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Identification of Isomeric Dicaffeoylquinic Acids from *Eleutherococcus senticosus* using HPLC-ESI/TOF/MS and ^{1}H-NMR Methods

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Liquid chromatography–electrospray time-of-flight mass spectrometry (HPLC-ESI/TOF/MS) and a novel NMR technique, developed to maximise the sensitivity obtained from the standard NMR spectrometer, have been applied to the identification of the phenolic constituents of *Eleutherococcus senticosus*. In addition, molecular modelling and dihedral bond angle calculations based on the vicinal $^3J_{HH}$-coupling constants have been used in the unambiguous assignment of signals in the ^{1}H-NMR spectra. 5′-*O*-Caffeoylquinic acid and three isomeric compounds, 1′,5′-*O*-dicaffeoylquinic acid, 3′,5′-*O*-dicaffeoylquinic acid and 4′,5′-*O*-dicaffeoylquinic acid, have been isolated and identified from a sample. The isolation and structure determination of the latter two compounds are reported for the first time from this plant. Copyright © 2002 John Wiley & Sons, Ltd.

Keywords: NMR; LC-MS; time-of-flight MS; dicaffeoylquinic acid; *Eleutherococcus senticosus*; *Acanthopanax senticosus*.

INTRODUCTION

The herb *Eleutherococcus senticosus* (Siberian ginseng; also known as *Acanthopanax senticosus*) belongs to the family Araliaceae and is a 1.5–2.6 m high bush that is widely spread throughout Asia. The herb is known for its normalising action on human health (Baranov, 1982), and the word adaptogen is often associated with it. The leaves and especially the roots, where the content of active constituent is greatest (ca. 0.8 wt%; Baranov, 1982), are used for medicinal purposes. Although *E. senticosus* is often compared with *Panax ginseng*, the chemical constituents of the latter are quite different (Dewick, 1997). Nevertheless, Siberian ginseng has been one of the 10 best-selling herbs in the US (Yat *et al*., 1998).

Research interest in the supposed active constituents of *E. senticosus* has focused mainly on eleutherosides B and E (Slacanin *et al*., 1991; Pietta *et al*., 1994; Yat *et al*., 1998), but 5′-*O*-caffeoylquinic acid (chlorogenic acid; **1**), 1′,5′-*O*-dicaffeoylquinic acid (**2**), lignan and triterpene glycosides, polysaccharides, and phenylpropane, oleanol and β-sitosterol derivatives have also been detected in the herb (Fang *et al*., 1985; Shao *et al*., 1988, 1989; Nishibe *et al*., 1990; Segiet-Kujawa and Kaloga, 1991; Slacanin *et al*., 1991; Umeyama *et al*., 1992; Dewick, 1997; Makarieva *et al*., 1997; Park *et al*., 2000). The biological effects of these constituents have been widely studied (Hacker and Medon, 1984; Nishibe *et al*., 1990; Shen *et al*., 1991; Fujikawa *et al*., 1996).

Together with **2**, the dicaffeoylquinic acids 3′,5′-*O*-dicaffeoylquinic acid (**3**) and 4′,5′-*O*-dicaffeoylquinic acid (**4**) have been isolated from many sources (Timmermann *et al*., 1983; Clifford, 1986; Wald *et al*., 1989; Baumer and Ruppel, 1996; Chuda *et al*., 1996; Schwarz *et al*., 1996; Fuchino *et al*., 1997; Azuma *et al*., 1999; Miketova *et al*., 1999). These compounds have been claimed to possess many pharmacological properties such as anti-oxidant (Chuda *et al*., 1996; Azuma *et al*., 1999), hepatoprotectant (Baset *et al*., 1996), anti-bacterial (Scholz *et al*., 1994), anti-histaminic (Kimura *et al*., 1985), anti-spasmodic (Trute *et al*., 1997) and, most importantly, anti-HIV (Robinson *et al*., 1996a,b; Mahmood *et al*., 1997). Through *in vivo* and *in vitro* studies, mono-, di- and tri-caffeoylquinic acid analogues have been shown to inhibit HIV-1 integrase, the enzyme that catalyses integration of the viral DNA into the host genome.

In this paper, sensitivity-improving NMR techniques, together with the high-resolution ESI/MS methods, are shown to be powerful tools in the analysis of 5′-*O*-caffeoylquinic acid (**1**) and the dicaffeoylquinic acid isomers (**2**–**4**), two of which (**3** and **4**) have not been detected previously in *E. senticosus*. It should be noted that, in some of the literature references cited in this paper, a different numbering system for **1**–**4** has been used; throughout this work, however, the preferred IUPAC numbering system will be employed.

EXPERIMENTAL

Chemicals. LiChrosolv GG (Merck, Darmstadt, Germany)-grade methanol and acetonitrile were used for HPLC analyses. Formic acid and trifluoroacetic acid (TFA) were obtained from BDH (Poole, England). Standard 5′-*O*-caffeoylquinic acid (chlorogenic acid; **1**) was purchased from Aldrich (St Louis, MO, USA) and quinic acid standard from Aldrich (Steinheim, Germany).

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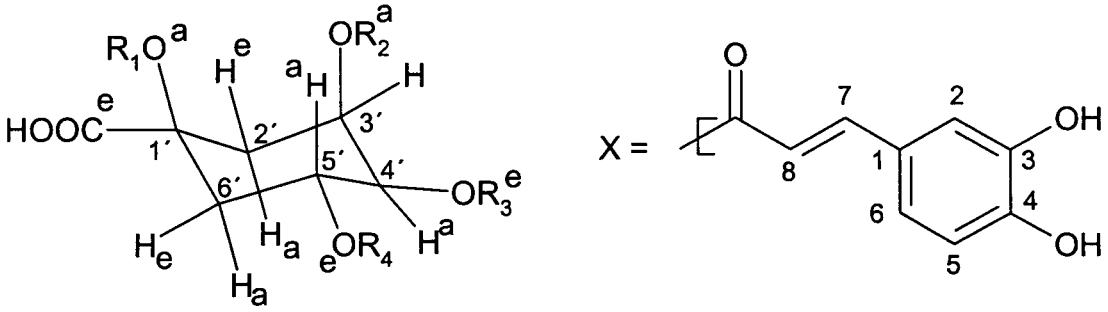

1: $R_1 = R_2 = R_3 = H$, $R_4 = X$
2: $R_2 = R_3 = H$, $R_1 = R_4 = X$
3: $R_1 = R_3 = H$, $R_2 = R_4 = X$
4: $R_1 = R_2 = H$, $R_3 = R_4 = X$

Laboratory water was purified with a Simplicity 185 (Millipore, Molsheim, France) water purifier. D_2O (99.9 atom % D) was from C/D/N Isotopes (Quebec, Canada).

Plant material. Seeds of *Eleutherococcus senticosus* were obtained from the Moscow Botanical Gardens of VILR in 1979, and mother plants (accession number 85-1326) have been grown outdoors in the Asia mountain section of the Botanical Gardens of the University of Oulu, Finland. The plant material used in the present study consisted of roots from a greenhouse-grown seedling and the material was collected in March 1999.

Extraction and isolation. Well-washed, dried roots of *E. senticosus* were powdered in a mortar. For quantitative analyses, 40–50 mg of root powder was weighed exactly and extracted for 10 min with methanol (1 mL) in a Branson (Danbury, CT, USA) model 200 (40 kHz) ultrasonic bath. The extract was filtered with a Gelman (Ann Arbor, MI, USA) syringe filter (GHP 13 mm; 0.2 μm) prior to HPLC injection. For the isolation procedures, 180–200 mg of root powder was employed for extraction: the filtered extract was dried from methanol and redissolved in 1 mL of 15% aqueous methanol in order to provide a larger injection volume. Isolation was performed with a Waters (Milford, MA, USA) model 2690 Alliance HPLC system equipped with a Waters Symmetry 300 C_{18} column (150 × 3.9 mm i.d.) maintained at 25°C. The column was eluted initially with water: acetonitrile: methanol (83:2:15; all eluents con-

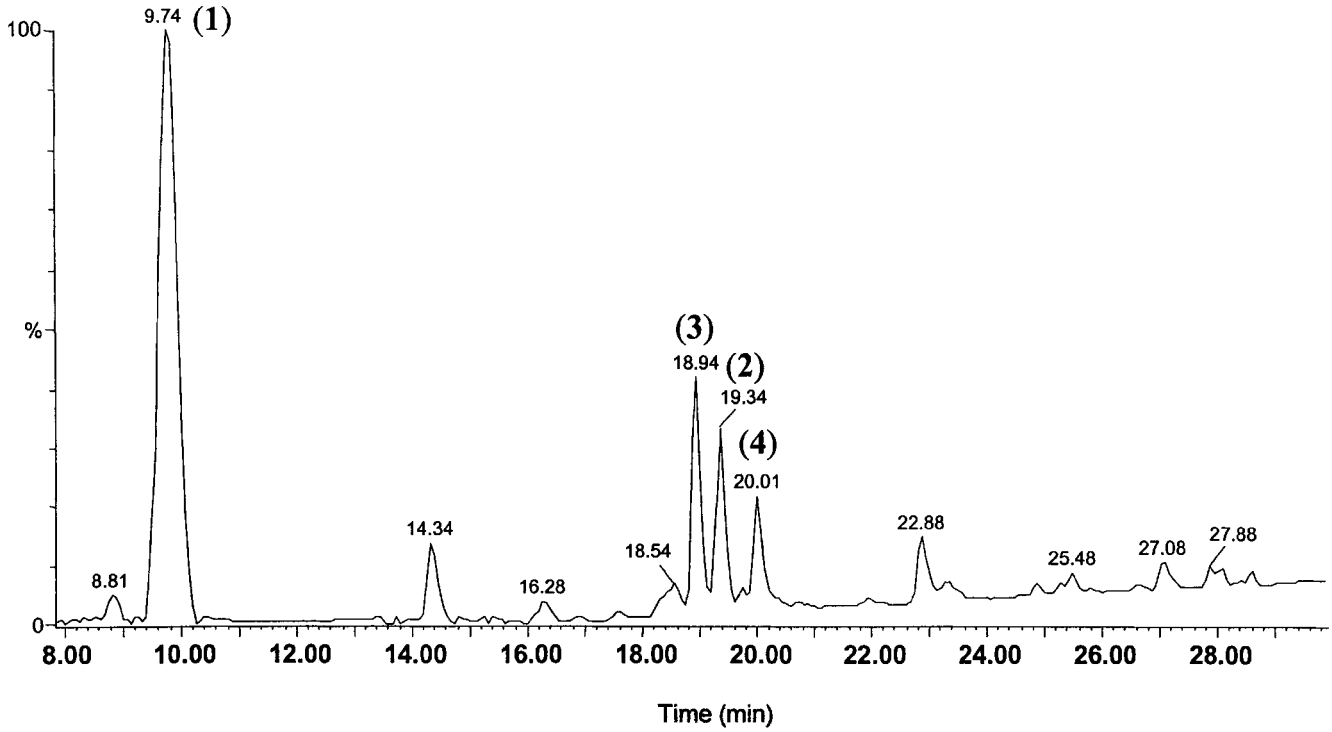

Figure 1. HPLC chromatogram of a methanol extract of *Eleutherococcus senticosus* (Siberian ginseng). Key to peak identities: **1**, 5′-*O*-caffeoylquinic acid (R_t 9.7 min); **2**, 1′,5′-*O*-dicaffeoylquinic acid (R_t 19.3 min); **3**, 3′,5′-*O*-dicaffeoylquinic acid (R_t 18.9 min); **4**, 4′,5′-*O*-dicaffeoylquinic acid (R_t 20.0 min). (For extraction and chromatographic protocols see the Experimental section.)

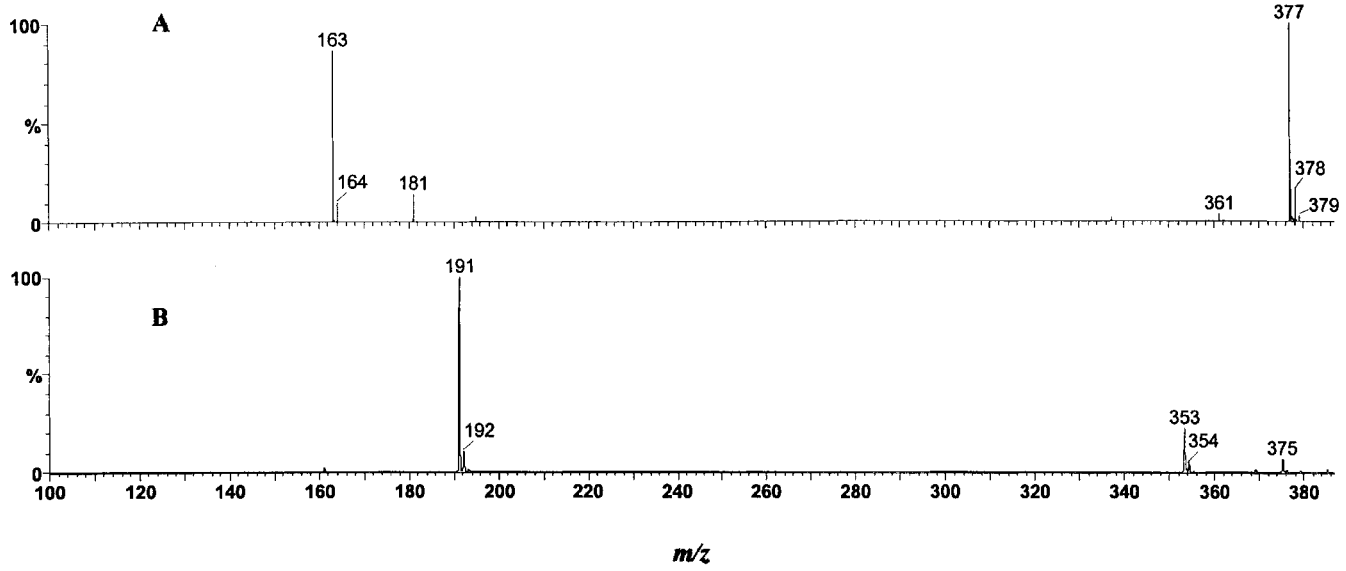

Figure 2. Positive ion (A) and negative ion (B) electrospray mass spectra of 5′-*O*-caffeoylquinic acid (**1**) (peak at R_t 9.7 min in Fig. 1).

tained 0.05% TFA) which was changed with a linear gradient to 62:2:36 in 16 min, and then in 1 min to 0:50:50 which was maintained for 4 min in order to clean the column. The flow-rate was 1.0 mL/min. Dicaffeoylquinic acids were collected with an Isco (Lincoln, NE, USA) Foxy 200 fraction collector from thirty HPLC runs each with a 100 µL injection volume. The 5′-*O*-caffeoylquinic acid was collected from 26 runs each with a 40 µL injection volume. The HPLC fractions collected were evaporated in an FTS (Stone Ridge, NY, USA) Flexi-dry freeze-drier.

Table 1. The exact masses of ions formed from isolated 5′-*O*-caffeoylquinic acid (1) (peak at R_t 9.7 min in Fig. 1) with positive electrospray ionisation

Measured mass (Da)	Calculated mass (Da)	Difference (mDa)	Molecular formula
377.0844	377.0849	0.5	$C_{16}H_{18}O_9Na$
181.0516	181.0501	1.5	$C_9H_9O_4$
163.0394	163.0395	0.1	$C_9H_7O_3$

HPLC–mass spectrometry. The HPLC analyses and HPLC-MS experiments were performed with a Waters 2690 Alliance HPLC system equipped with Waters Symmetry Shield C_8 column (50 mm × 2.1 mm i.d.) maintained at 35°C. The column was eluted initially with water:acetonitrile:methanol (95:2:3; all eluents contained 0.06% formic acid) for 2 min and then changed immediately to 88:2:10 which was maintained for

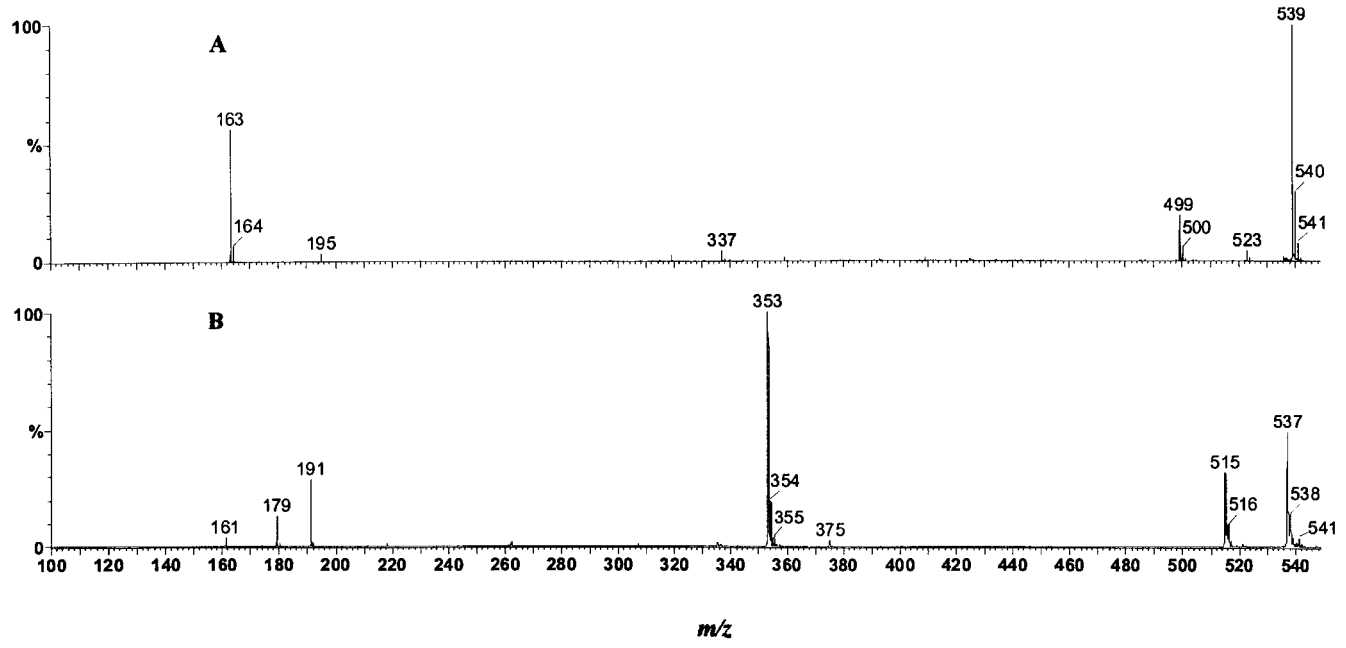

Figure 3. Positive ion (A) and negative ion (B) electrospray mass spectra of 3′,5′-*O*-dicaffeoylquinic acid (**3**) (peak at R_t 18.9 min in Fig. 1).

Table 2. The differences of measured and calculated exact masses of ions formed from the dicaffeoylquinic acids 2–4 with positive electrospray ionisation

	Calculated masses (Da)				
	$C_{25}H_{24}O_{12}Na$ 539.1165	$C_{25}H_{23}O_{11}$ 499.1240	$C_{16}H_{17}O_8$ 337.0923	$C_{10}H_{11}O_4$ 195.0657	$C_9H_7O_3$ 163.0395
Compound [R_t(min) as shown in Fig. 1]	Differences (mDa)				
1′,5′-*O*-Dicaffeoylquinic acid (**2**) [19.3]	2.8	0.2	2.4	3.2	1.5
3′,5′-*O*-Dicaffeoylquinic acid (**3**) [18.9]	3.1	2.3	2.1	3.3	0.1
4′,5′-*O*-Dicaffeoylquinic acid (**4**) [20.0]	3.2	0.5	3.2	0.7	0.6

6 min before being changed with a linear gradient to 0:2:98 in 20 min. The flow-rate was 0.3 mL/min and the injection volume was 10 μL. MS were measured with a Micromass (Wythenshawe, UK) model LCT time-of-flight mass spectrometer equipped with an electrospray ionisation source. The voltages used were 3000 V for the source capillary and 20 V for the extraction cone: the source temperature was 150°C and the de-solvatation temperature was 400°C.

NMR spectrometry. NMR spectra were acquired at 11.7 T on a Bruker (Fällanden, Switzerland) model DRX 500 spectrometer. For ^{1}H- and ^{13}C-NMR, RF-pulse power levels of 45 and 19 kHz, respectively, were used. For

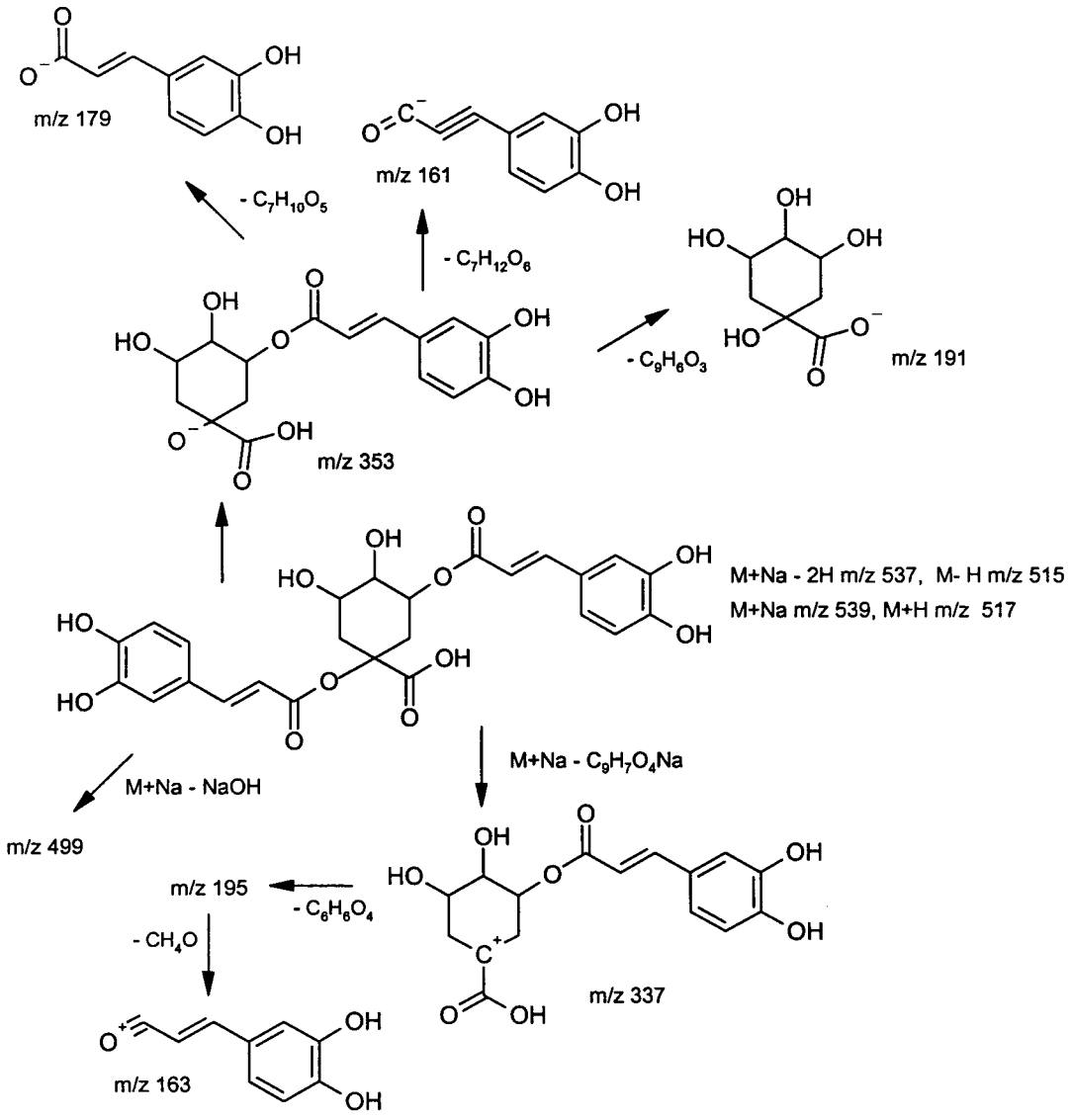

Figure 4. The proposed fragmentation of the dicaffeoylquinic acids in positive ion mode (below) and negative ion mode (above) ESI/TOF/MS experiments.

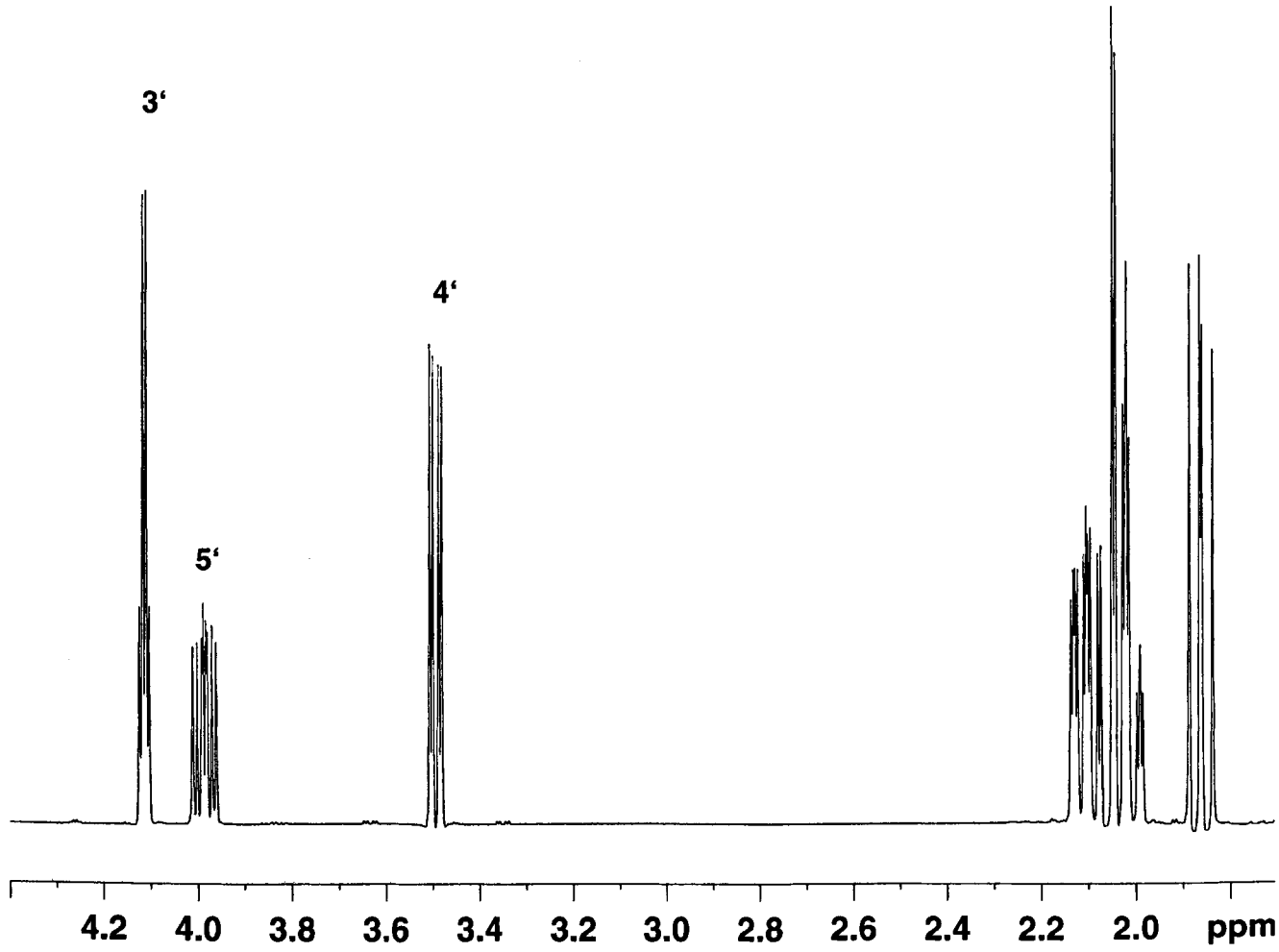

Figure 5. ^{1}H-NMR spectrum of a commercial sample of quinic acid measured in D_2O at 500.13 MHz.

TOCSY mixing, proton decoupling and carbon decoupling, power levels of 7, 2.5 and 3.5 kHz, respectively, were employed. Mixing time of 40 ms was used for TOCSY. Typically, a 0.5 s acquisition time was utilised together with 128 or 256 increments. The sample was dissolved in D_2O containing 0.06% non-deuterated formic acid and a 70 μL sample volume was analysed in Shigemi (Allison Park, PA, USA) NMR tubes (suscepti-bility matched to water) using a Bruker TXI triple resonance probe head equipped with *Z*-axis gradient coils. One-dimensional (1D) ^{1}H-NMR spectra were iterated using PERCH spectral analysis software (Laatikainen *et al*., 1996).

Molecular modelling. Molecular modelling was performed using an MM2-87 force field in a MacroModel software program (Schrödinger, Portland, OR, USA).

RESULTS AND DISCUSSION

The identification of compounds separated from the root extract of *Eleutherococcus senticosus* by HPLC was performed by MS and NMR techniques. Exact mass measurements of molecular and fragment ions of analytes were performed with a time-of-flight (TOF) mass spectrometer, and a sensitivity-improvement method for NMR measurements was developed in order to overcome the insensitivity of the method and the large and impractical sample amount requirements. With the NMR techniques used here, less than one-tenth of the sample was required to provide a spectrum of a quality at least similar to that obtained using standard methods. The key element in this technique was to reduce the sample volume from a standard 500–600 μL down to 60–80 μL. The excellent line shape and sensitivity from such a small sample volume was achieved using susceptibility-matched tubes, a special shimming technique producing a map of B_0 field strength along a sample volume (signal frequencies from different parts of the sample volume were compared), and the use of localised spectral acquisition only from the sample volume responding to the RF-coil area where the B_1 field was homogenous (Mattila, 2001).

Four main components of the extract with retention times (R_t) of 9.7, 18.9, 19.3 and 20.0 min in the HPLC chromatogram of the methanol extract (Fig. 1) were partially identified with the help of LC-MS. In the positive ion electrospray MS of caffeoylquinic acid (Fig. 2) the formation of a sodium adduct *m/z* 377 can readily be seen. Other fragment ions at *m/z* 181 and 163 in the spectrum correspond to the cleavage of quinic acid $[MH\text{-}174]^+$ followed by the loss of water $[MH\text{-}174\text{-}18]^+$.

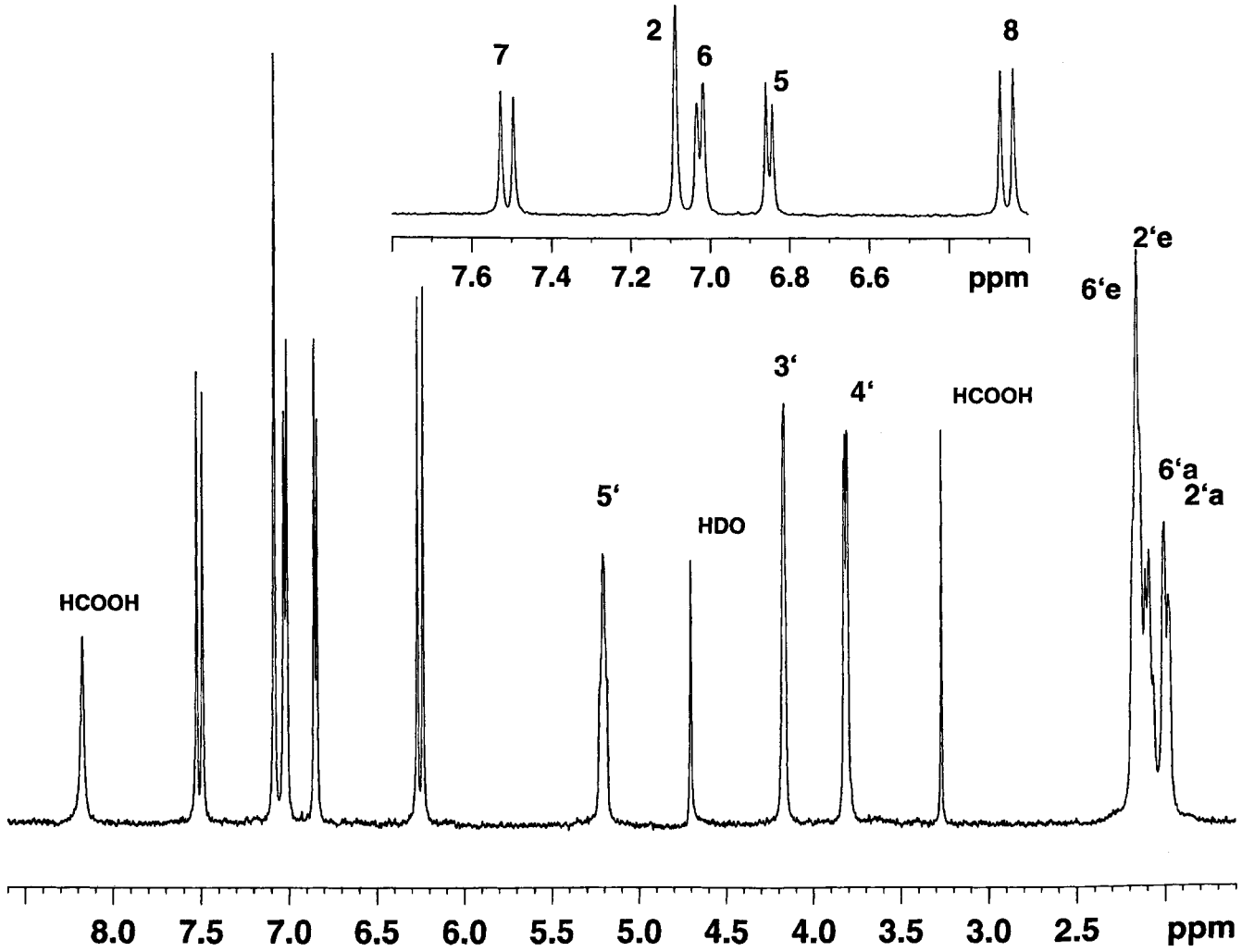

Figure 6. ^{1}H-NMR spectrum of isolated 5′-*O*-caffeoylquinic acid (**1**) (peak at R_t 9.7 min in Fig. 1) measured in D_2O at 500.13 MHz.

These fragmentations were confirmed by exact mass measurement of the ions presented in Table 1. In the negative ionisation mode, the deprotonated molecule at *m/z* 353 was observed and an ion at *m/z* 375 $[M + Na\text{-}2H]^-$ also appeared with an abundance of about 5%. The only fragment ion observed was formed from deprotonated quinic acid at *m/z* 191, which may be a result of the cleavage of the ester bond. As the position of the caffeoyl group in the ring could not be determined from MS data, acquisition of NMR spectra from isolated fractions was necessary.

The sodium adduct also dominated the MS of the dicaffeoylquinic acids. In Fig. 3, the spectra of the first dicaffeoylquinic acid isomer (R_t 18.9 min in Fig. 1) obtained with positive and negative electrospray ionisation are shown. The sodium adduct with *m/z* 539 $[M + Na]^+$ was the most intense; the ion at *m/z* 499 was formed by NaOH cleavage from the ion $[M + Na]^+$, or by water elimination from the protonated molecule. Ions at *m/z* 337 and 163 were also seen in the spectrum of 5′-*O*-caffeoylquinic acid (chlorogenic acid; **1**). The exact masses collected in Table 2 confirm the discussion above. Negative ionisation clearly showed the deprotonated molecule at *m/z* 515, the ion at *m/z* 537 $[M + Na\text{-}2H]^-$, and the ion at *m/z* 191, which could also be seen in the spectrum of 5′-*O*-caffeoylquinic acid. The ion with *m/z* 179 $[M\text{-}337]^-$ was formed from the deprotonated caffeoyl acid group, whilst the ion at *m/z* 161 $[M\text{-}355]^-$ originated from the 5′-*O*-caffeoylquinic acid cleavage of, or water elimination from, $[M\text{-}337]^-$. The positive and negative ESI-MS spectra of the other dicaffeoylquinic acid isomers were almost identical to the spectra shown in Fig. 3 such that the positions of the caffeoyl groups in the quinic acid ring of the dicaffeoylquinic acid isomers could not be solved by reference to MS alone but required NMR data from the isolated compounds. The proposed fragmentation pathways for the dicaffeoylquinic acids are shown in Fig. 4.

NMR spectra of the isolated pure compounds **2–4**, obtained by HPLC fractionation of the methanol extract of the plant and dissolved in D_2O under neutral conditions, showed that each pure compound converted in solution to more than one isomer. This isomerisation was observed to lead to a mixture of at least three different isomers and occurred only in neutral pH conditions. A similar phenomenon has been reported earlier for similar compounds in the pH range 5–8 by Haslam *et al.* (1964). NMR samples were thus prepared in D_2O containing 0.06% formic acid. When the pH of the solution was 2–3, the rearrangement of the caffeoyl groups in the ring did not occur and pure spectra could be obtained from which the positions of

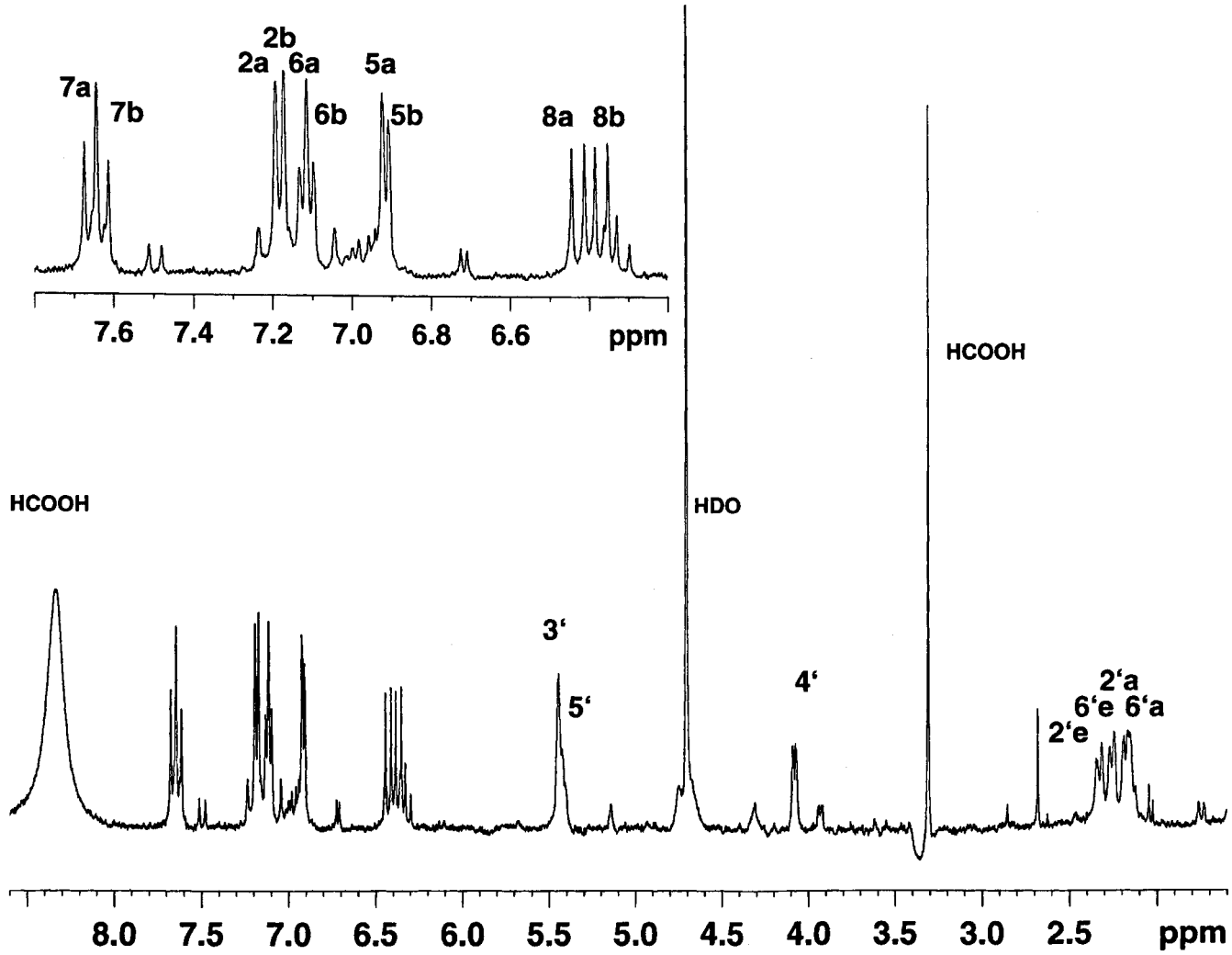

Figure 7. ^{1}H-NMR spectrum of isolated 3′,5′-*O*-dicaffeoylquinic acid (**3**) (peak at R_t 18.9 min in Fig. 1) measured in D_2O at 500.13 MHz.

the caffeoyl groups in the caffeoylquinic acids could be resolved.

The NMR spectrum of a commercial sample of quinic acid is shown in Fig. 5 for comparison purposes. Firstly, the mono-caffeoylquinic acid was identified as 5′-*O*-caffeoylquinic acid (chlorogenic acid; **1**) by comparing the ^{1}H-NMR spectrum (Fig. 6) with that of an authentic commercial sample. For the dicaffeoylquinic acids, a more comprehensive study was performed. After the initial assignment of the signals from a TOCSY experiment (short mixing time, 40 ms) (Table 3), the preliminary values of the proton–proton coupling constants were derived from 1D ^{1}H-NMR spectra (Figs 7–9). These values were later refined using PERCH (Laatikainen *et al.*, 1996) spectral analysis software. These coupling constants were also of assistance in the assignment of the signals. The most informative of the coupling constants were the two large J_{HH}-couplings ($^2J_{6'a6'e}$ and $^3J_{6'a5'a}$) of the axial quinic acid ring proton H-6′a, which allowed its signal to be distinguished from the signals of three other protons with similar chemical shifts, i.e. H-2′a, H-2′e and H-6′e. These three protons have a large coupling constant from geminal coupling but, because of the stereochemistry of the molecule, none of them can have large couplings with the H-3′ or H-5′. After identification of the H-6′a signal, the H-6′e signal could be identified by its TOCSY correlation peak with H-6′a. The other two high-field signals close to 2 ppm, having correlations with each other, are from H-2′a and H-2′e. One of the signals of H-3′, H-4′ and H-5′ shows a correlation in the TOCSY experiment to each of the others and can be recognized as that of H-4′. The signals of H-3′ and H-5′ can be identified by their correlation either to protons H-2′a and H-2′e or to H-6′a and H-6′e.

The iteration of 1D ^{1}H-NMR spectra resulted in unambiguous identification of the signals with exact coupling constants, chemical shifts and multiplicity patterns for each overlapping signal in the spectra. Several attempts to determine either heteronuclear multiple bond correlations from caffeoyl side chain protons to ring carbons, or vice versa (HMBC spectra), or the NOESY correlations between side chain and ring protons (data not shown) were made. However, none of these attempts were successful. The interactions on which these techniques are based are very weak in this structure, and these correlations were not found even in a very concentrated solution of commercial 5′-*O*-caffeoylquinic acid. The side chain positions had, therefore, to be determined using only proton chemical shifts data.

In the spectrum of quinic acid (Fig. 5; 500.13 MHz;

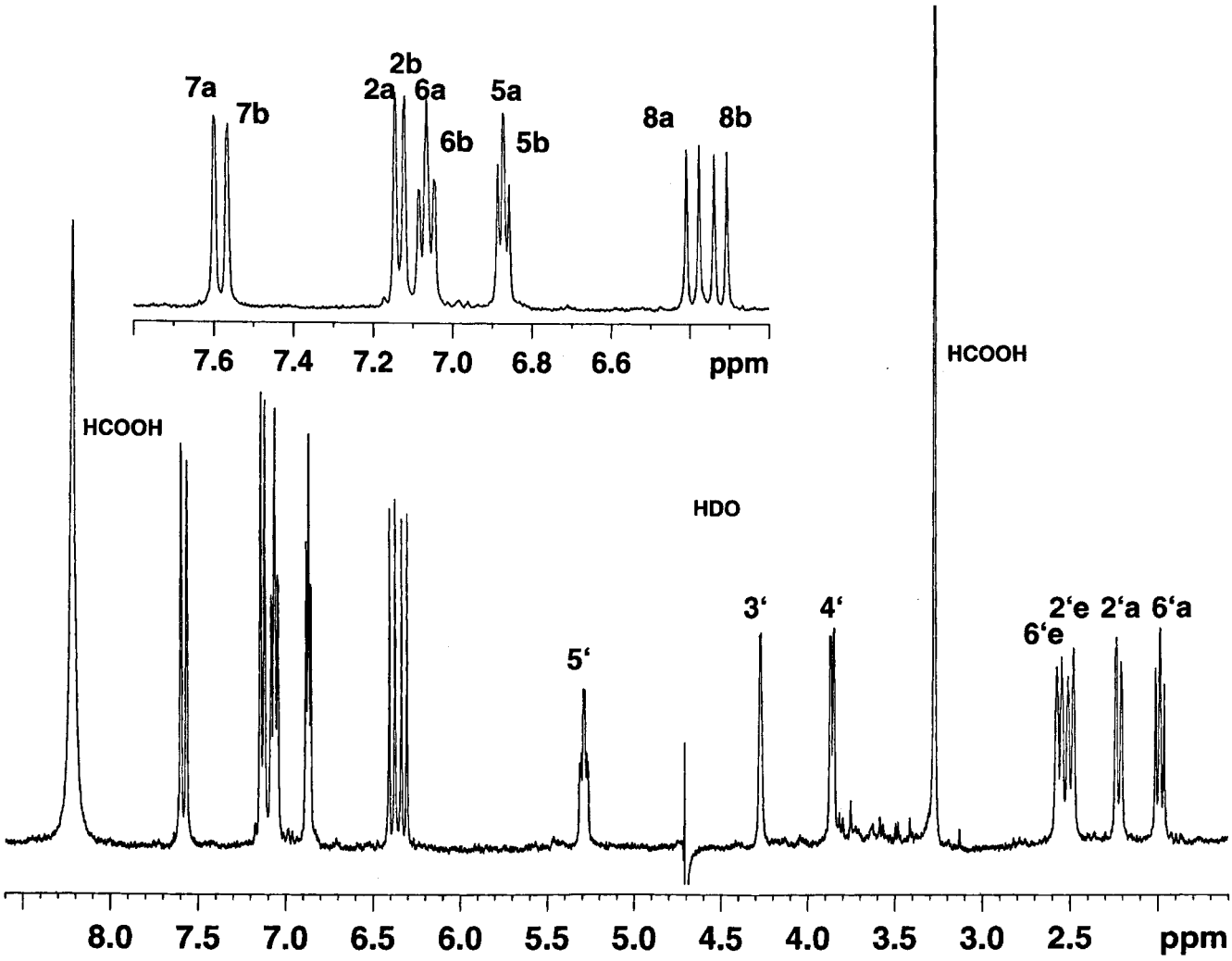

Figure 8. ^{1}H-NMR spectrum of isolated 1′,5′-*O*-dicaffeoylquinic acid (**2**) (peak at R_t 19.3 min in Fig. 1) measured in D_2O at 500.13 MHz.

sample dissolved in D_2O), the signals of protons at 3′, 4′ and 5′ have chemical shifts of 4.11, 3.49 and 3.99 ppm, respectively. In caffeoylquinic acids, the caffeoyl group forms an ester bond with one of the hydroxyl groups of quinic acid, deshielding the proton geminal to it, so that the signal of this proton is moved significantly downfield (Horman *et al.*, 1984; Clifford, 1986; Slacanin *et al.*, 1991). Correspondingly, the signals of the protons not having a geminal ester group do not change their position significantly when compared with the spectrum of quinic acid. After the signals in the ^{1}H-NMR spectra of dicaffeoylquinic acids were identified, the positions of the caffeoyl groups could be solved with the help of the chemical shifts. If only one of the signals of the quinic acid ring protons 3′, 4′ and 5′ moves significantly downfield, then the other caffeoyl group must be attached to the hydroxyl group at carbon 1′ of quinic acid.

The ^{1}H-NMR spectrum of the mono-caffeoylquinic acid (Fig. 6) clearly shows the presence of one caffeoyl group. The signal of H-5′ at 5.21 ppm is shifted 1.22 ppm downfield compared with the spectrum of quinic acid (Fig. 5) and this indicates the presence of a caffeoyl group as an ester at carbon 5′ of the quinic acid ring confirming the compound to be 5′-*O*-caffeoylquinic acid (chlorogenic acid; **1**).

In the ^{1}H-NMR spectra of compounds **2**–**4** (Figs 7–9), the presence of two caffeoyl groups in each can clearly be seen from the two separate signals for each caffeoyl proton. The large 16 Hz *J*-coupling constant between the caffeoyl group double bond protons indicates the *trans*-configuration of the double bond in all isolated compounds. In the spectrum of **3** (Fig. 7), the signal of H-5′ is shifted 1.43 ppm downfield and the signal of the H-3′ is 1.34 ppm downfield compared with the spectrum of quinic acid, indicating the presence of caffeoyl groups in positions 5′ and 3′. Accordingly, **3** is identified as 3′,5′-*O*-dicaffeoylquinic acid. In the spectrum of **2** (Fig. 8), the signal of H-5′ is shifted 1.30 ppm downfield with respect to the quinic acid spectrum, indicating the presence of a caffeoyl group in position 5′. The signals of H-3′ and H-4′ are not significantly deshielded, suggesting that the other caffeoyl group is attached to the hydroxyl group at carbon 1′ of quinic acid. Accordingly **2** is identified as 1′,5′-*O*-dicaffeoylquinic acid. In a like manner, **4** could be identified as 4′, 5′-*O*-dicaffeoylquinic acid by reason of the 1.57 ppm downfield shift of the H-5′ signal and the 1.65 ppm downfield shift of the signal of H-4′ (Fig. 9).

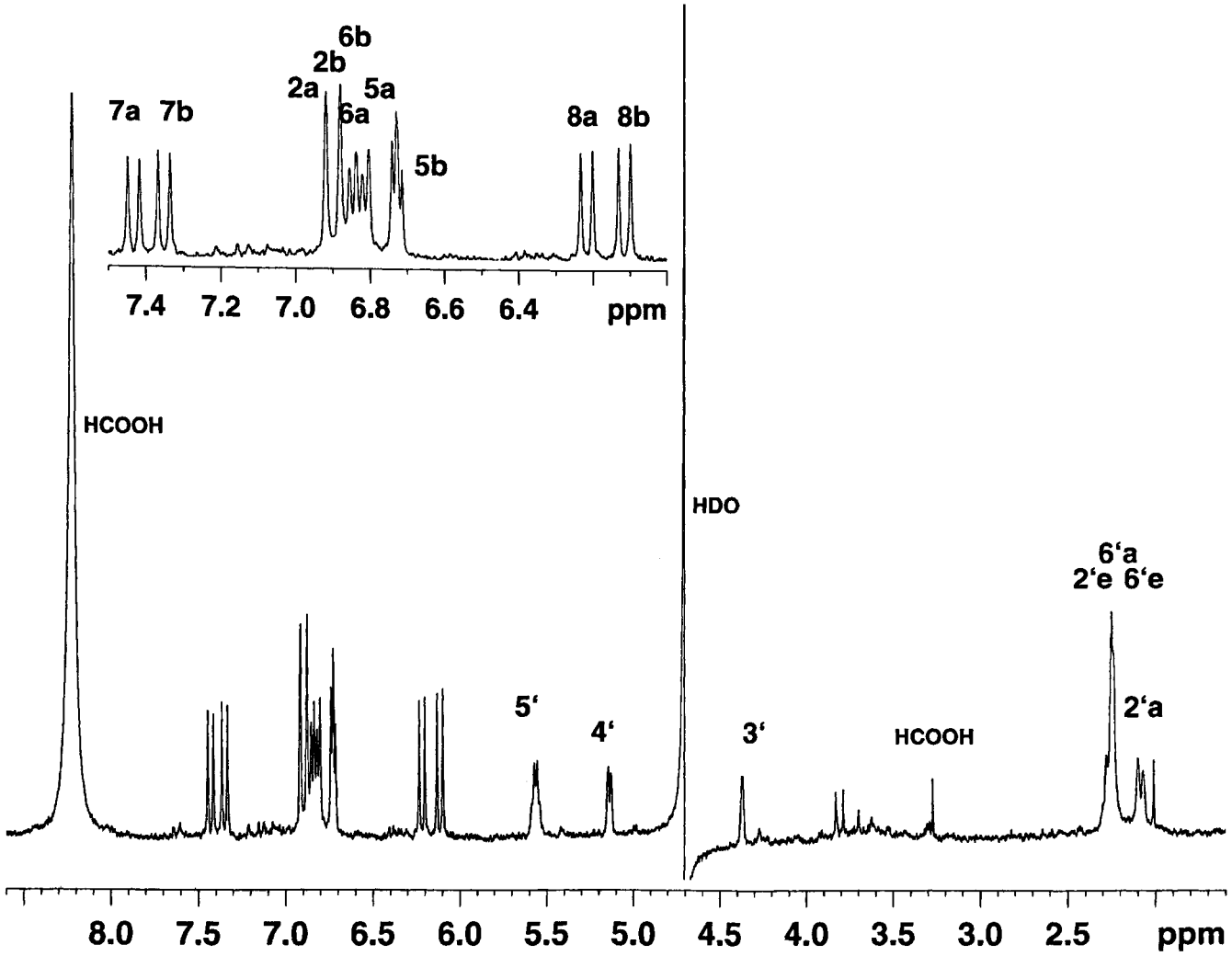

Figure 9. ^{1}H-NMR spectrum of isolated 4′,5′-*O*-dicaffeoylquinic acid (**4**) (peak at R_t 20.0 min in Fig. 1) measured in D_2O at 500.13 MHz.

Compounds **3** and **4** have not previously been isolated from *E. senticosus*.

In order to confirm the above results, the compatibility of the obtained iterated vicinal $^3J_{HH}$ coupling constants with identified structures was also examined. The dihedral bond angles (θ) between the quinic acid ring protons were calculated according to the Altona–Haasnoot equation (Haasnoot *et al.*, 1980; Altona *et al.*, 1994):

$$^3J_{HH} = 14.83\cos^2(\theta) - 0.78\cos(\theta) + 0.60 + \sum_i \lambda_i \times \{0.34 - 2.31\cos^2[s_i(\theta) + 18.4|\lambda_i|]\}$$

In this equation, which is estimated to give ca. 10% error in calculated bond angles, substituent effects are considered by using a group substituent parameter (λ) and sign factor (s_i) for each substituent that is attached to the same carbon atom as the coupling protons. However, there are no appropriate substituent parameters for the large caffeoyl groups and this will give lead to an inaccuracy in the calculated dihedral bond angles. The corresponding bond angles were also estimated by molecular modelling so that the results could be compared. The MM2-87 force field, which is very similar to that used by Altona *et al.* (1994) in their original prediction of the relationship between coupling constants and bond angles, was employed in molecular modelling.

It can be seen from Table 4 that the dihedral bond angles obtained by molecular modelling and from the Altona–Haasnoot equation are reasonably close to each other, confirming that the iterated coupling constants obtained fit the identified structures well. Whilst deviations between calculated and modelled bond angles of protons 4′–5′ and 5′–6′a were obtained, similar shifts in the same direction were also established in the results based on the spectrum of authentic 5′-*O*-caffeoylquinic acid, which demonstrates the restrictions in the general applicability of the Altona–Haasnoot equation.

5′-*O*-caffeoylquinic acid (chlorogenic acid; **1**) was obtained as a white powder and identified by comparison (MS, ^{1}H-NMR) with an authentic sample (Aldrich): ^{1}H-NMR (D_2O), δ (ppm) 1.99 (1H, dd, H-2′a, $J = 5.28$, $J = -14.52$), 2.09 (1H, dd, H-6′a, $J = 9.80$, $J = -13.77$), 2.15 (1H, ddd, H-2′e, $J = 3.40$, $J = -14.52$, $J = -1.53$), 2.17 (1H, ddd, H-6′e, $J = 4.15$, $J = -1.53$, $J = -13.77$), 3.81 (1H, dd, H-4′, $J = 8.62$, $J = 3.26$), 4.17 (1H, ddd, H-3′, $J = 3.26$, $J = 3.40$, $J = 5.28$), 5.21 (1H, ddd, H-5′, $J = 8.62$, $J = 4.15$, $J = 9.80$), 6.25 (1H, d, H-8, $J = 16.18$),

Table 3. Correlations in the TOCSY spectra of isolated dicaffeoylquinic acids 2–4

1′,5′-*O*-Dicaffeoylquinic acid (2)		3′,5′-*O*-Dicaffeoylquinic acid (3)		4′,5′-*O*-Dicaffeoylquinic acid (4)	
δ (ppm)	Correlations	δ (ppm)	Correlations	δ (ppm)	Correlations
1.99 H-6′a	2.56, 3.86, 5.29	2.15 H-6′a	2.25, 5.42, 4.08	2.08 H-2′a	2.26, 4.37
2.22 H-2′a	2.50, 4.27	2.17 H-2′a	2.33, 5.45	2.24 H-6′e	5.14, 5.56, (2.25)[c]
2.50 H-2′e	2.22, 4.27	2.25 H-6′e	2.15, 4.08, 5.42	2.25 H-6′a	5.14, 5.56, (2.24)[c]
2.56 H-6′e	1.99, 3.86, 5.29	2.33 H-2′e	2.17, 5.45,	2.26 H-2′e	2.08, 4.37
3.86 H-4′	1.99, 2.56, 4.27, 5.29	4.08 H-4′	2.25, 5.42, 5.45, 2.15	4.37 H-3′	2.08, 2.26, 5.14
4.27 H-3′	2.22, 2.50, 3.86	5.42 H-5′	2.15, 2.25, 4.08	5.14 H-4′	2.24, 2.25, 4.37, 5.56
5.29 H-5′	1.99, 2.56, 3.86	5.45 H-3′	2.17, 2.33, 4.08	5.56 H-5′	2.24, 2.25, 5.14
6.32 H-8b/a	(7.58/7.59)[a]	6.37 H-8b	7.63	6.12 H-8b	7.35
6.39 H-8a/b	(7.59/7.58)[a]	6.43 H-8a	7.66	6.22 H-8a	7.43
6.86 H-5b	7.05	6.91 H-5b/a	(7.10/7.12)[b]	6.72 H-5b	6.81, 6.92
6.88 H-5a	7.08	6.92 H-5a/b	(7.12/7.10)[b]	6.73 H-5a	6.85
7.05 H-6b	6.86	7.10 H-6b/a	(6.91/6.92)[b]	6.81 H-6b	6.72
7.08 H-6a	6.88	7.12 H-6a/b	(6.92/6.91)[b]	6.85 H-6a	6.73, 6.92
7.12 H-2b/a	—	7.17 H-2b/a	—	6.88 H-2b	—
7.15 H-2a/b	—	7.19 H-2a/b	—	6.92 H-2a	6.72, 6.85
7.58 H-7a/b	(6.32/6.32)[a]	7.63 H-7b	6.37	7.35 H-7b	6.12
7.59 H-7b/a	(6.39/6.32)[a]	7.66 H-7a	6.43	7.43 H-7a	6.22

[a] Signals of H-7a and H-7b show too much overlap to distinguish their correlations to H-8a and H-8b.
[b] Signals of H-5a and H-5b show too much overlap to distinguish their correlations to H-6a and H-6b.
[c] The correlation between H-6′a and H-6′e cannot be observed owing to strong overlap of the signals.

6.85 (1H, d, H-5, $J = 8.19$), 7.02 (1H, dd, H-6, $J = 8.19$, $J = 2.17$), 7.09 (1H, d, H-2, $J = 2.17$), 7.51 (1H, d, H-7, $J = 16.18$); ESI$^+$/TOF/MS, *m/z* (relative intensity percentage) 377 $[M + Na]^+$ (100), 181 $[MH\text{-}174]^+$ (7), 163 $[MH\text{-}174\text{-}18]^+$ (75); ESI$^-$/TOF/MS, *m/z* (relative intensity percentage) 375 $[M + Na\text{-}2H]^-$ (4), 353 $[M\text{-}H]^-$ (17), 191 $[M\text{-}163]^-$ (100).

1′,5′-*O*-dicaffeoylquinic acid (**2**) was obtained as a white powder: ^{1}H-NMR (D_2O), δ (ppm) 1.99 (1H, dd, H-6′a, $J = 10.79$, $J = -13.85$), 2.22 (1H, dd, H-2′a, $J = 3.55$, $J = -15.48$), 2.50 (1H, ddd, H-2′e, $J = 3.79$, $J = 2.77$, $J = -15.48$), 2.56 (1H, ddd, H-6′e, $J = 4.30$, $J = 2.77$, $J = -13.85$), 3.86 (1H, dd, H-4′, $J = 9.64$, $J = 3.47$), 4.27 (1H, ddd, H-3′, $J = 3.47$, $J = 3.79$, $J = 3.55$), 5.29 (1H, ddd, H-5′, $J = 9.64$, $J = 4.30$, $J = 10.79$), 6.32 (1H, d, H-8b, $J = 16.21$), 6.39 (1H, d, H-8a, $J = 16.13$), 6.86 (1H, d,

Table 4. Dihedral bond angles between the quinic acid ring protons of the caffeoylquinic acids 1–4 obtained by molecular modelling and from the Altona–Haasnoot equation

Compound	Protons	Observed vicinal couplings (Hz)	Altona–Haasnoot dihedral angle (°)	Modelled dihedral angle (MM2-87) (°)
5′-*O*-Caffeoylquinic acid (**1**)	3′e–2′a	5.28	45	53
	3′e–2′e	3.40	−64	−62
	3′e–4′a	3.26	−55	−54
	4′a–5′a	8.62	158	179
	5′a–6′a	9.80	−151	−178
	5′a–6′e	4.15	−61	−61
1′,5′-*O*-Dicaffeoylquinic acid (**2**)	3′e–2′a	3.55	54	57
	3′e–2′e	3.79	−62	−58
	3′e–4′a	3.47	−56	−53
	4′a–5′a	9.64	163	176
	5′a–6′a	10.79	−156	−177
	5′a–6′e	4.30	−60	−60
3′,5′-*O*-Dicaffeoylquinic acid (**3**)	3′e–2′a	4.01	50	53
	3′e–2′e	2.74	−69	−62
	3′e–4′a	3.33	−55	−48
	4′a–5′a	9.42	162	178
	5′a–6′a	10.58	−155	−177
	5′a–6′e	4.26	−60	−59
4′,5′-*O*-Dicaffeoylquinic acid (**4**)	3′e–2′a	4.27	50	54
	3′e–2′e	2.77	−68	−62
	3′e–4′a	3.11	−59	−61
	4′a–5′a	9.35	163	175
	5′a–6′a	8.93	−148	−176
	5′a–6′e	6.35	−50	−58

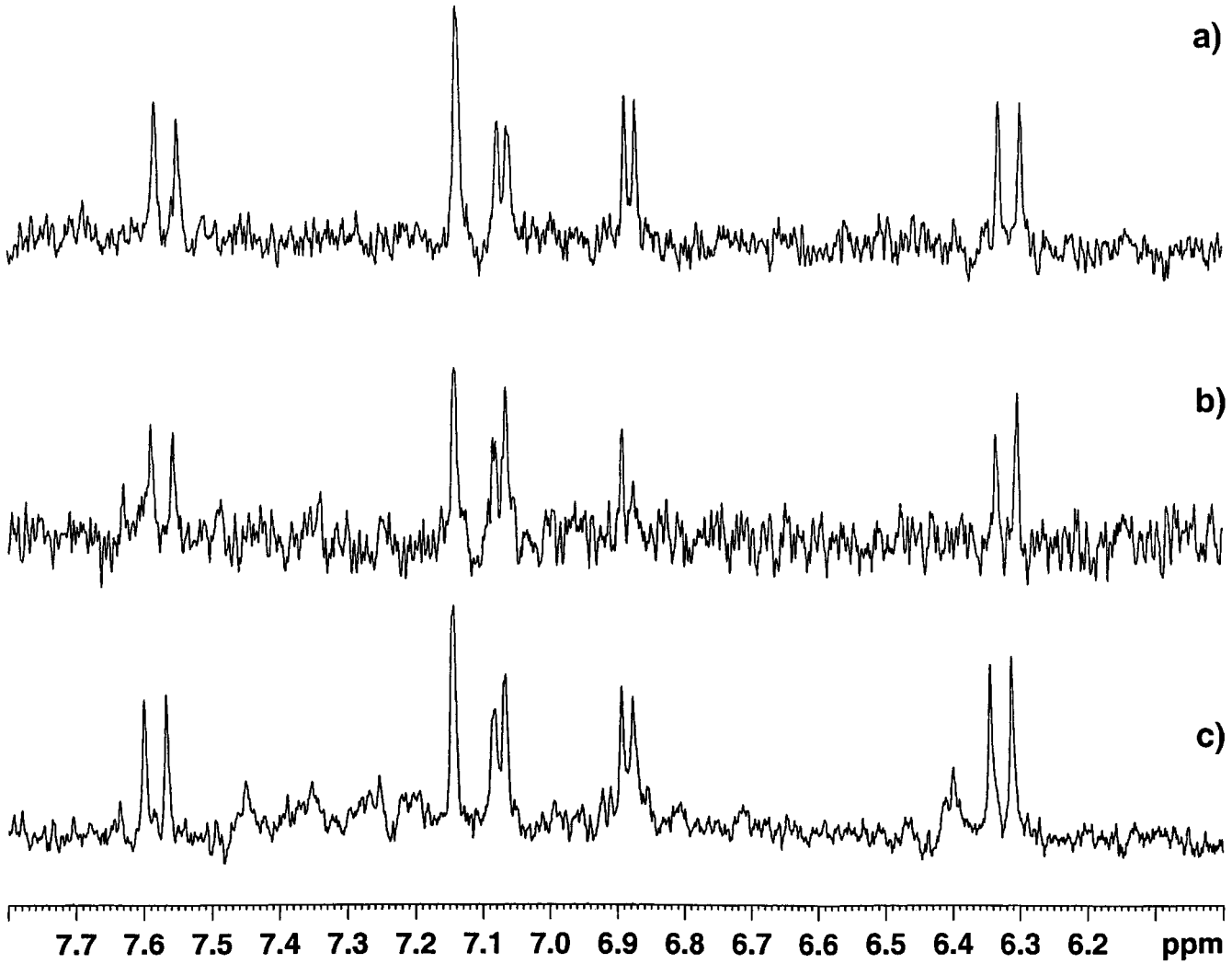

Figure 10. Aromatic regions of the ^{1}H-NMR spectra of isolated 5′-*O*-caffeoylquinic acid (**1**) acquired at concentrations of (a) 50 nmol (one pulse; s/n 9.56); (b) 3.7 nmol [128 pulses (3 min); s/n 5.1]; and (c) 225 pmol [45274 pulses (16 h); s/n 25].

H-5b, $J = 8.25$), 6.88 (1H, d, H-5a, $J = 8.24$), 7.05 (1H, dd, H-6b, $J = 8.25$, $J = 2.08$), 7.08 (1H, dd, H-6a, $J = 8.24$, $J = 2.07$), 7.12 (1H, d, H-2b, $J = 2.08$), 7.15 (1H, d, H-2a, $J = 2.07$), 7.58 (1H, d, H-7b, $J = 16.21$), 7.59 (1H, d, H-7a, $J = 16.13$); ESI$^+$/TOF/MS, *m/z* (relative intensity percentage) 539 $[M + Na]^+$ (100), 499 $[MH\text{-}18]^+$ (5), 377 $[M + Na\text{-}162]^+$ (4), 337 $[M\text{-}179]^+$ (5), 195 $[M\text{-}321]^+$ (6), 163 $[M\text{-}353]^+$ (57); ESI$^-$/TOF/MS, *m/z* (relative intensity percentage) 537 $[M + Na\text{-}2H]^-$ (44), 515 $[M\text{-}H]^-$ (72), 353 $[M\text{-}163]^-$ (48), 191 $[M\text{-}325]^-$ (100), 179 $[M\text{-}337]^-$ (9), 161 $[M\text{-}355]^-$ (3).

3′,5′-*O*-dicaffeoylquinic acid (**3**) was obtained as a white powder: ^{1}H-NMR (D_2O), δ (ppm) 2.15 (1H, dd, H-6′a, $J = 10.58$, $J = -13.16$), 2.17 (1H, dd, H-2′a, $J = 4.01$, $J = -15.00$), 2.25 (1H, ddd, H-6′e, $J = 4.26$, $J = 2.53$, $J = -13.16$), 2.33 (1H, ddd, H-2′e, $J = 2.74$, $J = 2.53$, $J = -15.00$), 4.08 (1H, dd, H-4′, $J = 9.42$, $J = 3.33$), 5.42 (1H, ddd, H-5′, $J = 9.42$, $J = 4.26$, $J = 10.58$), 5.45 (1H, ddd, H-3′, $J = 3.33$, $J = 2.74$, $J = 4.01$), 6.37 (1H, d, H-8b, $J = 16.14$), 6.43 (1H, d, H-8a, $J = 16.04$), 6.91 (1H, d, H-5b, $J = 8.26$), 6.92 (1H, d, H-5a, $J = 8.29$), 7.10 (1H, dd, H-6b, $J = 8.26$, $J = 2.00$), 7.12 (1H, dd, H-6a, $J = 8.29$, $J = 1.97$), 7.17 (1H, d, H-2b, $J = 2.00$), 7.19

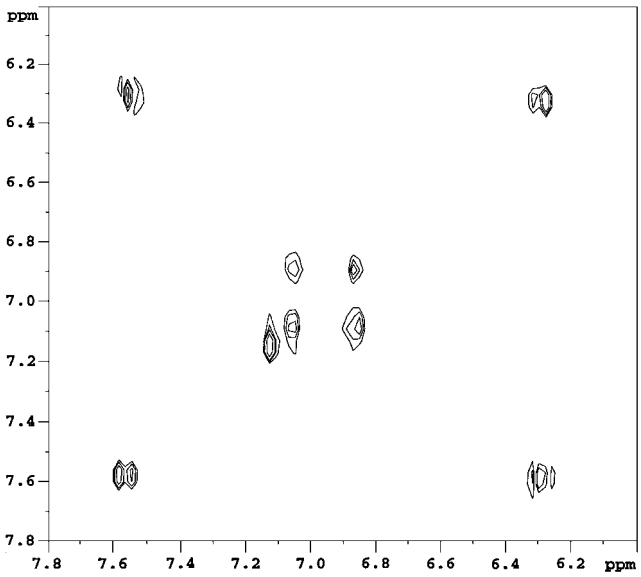

Figure 11. ^{1}H-TOCSY spectrum acquired within 9 min from 50 nmol of isolated 5′-*O*-caffeoylquinic acid (**1**).

(1H, d, H-2a, $J = 1.97$), 7.63 (1H, d, H-7b, $J = 16.14$), 7.66 (1H, d, H-7a, $J = 16.04$); ESI^+/TOF/MS, *m/z* (relative intensity percentage) 539 $[M + Na]^+$ (100), 499 $[MH\text{-}18]^+$ (17), 377 $[M + Na\text{-}162]^+$ (2), 337 $[M\text{-}179]^+$ (4), 195 $[M\text{-}321]^+$ (4), (2), 163 $[M\text{-}353]^+$ (35); ESI^-/TOF/MS, *m/z* (relative intensity percentage) 537 $[M + Na\text{-}2H]^-$ (54), 515 $[M\text{-}H]^-$ (33), 353 $[M\text{-}163]^-$ (100), 191 $[M\text{-}325]^-$ (17), 179 $[M\text{-}337]^-$ (8), 161 $[M\text{-}355]^-$ (2).

4′,5′-*O*-dicaffeoylquinic acid (**4**) was obtained as a white powder: ^{1}H-NMR (D_2O): δ (ppm) 2.08 (1H, dd, H-2′a, $J = 4.27$, $J = -15.22$), 2.24 (1H, dd, H-6′e, $J = 6.35$, $J = -11.28$), 2.25 (1H, dd, H-6′a, $J = 8.93$, $J = -11.28$), 2.26 (1H, dd, H-2′e, $J = 2.78$, $J = -15.22$), 4.37 (1H, ddd, H-3′, $J = 3.11$, $J = 2.78$, $J = 4.27$), 5.14 (1H, dd, H-4′, $J = 9.35$, $J = 3.11$), 5.56 (1H, ddd, H-5′, $J = 9.35$, $J = 8.93$, $J = 6.35$), 6.12 (1H, d, H-8b, $J = 16.20$), 6.22 (1H, d, H-8a, $J = 16.11$), 6.72 (1H, d, H-5b, $J = 8.24$), 6.73 (1H, d, H-5a, $J = 8.41$), 6.81 (1H, dd, H-6b, $J = 8.24$, $J = 2.30$), 6.85 (1H, dd, H-6a, $J = 8.41$, $J = 2.11$), 6.88 (1H, d, H-2b, $J = 2.30$), 6.92 (1H, d, H-2a, $J = 2.11$), 7.35 (1H, d, H-7b, $J = 16.20$ Hz), 7.43 (1H, d, H-7a, $J = 16.11$); ESI^+/TOF/MS, *m/z* (relative intensity percentage) 539 $[M + Na]^+$ (100), 499 $[MH\text{-}18]^+$ (3), 337 $[M\text{-}179]^+$ (2), 163 $[M\text{-}353]^+$ (39); ESI^-/TOF/MS, *m/z* (relative intensity percentage) 537 $[M + Na\text{-}2H]^-$ (22), 515 $[M\text{-}H]^-$ (100), 353 $[M\text{-}163]^-$ (31), 191 $[M\text{-}325]^-$ (1), 179 $[M\text{-}337]^-$ (4), 161 $[M\text{-}355]^-$ (4).

Quantification of **1** in *E. senticosus* was performed by HPLC using authentic 5′-*O*-caffeoylquinic acid at concentrations of 0.23, 0.091, 0.036 and 0.015 mg/mL for calibration. The results show that the root of this herb contained 1.7% of **1** [twice the amount reported earlier (Baranov, 1982)], indicating that this particular herb is rich in these constituents. In order to demonstrate the very high sensitivity of the NMR method developed, spectra were measured using consecutively more dilute solutions of **1** isolated from the plant extract following 26 preparative HPLC runs. First, the isolated sample was diluted to about 1/1000 (50 nmol **1**) and a basic 1D ^{1}H-NMR spectrum with a signal-to-noise (s/n) ratio of 9.56 was obtained with one pulse [Fig. 10(a)] and a TOCSY spectrum within 9 min (Fig. 11). This demonstrated that one HPLC isolation step with a normal analytical column would have been enough to provide far more of the compound than was needed to measure the NMR spectrum. The next dilution produced a solution containing 3.7 nmol of **1**; a 1D ^{1}H-NMR spectrum with s/n 5.1 was measured with 128 pulses in 3 min [Fig. 10(b)]. Finally, the sample was diluted to 225 pmol **1**, and even at this dilution the 1D ^{1}H-NMR spectrum was obtained in 16 h [Fig. 10(c)].

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