

Determination of Dihydromyricetin in *Cassia ferruginea* (Schr.) DC. by Quantitative ^1H NMR (qHNMR)

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Abstract: *Cassia ferruginea* (Schr.) DC. (Fabaceae) is a native plant widely used as an ornamental tree and for the restoration of degraded areas in Brazil. Although no previous ethnopharmacological or chemical information was available, bioprospecting studies on this species found out a high concentration of dihydromyricetin (DHM) in different parts of the plant. DHM, also known as (+)-ampelopsin, is a flavonoid that presents important pharmacological and biological activities, including anticancer, cardioprotective and hepatoprotective effects. The aim of this study was to develop and validate a simple method for the determination of DHM in hydroethanolic extracts of *C. ferruginea* by quantitative ^1H NMR (qHNMR) using the internal standard approach. The parameters as linearity, specificity and selectivity, accuracy, precision (repeatability and intermediate) and robustness showed satisfactory results. Five ethanolic extracts from different stages and organs of *C. ferruginea* were investigated, the DHM concentration ranges from 52.08 ± 3.95 to 201.83 ± 4.71 mg/g. The proposed method appears to be a suitable tool for fast quality control of herbal extracts, with no need of tedious sample preparation and chromatographic separations increasing the efficiency of natural products analysis. This is the first study reporting the presence and determination of DHM in *Cassia ferruginea* (Schr.) DC.

Key words: qHNMR, dihydromyricetin, *Cassia ferruginea*.

1. Introduction

The development of therapeutic products from plant bioactive compounds often faces problems related to costly and environmentally unfriendly syntheses, low content of the target phytocompound in the producing species or the difficulties in cultivation and domestication of wild plants. To overcome these issues, we have focused our chemical studies in Brazilian native plant species that are cultivated in seedlings nurseries for uses as landscaping or restoration of degraded areas. The investigation of these types of plants aims for the discovery of novel bioactive metabolites or known metabolites with commercial value.

Through this strategy, the flavonoid dihydromyricetin (Fig. 1) was identified in huge quantities in the hydroethanolic extracts from different

organs of *Cassia ferruginea* (Schr.) DC. (Casealpinaceae-Fabaceae), also known as “canafistula”, an ornamental Brazilian woody species, which had no previous ethnopharmacological or chemical studies [1].

The dihydromyricetin (DHM), also known as (+)-ampelopsin, is a flavonoid commonly found in the *Ampelopsis* genus used in the traditional Chinese medicine [2-5]. This flavanone presents versatile pharmacological and biological activities, including improved skeletal muscle insulin sensitivity, hepatoprotective activity, inhibits alcohol-induced muscular relaxation, counteracts alcohol intoxication and dependence, cough relief as well as anti-microbial, anti-hypertension, anti-oxidation, anti-carcinogenic and anti-inflammatory effects [6-8].

Few analytical methodologies have been published for the determination of DHM, usually by HPLC-DAD. [2, 9]. Although diode array detector (DAD) is widely used for quantitation of bioactive phytochemicals, this

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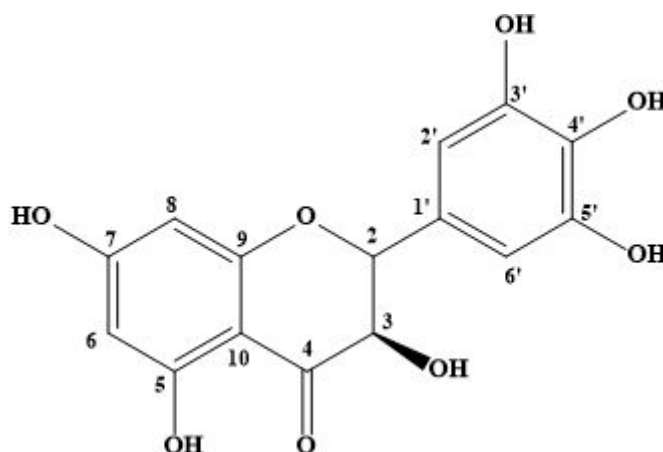


Fig. 1 Structure of dihydromyricetin (DHM).

procedure shows low sensitivity to complex matrices assays and unfriendly sample preparation.

A promising analytical tool to an accurate quantitation of target compounds in complex matrices, as plant extracts, is nuclear magnetic resonance (NMR) spectroscopy. Quantitative NMR (qNMR) provides tremendous benefits when compared to other techniques due to the non-dependence of any response factor, as the response of the NMR signal is directly proportional to the number of nuclei which generates a corresponding resonance line in the spectrum without the requirement of a separation step, for example when DAD, mass spectrometry (MS) and evaporative light scattering (ELSD) are used as detectors [10].

The method most frequently used in the determination of absolute values by qNMR is the internal standard (IS) approach [11]. By this approach, the IS is generally solubilized together with the sample in the same NMR tube and the NMR spectra of the sample and the sample with the IS are compared. Even the addition of IS is the most popular approach for qNMR, this procedure is especially challenging since it is difficult to find an adequate IS. There are several requirements for a compound to be suitable as an appropriate IS for qNMR, it should be stable and chemical inert, nonvolatile, nonhygroscopic, available in pure form, soluble in NMR solvents and have signal(s) which do not interfere with other signals in the spectrum and vice versa. However, the essential

requirement is that the signal(s) from the IS should have unique and isolated chemical shifts [12].

The assay of the analyte (P_X) is calculated directly from the NMR using a standard of known assay P_{IS} based on Eq. (1) [13]:

$$P_X = \frac{I_X}{I_{IS}} \frac{N_{IS}}{N_X} \frac{M_X}{M_{IS}} \frac{m_{IS}}{m} P_{IS} \quad (1)$$

where the subscript characters X and IS refer to the analyte and internal standard, respectively while I: integral of signal area, N: number of protons, M: molar mass, m: weight and P: purity.

The aim of this work was to develop and validate an analytical methodology for the determination of dihydromyricetin content in *Cassia ferruginea* by quantitative ^1H NMR (qHNMR) by the internal standard approach.

2. Materials and Methods

2.1 Chemicals

Benzyl benzoate (internal standard, IS, TraceCERT® grade, $99.43 \pm 0.16\%$) was purchased from Sigma-Aldrich (Inc. St. Louis, MO, USA) and methanol- d_4 (99.8 atom %D) was obtained from Acros Organics (Geel, Belgium).

2.2 Plant Material

Different parts of one adult plant and seedlings produced from its seeds were used in this study. The

seedlings were acquired in April of 2015 in a local retail plant nursery (Viveiro CamaráTM, Ibaté São Paulo State, Brazil certified by RENASEM No.SP-02032/2009, code 23694) and the different parts from the adult plant were collected in October of 2017 at Araraquara, São Paulo State, Brazil. A voucher specimen (IAC 56.308) was identified as *Cassia ferruginea* (Schrad.) DC and deposited in the “Herbarium of the Agronomic Institute of Campinas” (IAC).

2.3 Extraction

Leaves (2.7 g), flowers (5.1 g) and pods (3.2 g) from the adult plant and leaves (2.1 g), stems (2.6 g) and roots (2.6 g) from its seedlings, were dried and grounded to a fine powder. For the extraction, each sample was transferred to tubes with about 16 mL of EtOH/H₂O 7:3 v/v and sonicated for 10 min at approximately 30 °C. The samples were centrifuged at 3,600 rpm for 10 min and the supernatants were collected. This procedure was repeated seven times, the supernatants were combined and the fluid extracts were completely dried using a SpeedVac Concentrator (Thermo Scientific, Waltham, MA, USA).

2.4 Isolation and Identification of DHM

The crude extract (100 mg) obtained from the seedling leaves was used for the isolation of DHM by a preparative ShimadzuTM HPLC system (Tokyo, Japan), equipped with an LC-6AD binary gradient pumps, an injection valve model 7725i with an 8 mL loop (Rheodyne), an SPD-M20A diode array detector adjusted at 254 nm and LC solution software (Shimadzu). A C-18 column (Phenomenex, 21.20 × 150 mm, 5 µm) was used, the mobile phase consisted of water (A) and methanol (B) both acidified with 0.2% of formic acid using the following gradient elution mode: 30-50 % of B from 0 to 10 min, 50-100 % of B from 10 to 12 min, flow rate at 8.0 mL/min and injection volume of 100 µL.

Dihydromyricetin: t_R HPLC-DAD = 7.78 min;

$[\alpha]_D^{25} + 16.2$ ($c = 0.130$, CH₃OH); ^1H NMR (600 MHz, CD₃OD) δ : 4.46 (1H, *d*, $J = 11.4$ Hz, H-3), 4.83 (1H, *d*, $J = 11.4$ Hz, H-2), 5.88 (1H, *d*, $J = 2.1$ Hz, H-6), 5.92 (1H, *d*, $J = 2.1$ Hz, H-8), 6.53 (2H, *s*, H-2', H-6'), ^{13}C NMR (200 MHz, CD₃OD) δ : 73.7 (C-3), 85.3 (C-2), 96.2 (C-6), 97.2 (C-8), 101.8 (C-10), 108.0 (C-2', C-6'), 129.0 (C-1'), 134.9 (C-4'), 146.8 (C-3', C-5'), 164.4 (C-9), 165.3 (C-5), 168.7 (C-7), 198.3 (C-4); HRMS ((+)-ESI) m/z : 321.0611 [$M + H$]⁺.

2.5 Quantification of DHM by qHNMR

2.5.1 Sample Preparation to qHNMR

(1) IS Stock Solution

The stock solution was prepared by adding 38 µL of the internal standard into a 10-mL volumetric flask and filling up with CD₃OD, resulting in a solution of 20 mM benzyl benzoate.

(2) Sample Stock Solution

A working solution was prepared weighing precisely 30.0 mg of each powder crude extract and transferred to 10 mL volumetric flasks with 2.0 mL of the IS stock solution and then, filled up with 8.0 mL of CD₃OD. The mixture was vortexed and the final concentration was 4 mM of IS and 3.0 mg/mL of plant extract.

(3) Sample Solution (Preparation for qHNMR Internal Standard Method)

Samples of each plant material were prepared in triplicate. And 3 mg of each powder crude extract was precisely weighed in microtubes. For the flowers and pods from the adult plant, 30 µL of the IS stock solution and 570 µL of CD₃OD were added, resulting in a final concentration of 1 mM of IS. For leaves and stems from the seedlings and for leaves from the adult plant, 60 µL of the IS stock solution and 540 µL of CD₃OD were added, resulting in a final solution of 2 mM of IS. The mixture was vortexed and transferred to 5 mm NMR tubes and rapidly analyzed.

2.5.2 NMR Measurements

All experiments were performed at 600 and 150 MHz for ^1H and ^{13}C , respectively, on a Bruker Avance III HD 600 spectrometer operating at 14.1 Tesla,

equipped with a 5 mm direct observation multinuclear probe (BBFO-Z plus SmartProbe Broadband Observe). The ^1H NMR spectra were acquired with the *zgcprr* pulse sequence, which suppresses the solvent water signal at 4.69 ppm by presaturation. The pulse sequence was modified by the addition of a time parameter (d2) before the presaturation period to ensure the required time for complete spin relaxation between each scan ($d2 + d1 + \text{AQ} > 5 T_1$). The longitudinal relaxation time (T_1) was determined using the inversion-recovery sequence, T_1 of the slowest signal of interest was 4.8 s. The acquisition time and recycling time were 4.54 and 31.0 s ($d1 + d2$), respectively. Experiments were performed in automation mode, using a SampleXpress sample changer operated by Bruker IconNMR and ATMA (Automatic Tuning and Matching) command for automatic tuning. Also, TOPSHIM was used for automatically field shimming before the acquisitions. The 90° pulse width was automatically calibrated before each acquisition using pulsecal command. The temperature was controlled at 23°C . Spectra were processed with exponential multiplication (line broadening = 0.3 Hz), automatic baseline correction, manual phase correction and integration, using TopSpin v3.5pl7 (Bruker Biospin, Rheinstetten, Germany) and MestReNova (v6.0.2-5475, Mestrelab Research, Santiago de Compostela, Spain) softwares.

2.5.3 Method Validation by Internal Standard and Matrix Effect

The NMR method was validated according to the specificity and selectivity, linearity, accuracy, precision (repeatability and intermediate) and robustness, based on ANVISA [14]. For specificity and

selectivity assessment, the solvent (CD_3OD), internal standard (benzyl benzoate) and plant extracts were individually analyzed. To linearity and matrix effect evaluation, the IS stock solution and the working solutions were diluted in triplicate in five different concentrations of the IS: 0.5, 1.0, 2.0, 3.0, and 4.0 mM, resulting in six regression equations. The correlation coefficient (R), y-intercept and slope of the regression, as well as the analysis of variance (ANOVA) and standard errors were calculated to confirm the linear model. For the other parameters, three concentrations (top, middle and bottom) from the working solution prepared from the stems of the seedlings were analyzed in triplicate.

3. Results

During bioprospecting studies for the discovery of biologically active compounds and/or novel sources for useful phytochemicals, we found high levels of DHM in several organs of *Cassia ferruginea*. The weight and percentage yield (%) of hydroethanolic crude extracts are demonstrated in Table 1 for each organ and stage of the plant.

The initial chromatographic screening of crude extracts revealed a major peak in 7.5 min to all samples except for the roots seedlings which showed no peaks in this retention time (Fig. 2A) and the ^1H NMR spectrum corroborated with the HPLC data where a major compound was observed in all samples (Fig. 2B). After isolation and purification, the main compound was identified as DHM. From 100 mg of crude extract it was possible to obtain 11.8 mg of DHM. Consequently, an analytical method to quantify this analyte in the newly discovered botanical source

Table 1 Weight and percentage yield of hydroethanolic crude extracts of *C. ferruginea*.

Sample	Crude extract (mg)	Yield (%)
Leaves (seedlings)	1,207.6	57.4
Stems (seedlings)	585.5	22.3
Roots (seedlings)	506.3	19.1
Leaves (adult)	1,157.2	43.1
Pods (adult)	1,540.1	48.6
Flowers (adult)	2,685.1	52.8

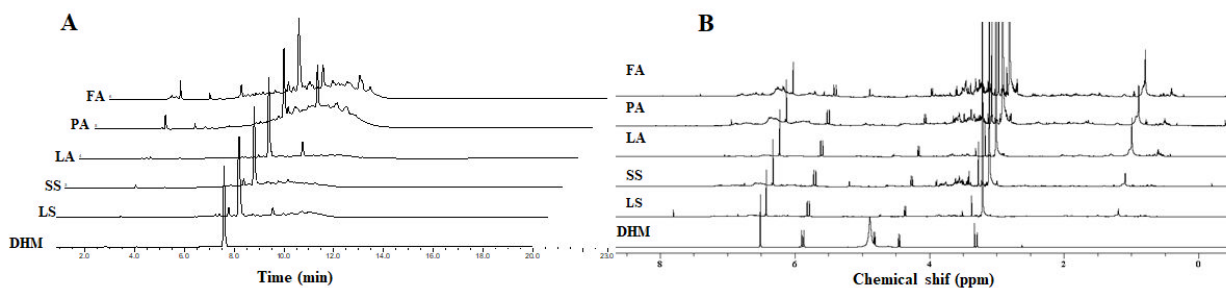


Fig. 2 (A): Chromatogram and (B) ^1H NMR (600 mhz, CD_3OD) of *C. ferruginea* crude extracts.

FA, PA and LA, represent flowers, pods and leaves from the adult plant. SS and LS, represent stems and leaves from the seedling, respectively.

quickly and with minimal sample preparation by qHNMR was proposed.

3.1 Method Validation

3.1.1 Specificity and Selectivity

In qNMR the aim of specificity and selectivity evaluation is to guarantee that the selected chemical shift position for quantitation unequivocally corresponds to the analyte of interest in a complex mixture, without interferences [15]. In order to check these parameters, the spectra from solvent, IS and extracts from *C. ferruginea* were individually evaluated. Considering the chemical shifts of the IS, no overlapping signals from the samples were observed in the range from 7.43 to 7.51 ppm in leaves (seedlings) and flowers (adult), corresponding to hydrogens H-4/H-6/H3'/H-7' from the IS. However, no overlapping signals from the samples were observed in the range from 8.00 to 8.07 ppm in stem (seedlings), leaves and pods (adult), corresponding to hydrogens H-3/H-7 from the IS, as shown in Fig. 3A. The selected chemical shifts of the IS are indicated in Table 2 with the corresponding attributions. For DHM determination, a clear region with no overlaps was observed in the range of 5.87 to 5.89 ppm corresponding to the hydrogen H-8 (Fig. 3C) and was therefore selected in the samples for quantitative measurements.

3.1.2 Linearity

The linearity of the method was evaluated by the analytical curves of five different molar ratios of the IS

diluted from the stock solution and from the working solutions containing the matrices. The first point of the regression was determined by the smallest peak to be integrated with a signal-to-noise (S/N) ratio above 250:1, that corresponds to 0.5 mM of IS. Table 2 shows the regression equations ($y=ax + b$) of the IS to each sample with all of them yielding correlation coefficients 0.99.

Additionally, an analysis of variance (ANOVA) was used in order to check the regression linearity objectively (Table 3). The F -test was applied for homoscedasticity testing, and F_1 value was calculated as the ratio between regression mean square (MSR) and residual mean square (MSr) and then, compared to the critical F_1 value (4.60). In the same way, for the evaluation of lack-of-fit, F_2 value was calculated as the ratio between lack of fit mean square (MSLF) and error mean square (MSE) and then, compared to the critical F_2 value (3.71). All regression experiments presented F_1 value > critical F_1 value and F_2 value critical F_2 value, therefore, data were considered to be statistically significant at a confidence level of 95%. Also, the plot of residuals showed points randomly scattered concluding that the data did not present heteroscedasticity or abnormalities.

3.1.3 Matrix Effect

The matrix effect can increase or decrease the detector response of an analyte present in a sample matrix compared to the same analyte in an organic solvent solution [16]. This matrix effect in the IS was calculated following Eq. (2).

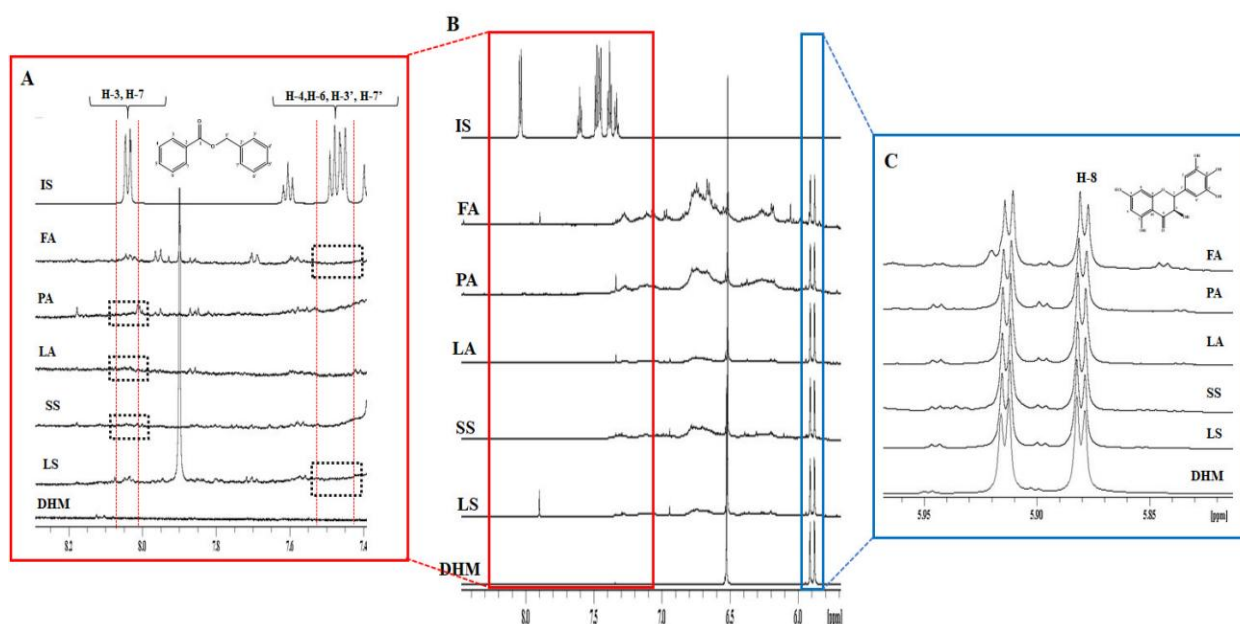


Fig. 3 ¹H NMR spectra (600 MHz, CD₃OD). (A): Partial spectra of the IS and samples with the absence of overlapping sample signals for H3/H7 and H4/H6/H3'/H7' chemical shifts from the IS. (B): Partial spectra of the hydrogen region of the IS and the DHM. (C): Partial spectra of samples demonstrating the absence of overlapping signals and the signal chosen for the quantitative determination of DHM (H-8).

FA, PA and LA, represent flowers, pods and leaves from the adult plant. SS and LS, represent stems and leaves from the seedling, respectively.

Table 2 IS chemical shifts, regression equation, correlation coefficients and matrix effect of *C. ferruginea* samples.

Sample	Signal IS (ppm)	Regression equation	r (correlation coefficient)	Matrix effect (%)
Internal standad	8.03 (2H, H3/H7)	$y = 8.35 \times 10^6 - 1.90 \times 10^5$	0.9992	-
Leaves from seedlings (LS)	7.47 (4H, H4/H6/H3'/H7')	$y = 8.93 \times 10^6 + 1.63 \times 10^5$	0.9970	10.22
Stems from seedlings (SS)	8.03 (2H, H3/H7)	$y = 1.76 \times 10^7 - 3.60 \times 10^5$	0.9990	6.14
Leaves from adult (LA)	8.03 (2H, H3/H7)	$y = 8.99 \times 10^6 - 6.14 \times 10^4$	0.9991	8.53
Pods from adult (PA)	8.03 (2H, H3/H7)	$y = 9.07 \times 10^6 + 5.34 \times 10^5$	0.9972	13.45
Flowers from adult (FA)	7.47 (4H, H4/H6/H3'/H7')	$y = 8.18 \times 10^6 + 1.17 \times 10^6$	0.9988	9.70

Table 3 Analysis of variance (ANOVA).

Source	DF	Internal standard		Leaves (seedling)		Stems (seedling)		Leaves (adult)		Pods (adult)		Flower (adult)	
		SS	MS	SS	MS	SS	MS	SS	MS	SS	MS	SS	MS
Regression	1	1.62 10 ¹⁵	1.62 10 ¹⁵	1.87 10 ¹⁵	1.87 10 ¹⁵	1.90 10 ¹⁵	1.90 10 ¹⁵	1.99 10 ¹⁵	1.99 10 ¹⁵	2.03 10 ¹⁵	2.03 10 ¹⁵	1.64 10 ¹⁵	1.64 10 ¹⁵
Residual	13	6.92 10 ¹²	5.32 10 ¹¹	1.08 10 ¹²	8.34 10 ¹⁰	3.54 10 ¹²	2.72 10 ¹¹	3.59 10 ¹²	2.76 10 ¹¹	1.12 10 ¹³	8.62 10 ¹¹	3.88 10 ¹²	2.98 10 ¹¹
Lack of fit	3	1.51 10 ¹¹	5.03 10 ¹⁰	6.07 10 ⁰⁹	2.02 10 ⁰⁹	1.84 10 ¹²	6.14 10 ¹¹	1.00 10 ¹¹	3.33 10 ¹⁰	9.26 10 ¹²	3.09 10 ¹²	3.66 10 ¹²	1.22 10 ¹²
Pure error	10	5.08 10 ¹³	5.08 10 ¹²	5.79 10 ¹³	5.79 10 ¹²	7.15 10 ¹³	7.15 10 ¹²	5.90 10 ¹³	5.90 10 ¹²	8.43 10 ¹³	8.43 10 ¹²	5.30 10 ¹³	5.30 10 ¹²
Total	14	1.63 10 ¹⁵	1.16 10 ¹⁴	1.88 10 ¹⁵	1.34 10 ¹⁴	1.90 10 ¹⁵	1.36 10 ¹⁴	1.99 10 ¹⁵	1.42 10 ¹⁴	2.04 10 ¹⁵	1.45 10 ¹⁴	1.65 10 ¹⁵	1.18 10 ¹⁴
MSR/MSr		3048.46 > f _{critical} 4.60		22471.54 > f _{critical} 4.60		6962.45 > f _{critical} 4.60		7190.33 > f _{critical} 4.60		2349.84 > f _{critical} 4.60		5511.74 > f _{critical} 4.60	
MSLF/MSE		0.01 < f _{critical} 3.71		0.0003 < f _{critical} 3.71		0.09 < f _{critical} 3.71		0.01 < f _{critical} 3.71		0.37 < f _{critical} 3.71		0.23 < f _{critical} 3.71	

DF: deegres of freedom; SS: sums of squares; MS: mean squares; MSR: regression mean square; MSr: residual mean square; MSLF: lack of fit mean square; MQE: error mean square.

Matrix effect (%) =

$$\frac{\text{Response (matrix)} - \text{Response (solvent)}}{\text{Response (solvent)}} \times 100 \quad (2)$$

The matrix effects are shown in Table 2. According to Zrostl kova et al. [17] a variation of 20% between the response of the same curves is feasible. There are no observed matrix effects for any of the five tested matrices, it is worth highlighting that the analytical curve elaborated in the matrix has the advantage of incorporating a recovery correction in the obtained

results and thus demonstrating a value closer to the real. The analytical curves of the IS in the solvent and matrix for the five hydroethanolic extracts are graphically shown in Fig. 4. For the IS, there is no significant difference and the curves practically overlap.

For evaluation of precision (repeatability and intermediate), accuracy and robustness, the working solution of stems from seedlings (SS) was chosen because all samples presented a similar complexity.

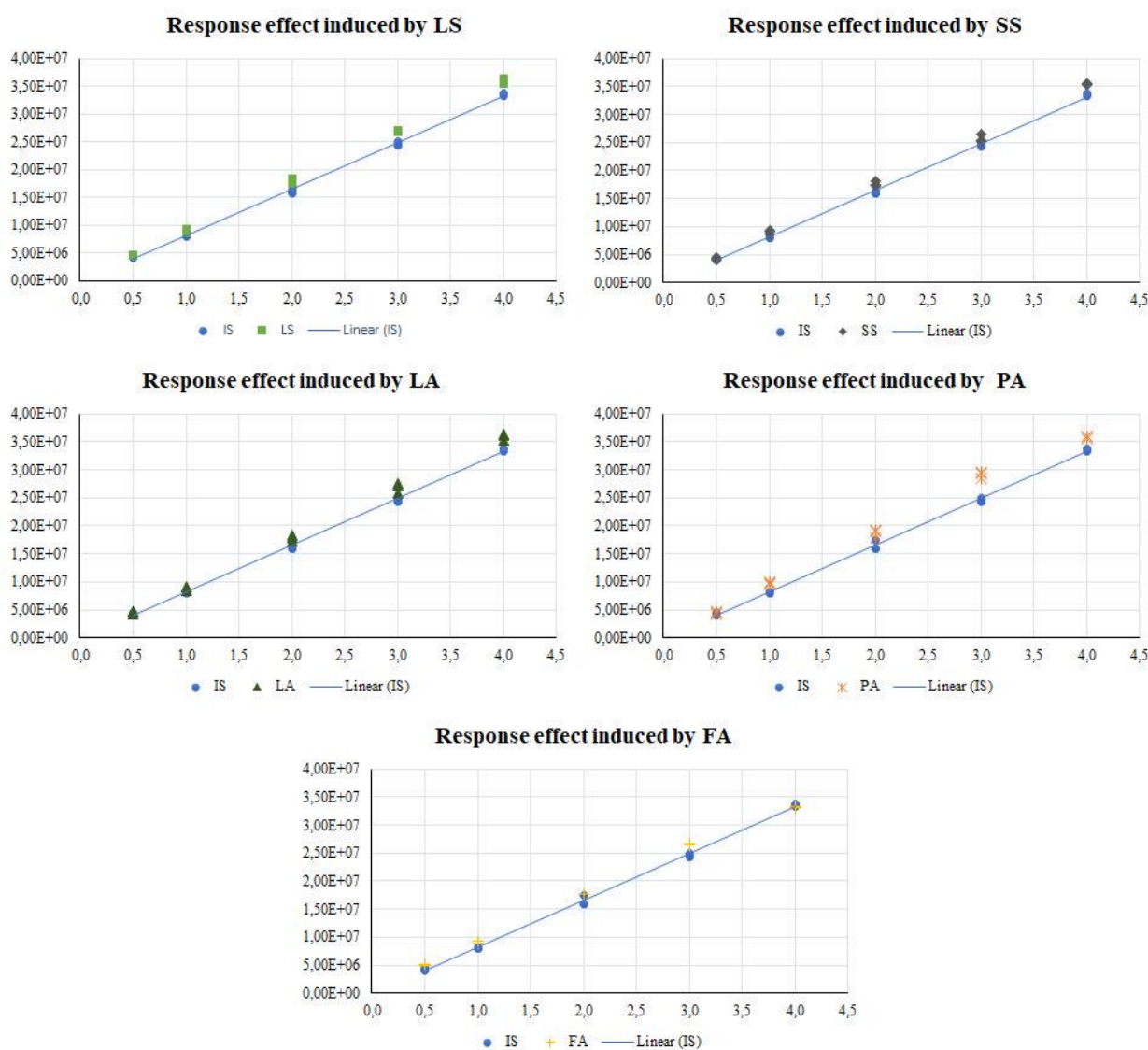


Fig. 4 Graphical representation of analytical curves for the IS (benzyl benzoate) in the solvent and the matrix of samples. FA, PA and LA, represent flowers, pods and leaves from the adult plant. SS and LS, represent stems and leaves from the seedling, respectively.

3.1.4 Precision

The repeatability of the method was evaluated repeating the entire method ($n = 3$) in three different concentrations (0.5; 2.0 and 4.0 mM) by the same analyst, on the same day, at the same laboratory. The intermediate precision was done in the same instrument, by a second analyst, on different days (0; 24 and 48 hours). The results of repeatability and intermediate precision presented a relative standard deviation (RSD) of 1.42 and 2.88%, respectively. By this way, both tests were considered satisfactory.

3.1.5 Accuracy

The accuracy shows the proximity of the measured results to the actual (true) value. It is expressed by the ratio between experimental mean concentration and theoretical mean concentration, following Eq. (3).

$$\text{Accuracy (\%)} = \frac{\text{observed value}}{\text{theoretical value}} \times 100 \quad (3)$$

The obtained results of the nine samples were compared and accuracy of 102.2% (RSD 2.95%) was obtained and was considered satisfactory.

3.1.6 Robustness

For robustness evaluation of the method, three important parameters were stepwisely varied to influence signal-to-noise (S/N) ratio since this is the most critical point for qNMR. These factors were individually evaluated and the other acquisition parameters were kept constant. By this way, the least concentrated working solution (0.5 mM) was chosen due to being the more likely to feel variations on S/N. The first variable was number of scans (ns) from 16 to 8, but RSD for 8 is bigger than the adopted value and could not be used in this method. The other variables were temperature and software used for data processing. The temperature was changed from 23.05 °C to ± 3.0 °C. For data processing MestReNova was tested against TopSpin, previously used. Both temperature and software parameters did not present significant differences (RSD acceptable), concluding that the method was not influenced by small operational variations.

3.2 Quantification of DHM

Hydroethanolic extracts from *C. ferruginea* seedlings (leaves and stems, abbreviated by LS and SS, respectively) and from the adult plant (leaves, pods and flowers, abbreviated by LA, PA and FA, respectively), were submitted to DHM determination by qHNMR. Quantification of this compound was performed in relation to benzyl benzoate (MM = 212.24 g/mol), used as an internal standard (IS). The ^1H NMR spectra of DHM (MM = 320.251 g/mol) is represented in Fig. 5A while the acquired ^1H spectrum, chemical structure and attribution of the hydrogen signals of this compound are illustrated in Fig. 5B. The signal employed for the quantification of DHM, at 5.88 ppm, refers to hydrogen H-8 (1H). For the IS, the signals chosen were 8.03 ppm or 7.53 ppm, referring to the aromatic hydrogens H-3 and H-7(2H) or H-4, H-6, H3' and H-7'(4H), respectively.

Experimental mean purity values (content in %, w/w) were calculated from Eq. (1) and the results are described in Table 4. All samples presented outstanding results for the DHM content except for flowers as a concentration below the working range was found and could not be quantified by the tested qHNMR method. The DHM content encountered in *C. ferruginea* samples ranges from 52.08-201.83 mg DHM/g extract that significantly differs from previous results reported in the literature. By the HPLC-DAD methodology, methanolic extracts of Yemputaoteng, which is the dried ground part of *Ampelopsis sinica* [2], showed a range of 0.010-0.015 mg DHM/g extract whilst water extracts of *Hovenia dulcis* fruits [9] showed a range of 0.88-1.19 mg DHM/g extract. The results obtained using the IS methodology were very satisfactory, besides it contributes to an efficient, simple and fast quality control of complex samples.

4. Conclusions

The proposed qHNMR method using the IS approach was validated according to the most indicated

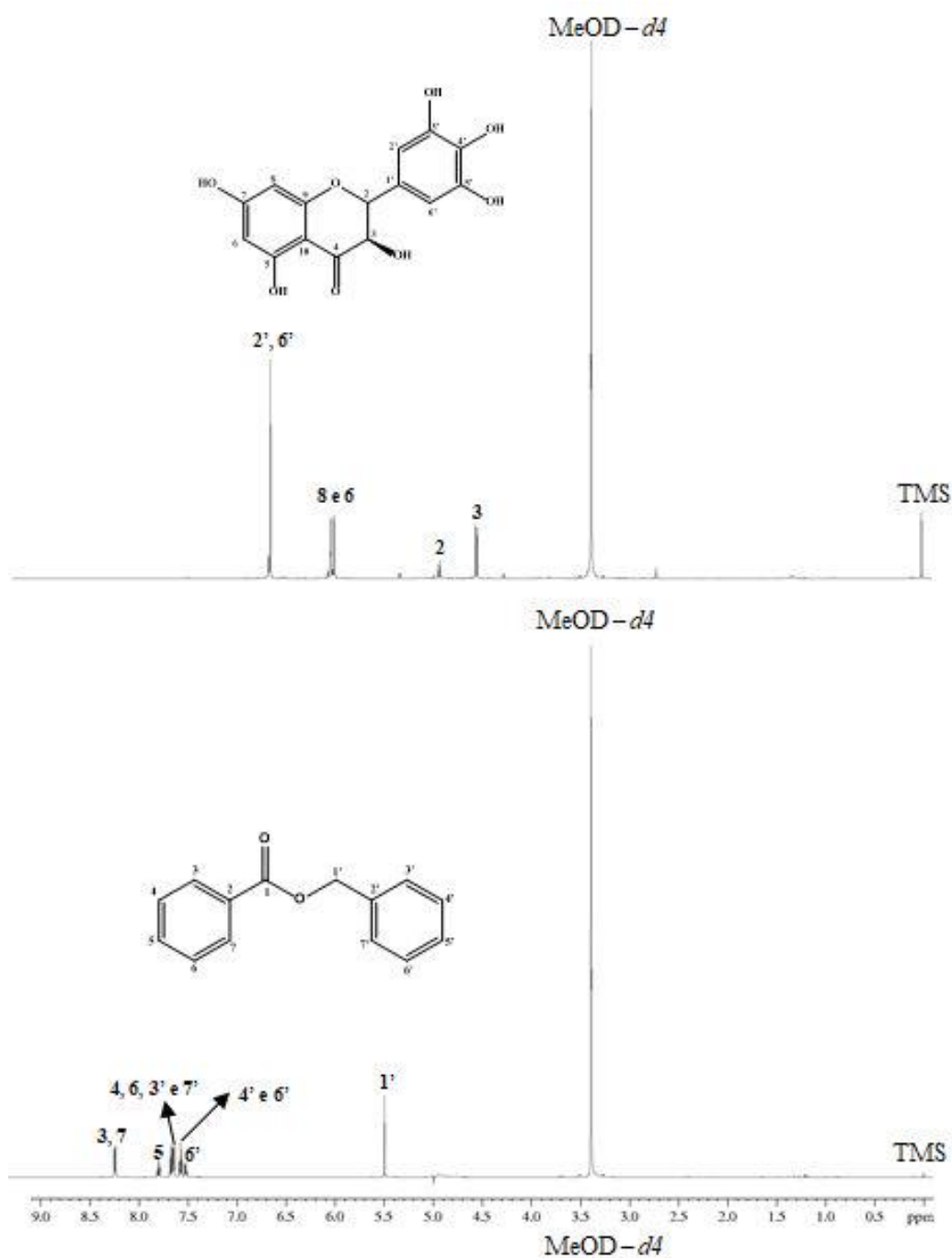


Fig. 5 ^1H -NMR spectra (600 MHz, $\text{MeOD}-d_4$): (A) DHM and (B) benzyl benzoate.

Table 4 DHM concentrations measured by qHNMR for each *C. ferruginea* extract.

Sample	Content (mg/g extract)*
Leaves (seedling)	201.83 ± 4.71
Stems (seedling)	83.36 ± 1.40
Leaves (adult)	166.98 ± 3.58
Pods (adult)	52.08 ± 3.95

* Average $\pm$ standard deviation (RSD), $n = 3$.

parameters for quantitative evaluations. Employing the validated method, it was possible to quantify DHM in four hydroethanolic extracts of *C. ferruginea* while only the flower extract could not be determined as the obtained concentration was outside the working range. Additionally, the results reveal that we have discovered a new botanical source that produces high amounts of DHM both in the seedlings and adult plants.

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