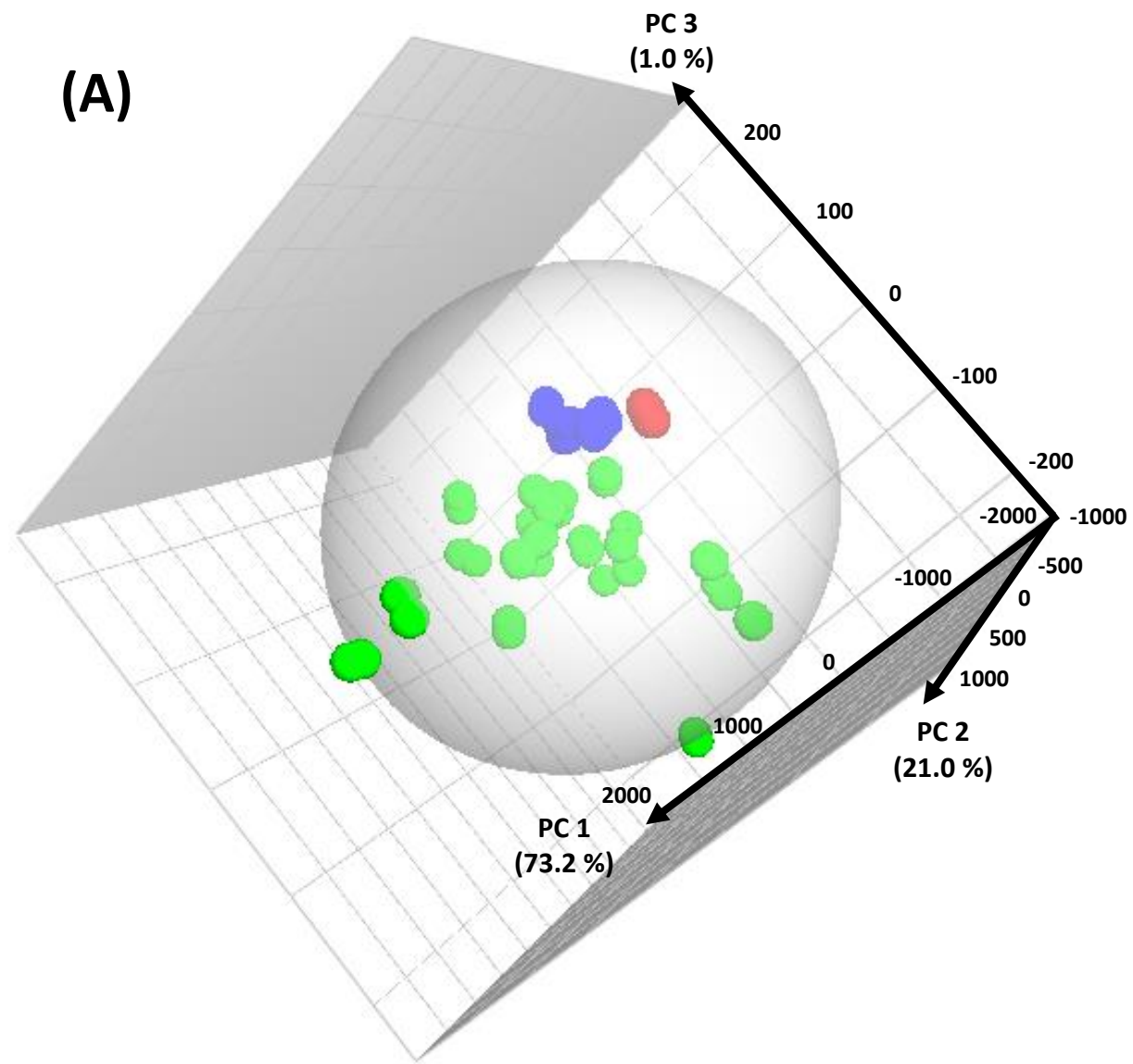


Figure 4

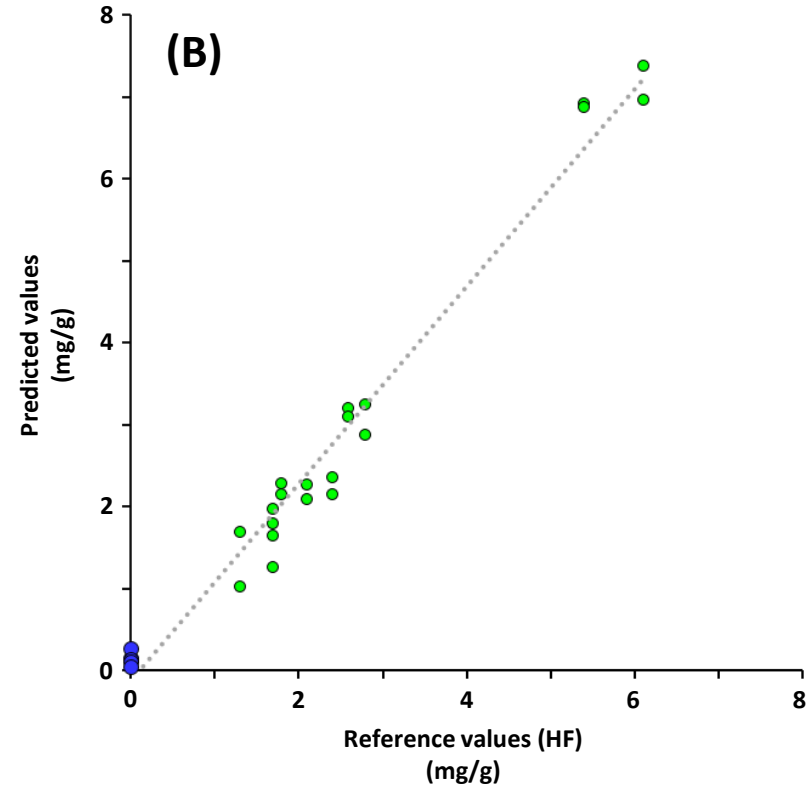
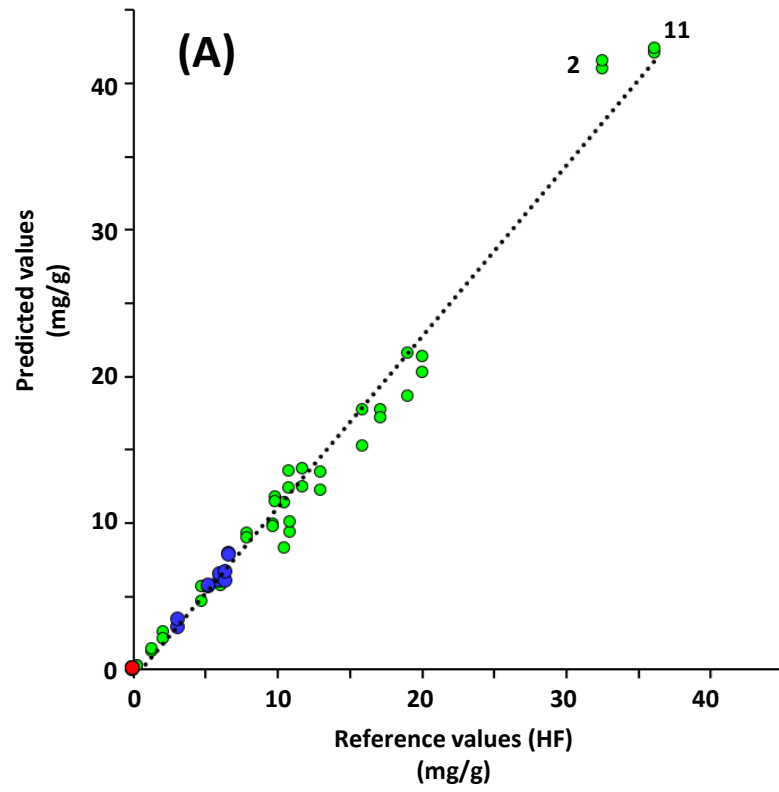


Figure 4

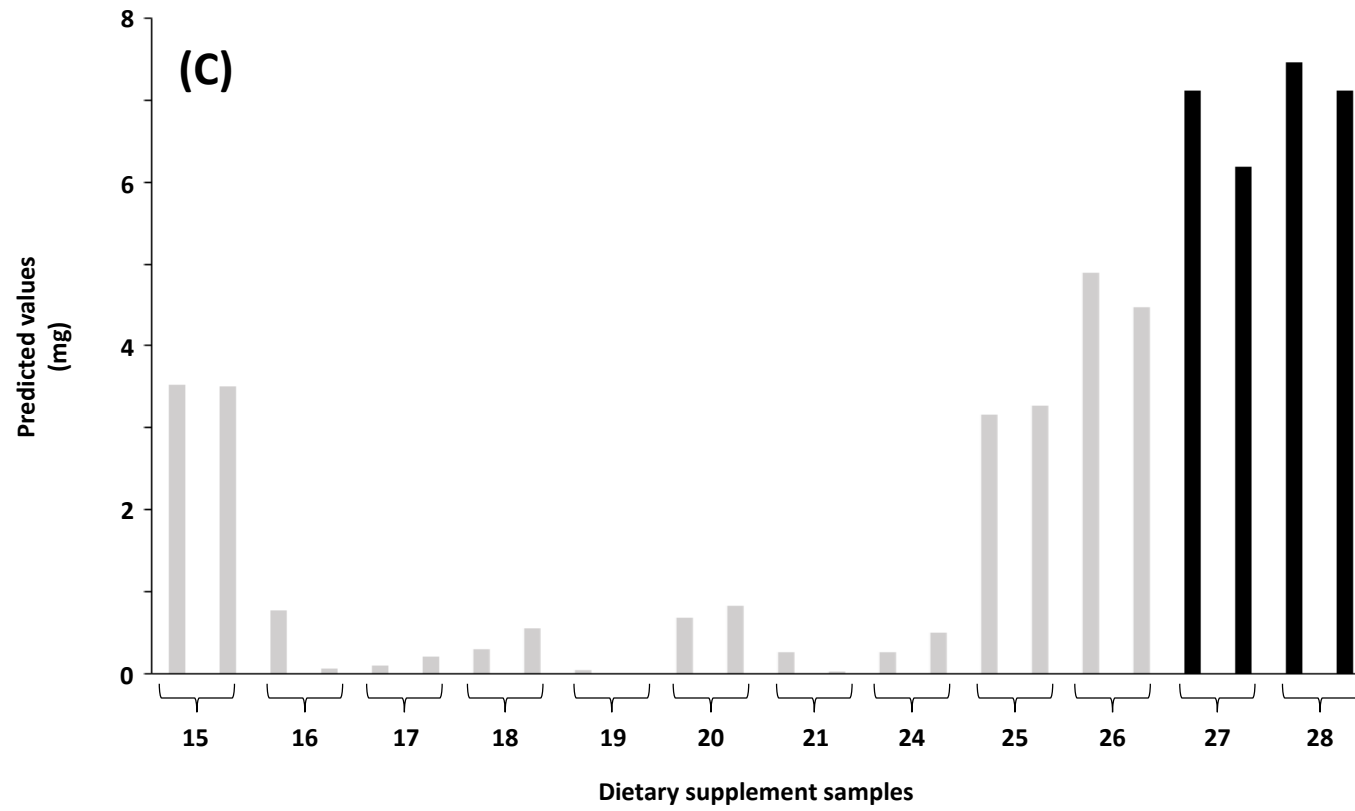


Table 1. Quantitative data of cinnamon commercial samples from HF NMR analysis (n=2)^a and comparison of labelled information to the proposed classification from HF NMR data.

	Sample	(E)- cinnamaldehyde	Coumarin	Eugenol	o-methoxy- cinnamaldehyde	Cinnamyl alcohol	(E)- cinnamic acid	Commercial information about origin (when available)	Proposed classification	Daily dose of coumarin ^b (mg)
	mg/g ± SD									
FOOD	1	15.8 ± 0.3	1.7 ± 0.1	-	0.16 ± 0.01	0.24 ± 0.02	0.53 ± 0.03	Southeast Asia Cinnamon	Cassia	
	2	32.5 ± 0.2	6.1 ± 0.1	-	0.94 ± 0.01	0.24 ± 0.03	0.98 ± 0.07	Vietnam Cinnamon	Cassia	
	3	7.8 ± 0.1	1.8 ± 0.1	-	-	-	0.65 ± 0.02	-	Cassia	
	4	4.7 ± 0.1	1.3 ± 0.1	-	-	-	0.51 ± 0.01	-	Cassia	
	5	6.6 ± 0.1	-	0.08 ± 0.01	0.28 ± 0.02	0.12 ± 0.01	0.18 ± 0.01	-	<i>C. verum</i>	
	6	6.0 ± 0.1	-	0.12 ± 0.01	0.23 ± 0.01	0.12 ± 0.01	0.19 ± 0.01	Ceylon Cinnamon, Madagascar	<i>C. verum</i>	
	7	10.4 ± 0.1	-	-	-	-	1.39 ± 0.04	-	Unclassified	
	8	10.7 ± 0.1	2.1 ± 0.1	-	-	-	0.55 ± 0.03	-	Cassia	
	9	5.2 ± 0.3	-	0.11 ± 0.02	0.22 ± 0.01	-	0.21 ± 0.01	Ceylon Cinnamon	<i>C. verum</i>	
	10	11.7 ± 0.1	2.8 ± 0.1	-	-	-	0.43 ± 0.04	<i>Cinnamomum burmannii</i> , Indonesia	Cassia	
	11	36.1 ± 0.8	5.4 ± 0.1	-	1.70 ± 0.10	0.20 ± 0.01	0.78 ± 0.07	Vietnam Cinnamon	Cassia	
	12	9.6 ± 0.4	2.4 ± 0.1	-	-	-	0.57 ± 0.01	<i>Cinnamomum verum</i>	Cassia ^c	
	13	10.8 ± 0.2	1.7 ± 0.1	-	-	0.33 ± 0.01	0.79 ± 0.05	Ceylon Cinnamon	Cassia ^c	
	14	9.8 ± 0.2	2.6 ± 0.1	-	0.31 ± 0.04	-	0.87 ± 0.02	Cinnamon Cassia, Thailand	Cassia	
DIETARY SUPPLEMENTS	15	0.2 ± 0.1	1.5 ± 0.1	-	-	-	0.62 ± 0.04	<i>Cinnamomum zeylanicum</i>	Cassia	2.4
	16	-	-	-	-	-	-	<i>Cinnamomum verum</i>	No cinnamon detected	
	17	6.4 ± 0.1	-	0.08 ± 0.01	0.27 ± 0.02	-	0.28 ± 0.02	<i>Cinnamomum verum</i>	<i>C. verum</i>	
	18	3.1 ± 0.1	-	0.05 ± 0.01	0.14 ± 0.01	0.13 ± 0.02	0.18 ± 0.01	Ceylon Cinnamon	<i>C. verum</i>	
	19	-	-	-	-	-	-	<i>Cinnamomum zeylanicum</i> , <i>C. cassia</i>	No cinnamon detected	
	20	6.0 ± 0.1	1.2 ± 0.1	-	-	-	0.20 ± 0.01	<i>Cinnamomum cassia</i>	Cassia	0.65
	21	-	-	-	-	-	-	Ceylon Cinnamon	No cinnamon detected	
	22	1.2 ± 0.1	-	-	-	-	4.10 ± 0.31	<i>Cinnamomum cassia Presl</i>	Unclassified	
	23	2.0 ± 0.1	-	-	-	-	3.74 ± 0.20	<i>Cinnamomum cassia Presl</i>	Unclassified	
	24	-	-	-	-	-	-	Ceylon Cinnamon	No cinnamon detected	
	25	20.0 ± 0.7	3.4 ± 0.1	-	-	-	0.50 ± 0.08	<i>Cinnamomum burmannii</i> , Indonesia	Cassia	3.5
	26	12.9 ± 0.2	2.5 ± 0.1	-	-	0.20 ± 0.03	0.43 ± 0.02	Ceylon Cinnamon	Cassia ^c	4.8
	27	19.0 ± 0.1	2.7 ± 0.1	-	-	-	0.50 ± 0.06	<i>Cinnamomum cassia</i>	Cassia	7.7
	28	17.1 ± 0.1	3.2 ± 0.1	-	-	0.19 ± 0.01	0.67 ± 0.03	<i>Cinnamomum cassia</i>	Cassia	8.4

^a Some samples were extracted in triplicate.

^b Calculated from the HF NMR quantification according to the recommended dosage on the dietary supplement label.

^c For these 3 samples, the commercial information does not fit with the NMR analysis; Ceylon cinnamon was replaced by cassia cinnamon.