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# Identification of *Xanthium Sibiricum* Components using LC-SPE-NMR-MS Hyphenated System

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**Abstract** *Xanthium sibiricum* is used as a traditional folk medicine for the treatment of cancer, fever, headache, nasal sinusitis, and skin pruritus. This study aimed to identify components from *Xanthium sibiricum* extracts using an SPE-800MHz NMR-MS hyphenated system. The simultaneous acquisition of MS and NMR spectra from the same chromatographic peaks significantly increases the depth of information acquired for the compound and allows the elucidation of structures that would not be possible using MS or NMR data alone. LC -NMR analysis was conducted using a HPLC separation system coupled to 800 MHz spectrometer equipped with a cryoprobe, and a SPE unit was used to automatically trap chromatographic peaks using a HPLC pump. LC-MS analysis was conducted with a Q-TOF MS instrument using ESI ionization in the negative ion mode. Using the hyphenated analysis, several secondary metabolites were identified, such as 3',5'-O-dicaffeoylquinic acid, 1',5'-O-dicaffeoyl- quinic acid, and ethyl caffeate. These results demonstrate that the SPE-800MHz NMR-MS hyphenated system can be used to identify metabolites within natural products that have complex mixtures.

**Keywords** LC-SPE-NMR-MS, Hyphenated system, *Xanthium sibiricum*, $^{1}$H NMR, 2D NMR

## Introduction

*Xanthium sibiricum* is the dried fruit of *Xanthium strumarium* and is used as a traditional folk medicine for the treatment of cancer, fever, headache, sinusitis, and skin pruritus.[1] Previous chemical studies conducted on *Xanthium sibiricum* have reported the presence of carboxyatractyloside, xanthanol, isoxanthanol, hydroquinone, alkaloids, thiazinedoine, in addition to several fatty acids such as linoleic acid and oleic acid.[2-6] Although online spectroscopic methods like liquid chromatography (LC)–UV and LC– mass spectrometry (MS) are very sensitive and useful methods, nuclear magnetic resonance (NMR) analysis is subsequently required for accurate structural identification. In metabolomic study, NMR spectroscopy has been used to identify xenobiotic metabolites directly in complex matrixes, such as urine and plasma,[7-14] and/or determine the structures of unknown components. However, to obtain structural information on the individual components of natural product, each of the compounds needs firstly to be homogeneously purified before determining the structure using several analytical methods, including NMR and MS.

The use of LC–NMR is a logical development in this respect, but sensitivity is currently a problem.[15-17]

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One solution proposed to overcome the limitations of directly coupled LC-NMR is to integrate solid-phase extraction (SPE) and MS within the system. An integrated combination of LC, SPE, NMR, and MS is a rational way of enhancing LC-NMR, as it significantly expands the applications of this hyphenated technique.[18,19] This type of modular system combines the benefits of LC-NMR and the peak enrichment capabilities of SPE with peak identification by MS.[20] The subsequent simultaneous acquisition of MS and NMR spectra from the same chromatographic peaks of one sample thus significantly increases the depth of information acquired and allows the elucidation of structures, whereas the use of MS or NMR data alone would not be sufficient.

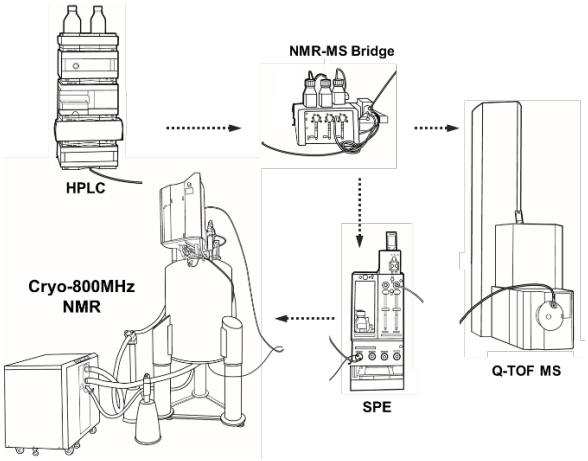
In this study, the SPE-800MHz NMR-MS hyphenated system was used to determine metabolites from *Xanthium sibiricum* extracts. The semipolar metabolite contents of *Xanthium sibiricum* were extracted using ethanol as an extraction solvent, and extracts were analyzed using $^{1}$H NMR and MS to detect secondary metabolites of *Xanthium sibiricum*.

## Experimental Methods

*Plant Material and Chemicals*- *Xanthium sibiricum* was obtained from South Korea and stored at -80 ℃ until required for analysis. Ethanol (99.8%) purchased from Sigma-Aldrich was used to extract *Xanthium sibiricum*; acetonitrile-d3 (99.9%) purchased from Cambridge Isotope Laboratories, Inc. was used as the NMR solvent, acetonitrile and water (HPLC grade) were purchased from Burdick & Jackson, and formic acid was obtained from Sigma- Aldrich.

*Sample Preparation for NMR and MS Analyses*- *Xanthium sibiricum* was freeze-dried and ground to a fine powder. Subsequently, 1 ml of 70% ethanol was added to 20 mg of dried powder as an extraction solvent. Extracts were sonicated for 30 min and then centrifuged (10 min, 16609 g) at room temperature. Prior to analysis, filtration through a 0.2 μm membrane filter was performed to protect the HPLC column used under high pressure.

*LC-SPE-NMR/MS System*- Separation, UV detection, and MS and NMR analyzes were conducted using the system schematically represented in Scheme 1. Chromatographic separation of *Xanthium sibiricum* extract was performed using an Agilent 1260 Infinity HPLC; separated peaks were then split into two portions and sent to the NMR and MS system by the NMR-MS bridge device. Portions sent to the MS system were analyzed by an Impact high definition (HD) MS system (Bruker Daltonics GmbH, Bremen), and portions sent to the NMR system were automatically trapped by Hysphere Resin GP cartridges (2 mm i.d, particle size 10–12 μm) using the Bruker/Spark Prospekt 2 SPE unit (Bruker BioSpin and Spark, Emmen, The Netherlands), after the post-column addition of water using a Knauer K100 HPLC pump (Berlin, Germany). The trapped peaks were then dried with nitrogen gas for 30 min and eluted with deuterated acetonitrile into a 800 MHz NMR spectrometer equipped with a 60 ul flow-probe (Bruker BioSpin Co., Billerica, MA).




**Scheme 1.** Schematic of SPE-800MHz NMR/MS system.

*LC Conditions*- LC analyses were conducted using a 1260 Infinity HPLC system (Agilent Technology Inc., Santa Clara, CA) comprising a G1311B solvent delivery gradient pump, G1329B Auto sampler, and a DAD UV detector (Agilent Technology Inc., Santa Clara, CA, USA). Chromatographic separation was

conducted using a ProntoSIL™ SC-04 Eurobond C18 column (125 × 4 mm id, 5 μm; BISCHOFF Chromatography, Leonberg, Germany). The mobile phase consisted of 0.1% formic acid in acetonitrile and 0.1% formic acid in water as solvent A and B, respectively. Separation was performed by gradient elution with 5% A for 0 min, 5% A for 3 min, 17% A for 8 min, 30% A for 20 min, 33% A for 22 min, 46% A for 23 min, 99% A for 26 min, 99% A for 28 min, 5% A for 31 min, and 5% A for 35 min at a flow rate of 1.5 mL/min. The sample injection volume was 5 μL.

*MS Conditions*- Accurate mass measurements and MS/MS fragmentation analyzes were conducted using an Impact high definition (HD) mass spectrometer (Bruker Daltonics GmbH, Bremen). Electrospray ionization (ESI) mass spectra were acquired in negative ionization modes by scanning over a m/z range of 50 to 1000 with a sampling rate of 2 Hz. In the negative ionization mode, the capillary was set at +4500 V, the end plate offset was set at -500 V, the nebulizer gas was set at 1.5 bar, and the dry gas was set at 5 L/min at 200°C. MS/MS analyses were acquired by automatic fragmentation, in which five most intense mass peaks were fragmented. Collision energy values for the MS/MS experiments were adjusted as follows: m/z 100, 10 eV; m/z 300, 15 eV; m/z 500, 20 eV; and m/z 1000, 35 eV. Nitrogen was used as the drying, nebulizing, and collision gas; the ESI capillary voltage was set at 3.1 kV, and temperatures of the electrospray source and desolvation gas were 100 °C and 300 °C, respectively.

*NMR Spectroscopy*- $^{1}$H NMR and 2D NMR spectra were acquired on a Bruker AVANCE Ⅲ HD 800 MHz FT-NMR Spectrometer at 298K using a 5mm Triple-resonance Inverse (TCI) cryoprobe with Z-Gradients (Bruker BioSpin Co., Billerica, MA). Time-shared double presaturation (pulse program "lc1prf2" from Bruker pulse program library) was applied to suppress residual water (δH = 2.1 ppm for HOD) and organic solvent signal (δH = 1.93 ppm for CD2HCN). Furthermore, 256 scans were collected into 32768 data points using a spectral width of 16025.6 Hz, a relaxation delay of 2.4 s, and an acquisition time of 1.0 s. The 1 Hz line-broadening function was applied to all spectra for Fourier transform (FT) followed by phasing and baseline correction. Signal assignment was achieved using two dimensional (2D) correlated spectroscopy (COSY), heteronuclear single quantum correlation (HSQC), and a comparison with literature values.

**Results**

The on-line LC-SPE-NMR/MS system is characterized in that after the injected sample is separated through LC system, the NMR and MS spectra can be obtained from same chromatographic peaks. In the SPE-NMR/MS system, the NMR-MS bridge device simultaneously distributes the components separated from the LC column to the MS and NMR system with a separation ratio in the bridge of approximately 1:20. As the sensitivity of MS is higher than that of NMR, more samples are sent to the NMR side.

In this study, *Xanthium sibiricum* extract was separated using the HPLC system (UV chromatogram resulting from HPLC elution at 262 nm wavelength are shown in Figure 1a). The MS and MS/MS spectra of each component from *Xanthium sibiricum* extracts were acquired in the negative ionization mode, and the total ion chromatogram (TIC) are shown in Figure 1b. Three major peaks of the extract with retention times (RT) of 25.2, 25.5, and 32.3 min were trapped in the HPLC chromatogram and dried by SPE cartridges, and $^{1}$H NMR analyses of these peaks were subsequently conducted. However, as none of the $^{1}$H NMR spectra had a high signal-to-noise ratio (S/N), the same peak was multi-trapped using repeated LC injections with the aim of improving the S/N. After conducting trapping 10 times, $^{1}$H NMR spectra and 2D NMR spectra (COSY and HSQC) with a significantly increased S/N were obtained.

A comparison between the NMR and MS spectra showed peaks corresponding to the RT described above at 3', 5'-O-dicaffeoylquinic acid (# 1, RT 25.2 min), 1', 5'-O-dicaffeoylquinic acid (# 2, RT 25.5 min), and ethyl caffeine (# 3, RT 32.3 min) (Figure 1c).

These compounds are summarized in Table 1 with corresponding chromatograph peak numbers, retention times, and assignment of various signals in associated $^{1}$H NMR spectra.

**Table 1**. Chromatographic and NMR data for compounds identified in *Xanthium sibiricum* by LC-SPE-NMR.

| LC peak / Structure | RT (min) | Compound | NMR signals δ (ppm) (multiplicity, Assignment) |
|---|---|---|---|
| #1 | 25.2 | 3',5'-O-dicaffeoylquinic acid | 5.38 (ddd, H3'), 3.88 (dd, H4'), 5.33 (ddd, H5'), 7.12 (d, H2), 7.14 (d, H2), 6.84 (d, H5), 6.86 (d, H5), 7.03 (dd, H6), 7.05 (dd, H6), 7.57 (d, H7), 7.59 (d, H7), 6.29 (d, H8), 6.35 (d, H8) |
| #2 | 25.5 | 1',5'-O-dicaffeoylquinic acid | 4.17 (ddd, H3'), 3.70 (dd, H4'), 5.31 (ddd, H5'), 7.13 (d, H2), 6.85 (d, H5), 7.03 (dd, H6), 7.05 (dd, H6), 7.58 (d, H7), 7.59 (d, H7), 6.26 (d, H8), 6.32 (d, H8) |
| #3 | 32.3 | Ethyl caffeate | 7.10 (d, H2), 6.84 (d, H5), 7.02 (dd, H6), 7.54 (d, H7), 6.29 (d, H8), 4.18 (q, H9), 1.27 (t, H10) |

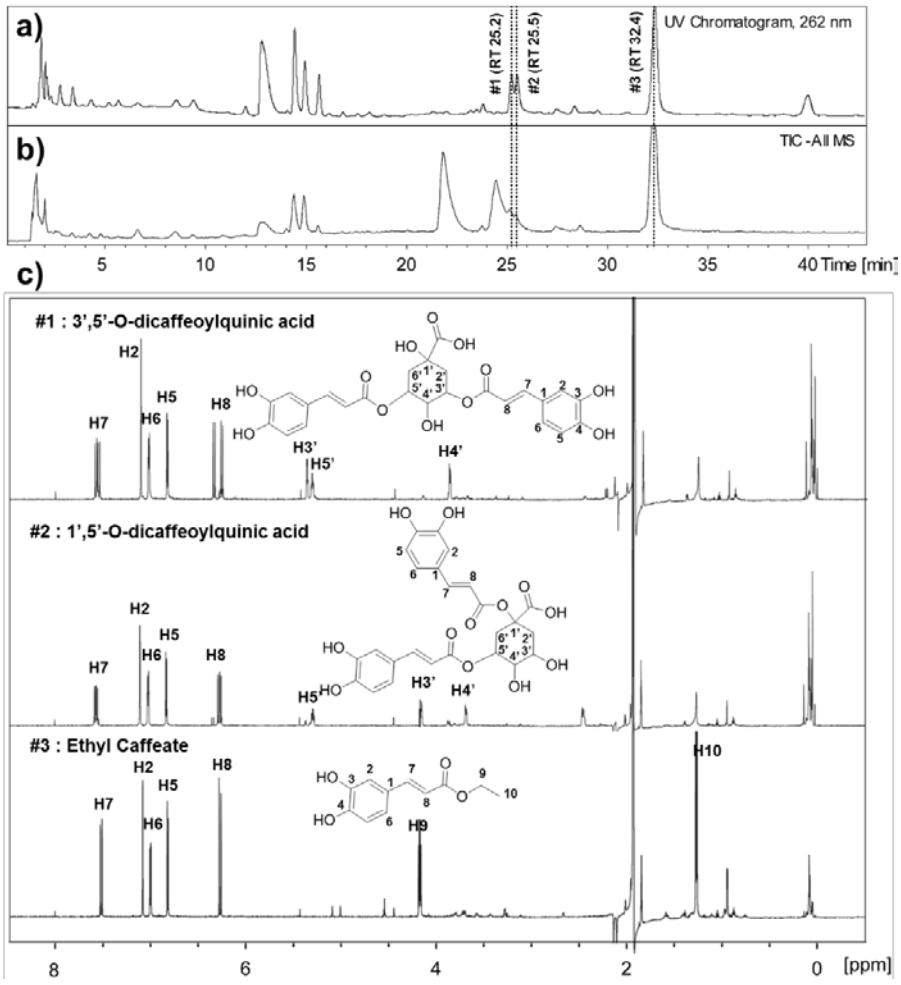



**Figure 1.** (a) Chromatographic profile at 262nm; (b) total ion chromatogram (TIC) with negative ion mode ESI/MS; (c) $^{1}$H NMR spectra of importantly identified chromatographic peaks of *Xanthium sibiricum* extract.

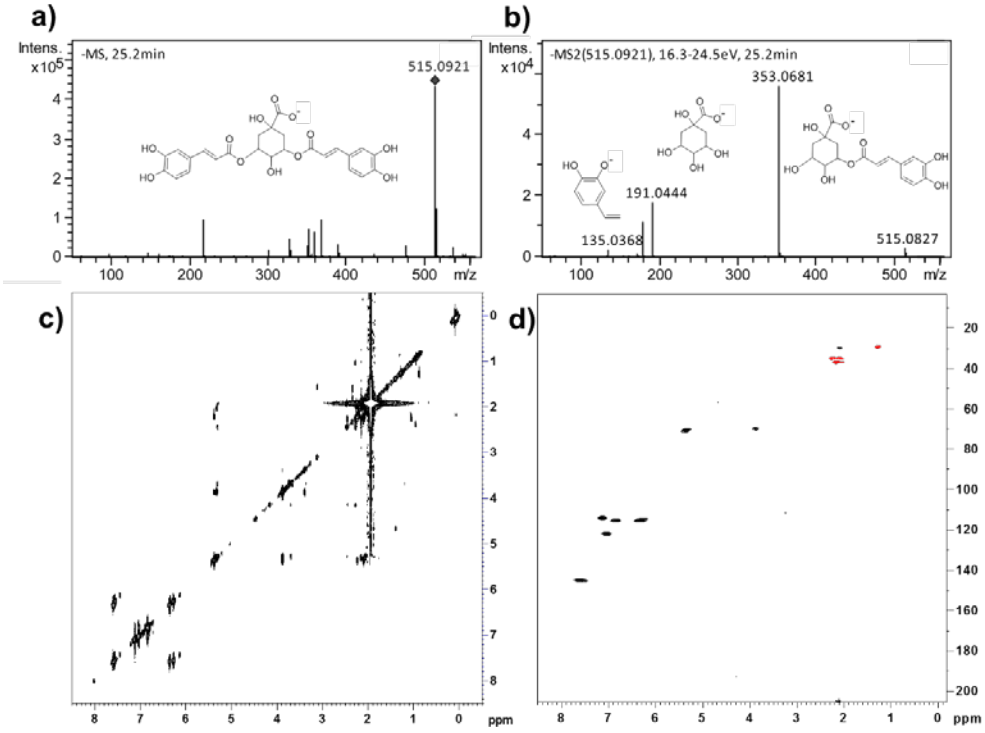



**Figure 2.** (a) MS and (b) MS/MS spectra of chromatographic peak #1 attributed to 3',5'-O-dicaffeoylquinic acid; (c) COSY and (d) HSQC NMR spectra of chromatographic peak #1 attributed to 3',5'-O-dicaffeoylquinic acid.

The ESI MS and MS/MS fragmentation spectra were useful for identifying the three caffeic acid derivatives, and these are shown in Figure 2-4. For 3',5'-O-dicaffeoylquinic acid, a precursor ion were identified at m/z 515 [M–H]- (Figure 2a) and the fragment ions at m/z 353 [M–H–$C_9H_6O_3$]-, 191 [M–H–$C_{18}H_{12}O_6$]-, and 135 [M–H–$C_{17}H_{16}O_{10}$]- were prominent in the MS/MS spectrum of the ion at m/z 515 (Figure 2b)[21-24]. 1',5'-O-dicaffeoylquinic acid exhibited a precursor ion at m/z 515 [M–H]- (Figure 3a) with fragment ions at m/z 353 [M–H–$C_9H_6O_3$]-, and 191 [M–H–$C_{18}H_{12}O_6$]- (Figure 3b),[21-23,25] and ethyl caffeate displayed a precursor ion at m/z 207 [M–H]- (Figure 4a) and fragment ions at m/z 179 [M–H–$C_2H_4$]- and 135 [M–H–$C_3H_4O_2$]- (Figure 4b).[24] However, the MS and MS/MS spectra of 3',5'-O-dicaffeoylquinic acid were almost identical to those of 1',5'-O-dicaffeoylquinic acid. In this respect, although MS is a rapid, effective, and convenient analytical method for the detection of compounds present in plant extracts, there are limitations associated with its ability to accurately identify isomers. Therefore, to determine the position of caffeoyl groups in the quinic acid ring of the dicaffeoylquinic acid isomers, $^1$H and 2D NMR were obtained together from peaks separated from the HPLC.

For diacaffeoylquinic acids, the proton NMR assignment was derived in the 1D $^1$H-NMR spectrum (Figure 1c) after the first assignment of signals in the COSY and HSQC experiment. As can be seen from Figures 2c and 3c, the signals of H-3', H-4', and H-5' show correlations with each other's peaks in the COSY experiment. Figure 1c shows the presence of two caffeoyl groups in each isomer, which are evident from the two separate signals for each caffeoyl proton. The results of a large J-coupling constant between the caffeoyl group double bond protons suggest trans-configuration of the double bond in all isolated compounds. In addition, the signals of H-3' and H-5' shown in $^1$H spectrum #1 in Figure 1c are shifted downfield compared to the quinic acid spectrum,[21,26]

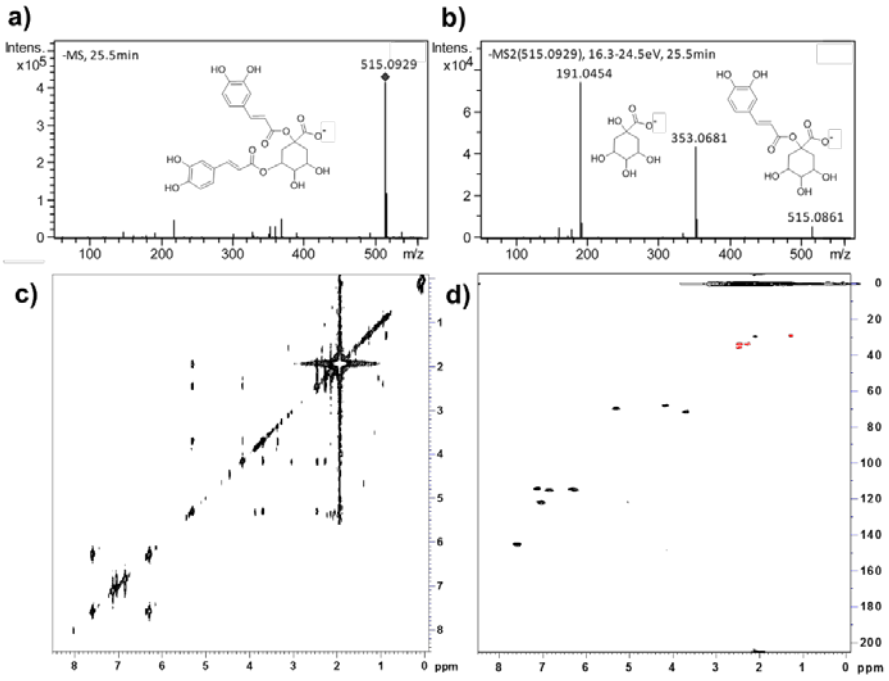



**Figure 3.** (a) MS and (b) MS/MS spectra of chromatographic peak #2 attributed to 1',5'-O-dicaffeoylquinic acid; (c) COSY and (d) HSQC NMR spectra of chromatographic peak #2 attributed to 1',5'-O-dicaffeoylquinic acid.

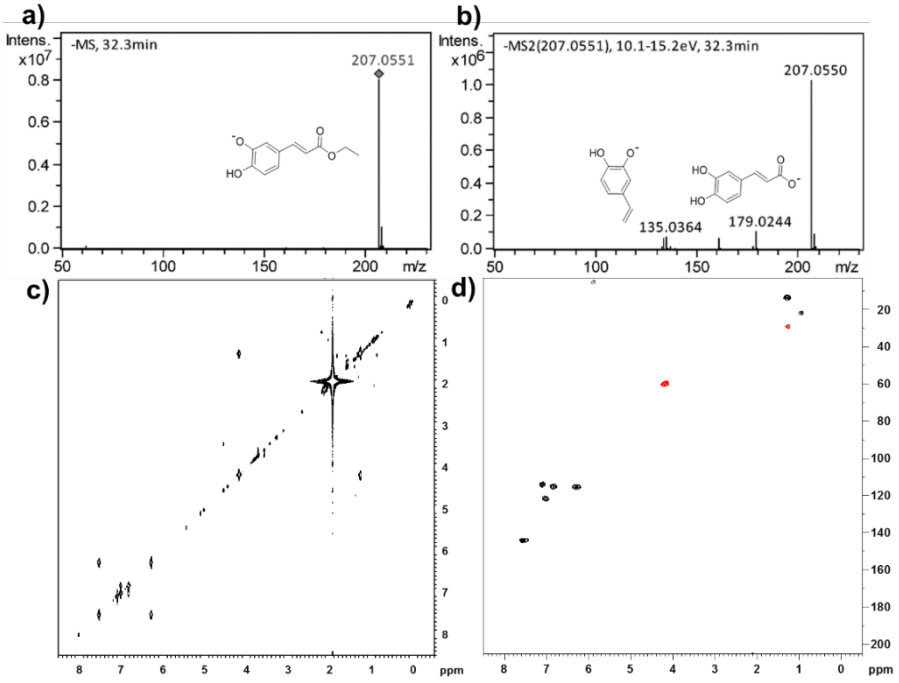



**Figure 4.** (a) MS and (b) MS/MS spectra of chromatographic peak #3 attributed to ethyl caffeate; (c) COSY and (d) HSQC NMR spectra of chromatographic peak #3 attributed to ethyl caffeate.

which indicates the presence of caffeoyl groups in H-3', and H-5' positions. Therefore, #1 is confirmed as 3',5'-O-dicaffeoylquinic acid.

In the $^{1}$H spectrum of #2, the signal of H-5' is shifted downfield with respect to the spectrum of quinic acid, suggesting the presence of a caffeoyl group in position 5'. However, the H-3'and H-4'signals in 1',5'-O-dicaffeoylquinic acid are distinct from the other isomer as the signals are not significantly deshielded, which indicates that the other caffeoyl group is attached to the hydroxyl group at carbon 1' of quinic acid. Therefore, #2 is identified as 1',5'-O-dicaffeoylquinic acid.[21,27] The spectroscopic results for these dicaffeoylquinic acids compounds agree very closely with those reported in literature.[21,26,27]

According to the $^{1}$H NMR spectrum of ethyl caffeate shown in Figure 1c, the signals of protons at 2, 5, 6, 7, 8, 9, and 10 have chemical shifts of 7.10, 6.84, 7.02, 7.54, 6.29, 4.18, and 1.27 ppm, respectively. These results indicate that the chemical shifts of the ethyl caffeate are in good agreement with those reported in the literature.[28]

**Discussion**

In the present study, the major constituents of the *Xanthium sibiricum*, 3',5'- O-dicaffeoylquinic acid, 1',5'-O-dicaffeoylquinic acid, and ethyl caffeate, were identified by the combined use of UV, MS, MS/MS spectra, and $^{1}$H and 2D NMR data. The high sensitivity of MS was suitable for use with a very limited quantity of samples, and thus NMR spectroscopy was used to obtain stereochemical information that enabled identification of isomers such as dicaffeoylquinic acids. One of major disadvantages of NMR analysis is its low sensitivity, but this problem was overcome by using high-sensitivity NMR equipped with a cryoprobe and a sample concentration where the same peak was multi-trapped with repeated LC injections. Consequently, our results provide insight into identification of metabolite components using a SPE-800MHz NMR-MS hyphenated system, which is evidenced to be a reliable and complete approach for providing complementary information about natural products.

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