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# Efficient Identification and Structural Elucidation of Metabolites Using HPLC-DAD-SPE-CryoNMR-MS Method

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**Abstract**: Rapid identification and efficient structural elucidation of metabolites are vitally important in metabonomics, phytochemistry and drug discoveries from the natural sources. In this work, the potentials of the hyphenated HPLC-DAD-SPE-CryoNMR-MS techniques were demonstrated in characterization and structural elucidation using the complex extracts of rosemary (*Rosmarinus officinalis* L.) as an example. 6 metabolites, including 1 phenolic diterpene (carnosol), 2 phenolic acids (rosmarinic acid and caffeic acid), 2 flavonoids (6-methoxyluteolin-7-glucoside and homoplantaginin) and 1 coumaric acid (*cis*-4-glucosyloxycinnamic acid), were successfully identified from two rosemary extracts with chloroform-methanol ($v/v$, 3/1) and 50% aqueous methanol on the basis of HPLC, UV, NMR and MS data. Such structural determination benefited from the capability of acquiring the high-quality homo- and hetero-nuclear 2D NMR spectra with the analytical HPLC separation. The results have not only provided whole set spectroscopic data for these metabolites, but also proved the hyphenated HPLC-DAD-SPE-CryoNMR-MS method to be an important structural elucidation platform for the components in natural products and probably other complex mixtures, even when multiple components in the same chromatographic peak are present with concentration differences in an order of magnitude.

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## Introduction

The structural determination of natural products is crucial for metabonomics[1], phytochemistry[2-4] and drug discoveries[5-7] from the natural sources. Traditionally, four major analytical techniques were applied to elucidate the structures of constituents in the phytochemistry researches, including high performance liquid chromatography (HPLC), ultraviolet-visible spectra (UV-Vis), mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy. However, the analytes have to be isolated and purified to milligram levels since the plant extracts are often complex containing many chemically diverse constituents with variable concentrations. Analysis of these inherent complex mixtures, therefore, demands some powerful and efficient tools, especially for the identification and structural elucidation of the unknown chemical constituents.

So far, the combination of HPLC, diode-array detector (DAD), solid-phase-extraction (SPE), NMR with cryogenic probes (CryoNMR) and MS in the hyphenated form (HPLC-DAD-SPE-CryoNMR-MS)[8,9] represents the most powerful method for analysis of the plant extracts. This is because the hyphenated technology integrates the advantages of multiple analytical techniques including HPLC, UV-Vis, NMR and MS, and offers comprehensive information for analytes including hydrophobicity, UV-visible absorption, atomic connectivity (from NMR) as well as molecular mass and fragments. Furthermore, this method not only overcomes the shortcomings of the traditional LC-MS[10,11] methods by providing much more detailed structural information, but also improves LC-NMR methods[12,13] by increasing the signal-to-noise ratio and enabling the acquisition of a catalogue of 2D NMR spectra. Moreover, the introduction of the cryogenic probe technology and solid-phase-extraction (SPE) to this hyphenated system significantly increases the resultant sensitivity of NMR detection and lowers the amount of analytes required to microgram level. The powerfulness and advantages of this hyphenated methods have already been demonstrated in some preliminary studies of flavonoids[14] and diterpenes[15].

Rosemary (*Rosmarinus officinalis* L.), an evergreen shrub in the lamiaceae family, was grown widely in Mediterranean basin and part of Europe. Rosemary extracts were commonly added to food, cosmetic and pharmaceutical products to inhibit lipid oxidation[16] owing to their potent antioxidant activity[17]. Many studies[17-22] showed that the rosemary extracts were chemically complex and contained a large amount of secondary metabolites such as polyphenolic acids, flavonoids, phenolic diterpenes and essential

oil. In our recent systematic characterization of rosemary extracts using NMR[23], we identified 33 abundant primary and secondary metabolites, amongst which 3 metabolites were found for the first time. We also found that the chemical composition of the rosemary extracts was remarkably dependent on the extraction solvents, harvest seasons and post-harvest drying processes[23]. In this work, we employed two rosemary extracts from chloroform-methanol (3/1, *v*/*v*) and 50% aqueous methanol as examples and demonstrated the applicability of the hyphenated HPLC-DAD-SPE-CryoNMR-MS technique in the rapid and efficient constituent characterization of plant extracts.

# 1 Experimental section

## 1.1 Chemicals

Methanol, chloroform and acetonitrile were obtained from J. T. Baker Pharmaceuticals Company (USA) as analytical or HPLC grades and were used as received. Deionized water was purified by Millipore system, and methanol-$D_4$ ($CD_3OD$, 99.9% in D) and acetonitrile-$D_3$ ($CD_3CN$, 99.9% in D) were obtained from Cambridge Isotope Laboratories (MA, USA).

## 1.2 Sample collection and extraction

Fresh rosemary materials were collected as reported previously[23]. Briefly, the top of plant shoots (5～10 cm) including leaves and stems harvested from a herbal garden in southern London, U.K., in 2004 were snap frozen in liquid nitrogen followed with lyophilization in a freeze-drier for 48 hours. They were then sealed in plastic bags respectively and stored dry in darkness until analysis. Careful checking was done before analysis and no dampening was found for all samples.

The dried rosemary materials were ground with a coffee blender and sieved through a 2 mm sieve. The ground powder samples (300 mg) were extracted with chloroform-methanol (3/1, *v*/*v*) and 50% aqueous methanol (each 5 mL), respectively, by vortexing for 30 s followed with the discontinuous ultra-sonication for 30 min (i.e., 1 min sonication with 1 min break). Temperature increase induced by sonication was less than 5 ℃. The raw materials were extracted three times sequentially, and the resultant 3 stock solutions were combined and span at 10 000 rpm for 10 min. The supernatants were condensed at 30 ℃ with a rotary evaporator to remove the organic solvents before lyophilization. The dried powder extracts (2 mg) were dissolved in chloroform-methanol (3/1) and 50% aqueous methanol (each 1 mL) for HPLC-DAD-SPE-CryoNMR-MS analysis.

## 1.3 HPLC-DAD-SPE-CryoNMR-MS measurements

The hyphenated analysis was performed on a Bruker Metabolic Profiler consisting of an HPLC system (Agilent Series 1200, Waldbronm, Germany) with a quaternary pump, an autosampler, a column oven and a diode-array detector (DAD), a Prospekt II SPE unit (Spark Holland), a quadrupole time-of-flight mass spectrometer with an elec-

trospray ionization source (Bruker Daltonics, Bremen, Germany), a Bruker AVIII 600 MHz spectrometer equipped with inverse cryogenic probe and a 60-μL LC CryoFit (Bruker BioSpin) insert. Five percent of the eluent was directed to MS using a BNMI unit (Bruker BioSpin). The HySphere C18 HD SPE cartridges (10×2 mm i. d., Spark Holland) were used for peak trapping monitored with the UV absorbance at 254 nm.

The chromatographic separation was carried out on an Ace 5 C18-HL column (250 mm × 4.6 mm; ACT, Scotland) at 25 ℃ with the injection volume of 50 μL (2 mg/mL). The elution was performed using water (containing 0.1% *v*/*v* formic acid) and acetonitrile with a linear gradient lasting 55 min at a flow rate of 1 mL/min. The elution gradient was from 5% to 70% acetonitrile for aqueous methanol extract and from 10% to 90% acetonitrile for the chloroform-methanol extract. The ESI source was operated with a nebulizer pressure of 0.8 bar while the drying gas was delivered at a flow rate of 10 L/min at 180 ℃. The capillary voltage was 4 000 V and the collision energy level was −10.0 eV/z. MS data were acquired in the scan range between 50 and 1 000 Daltons under the negative and positive ionization mode. The SPE cartridges were conditioned with 500 μL of $CH_3CN$ followed by 500 μL of water at 6 mL/min prior to the trappings. The peak loaded cartridges were dried with a stream of nitrogen gas for 30 min and the analytes eluted with 450 μL of $CD_3OD$ or $CD_3CN$ into the NMR probe for data acquisition.

The $^1H$ NMR spectra were acquired using standard one dimensional pulse sequence (*RD*-90°-$t_1$-90°-$t_m$-90°-acquisition) with the presaturation pulses during the recycle delay (*RD*, 2.4 s) and the mixing time ($t_m$, 0.1 s) to suppress residual solvent signals. $t_1$ was set to 4 μs and 90° pulse length was adjusted to about 10 μs for each sample. 16-512 transients (as appropriate) were collected into 16 k data points for each spectrum with a spectral width of δ 20. All free induction decays (FID) were multiplied by an exponential function with 1.0 Hz line broadening factor prior to Fourier transformation (FT). Typically for correlation spectroscopy ($^1H$-$^1H$ COSY 2D NMR) and total correlation spectroscopy ($^1H$-$^1H$ TOCSY 2D NMR) experiments, 4-64 transients per increment (as appropriate) and 128-400 (as appropriate) increments were collected into 2 048 data points with the spectral width of δ 10 for both dimensions. MLEV-17 was employed in TOCSY as a spin-lock scheme with the mixing time of 60～90 ms (as appropriate). Gradient-selected heteronuclear single quantum coherence ($^1H$-$^{13}C$ HSQC) 2D NMR spectra were acquired with spectral widths of δ 10 for $^1H$ and δ 160 for $^{13}C$, 8 scans were collected into 1 024 data points for each of 256 increments, composite pulse decoupling (GARP) was employed on $^{13}C$ decoupling during the acquisition period. For gradient-selected heteronuclear multiple bond correlation ($^1H$-$^{13}C$ HMBC) 2D NMR spectra, the spectral width was δ 10 in the $^1H$ dimension and δ 220 in the $^{13}C$ dimension, and 32 transients were collected into 2 048 data points for each of 256 increments. All the 2D NMR spectra were performed with WET solvent suppression including standard 4 selective ex-

citation with 20 ms sinusoidal pulses flanked by 1 ms gradient pulses. For both dimensions, the NMR data were zero filled to 2 048 and a sine or a shifted sinebell-squared function was applied to FIDs prior to FT.

## 2 Results and Discussion

In the HPLC profiles of the rosemary extracts from chloroform-methanol (Fig. 1(a)) and 50% aqueous methanol (Fig. 1(b)), 5 selected peaks (labeled 1～5) representing constituents with various concentrations were trapped with SPE cartridges in HPLC-DAD-SPE-CryoNMR-MS experiments and subjected to detailed analysis.

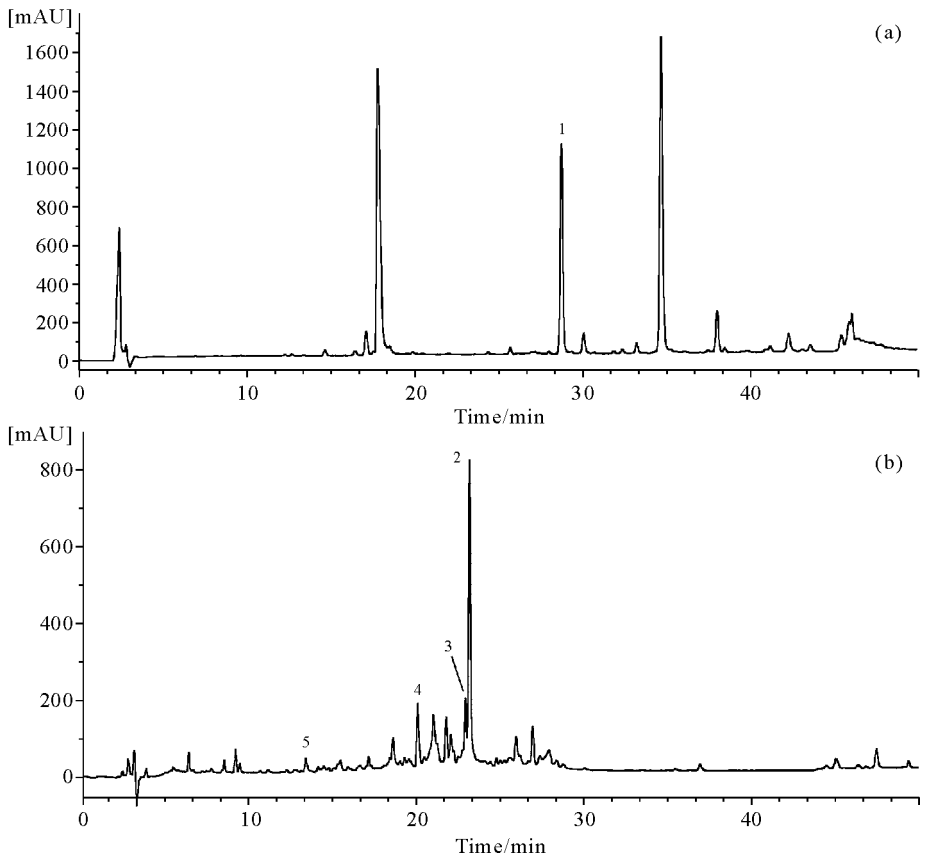


Fig. 1　The HPLC chromatograms of rosemary extracts from (a) chloroform-methanol (3/1, *v*/*v*) and (b) 50% aqueous methanol. The chromatograms were recorded at the detection wave length of 254 nm and the peaks used for HPLC-DAD-SPE-CryoNMR-MS were labeled as 1～5

Peak 1 has an HPLC retention time ($T_R$) of 28.9 min indicating that this component has intermediate hydrophobicity. The $^1$H NMR spectrum (Fig. 2(a)) of this component showed some features from a diterpene compound; the chemical shift data (Table 1) and coupling patterns were similar to those of carnosol reported in the literature[15]. Direct H-C bonding patterns from $^1$H-$^{13}$C HSQC spectrum (Fig. 2(d)) showed the

presence of 3 methylene groups ($\delta$ 2.92, 2.40, m; $\delta$ 2.04, 1.69, m; $\delta$ 1.55, 1.29, m), 2 methine groups ($\delta$ 1.73, m; $\delta$ 5.41, m) and 2 methyl groups ($\delta$ 0.87, s; $\delta$ 0.91, s) (see Table 1 for details). Careful analysis of the $^1$H-$^1$H COSY spectrum (Fig. 2(b)) confirmed the presence of an isopropyl group with the couplings between the methine proton ($\delta$ 3.10, seq) and the 2 methyl protons ($\delta$ 1.18, d; $\delta$ 1.20, d). In $^1$H-$^1$H TOCSY spectrum (Fig. 2(c)), 2 additional coupling systems were clearly observed, namely, between 3 methylene groups ($\delta$ 2.92, 2.40, m; $\delta$ 2.04, 1.69, m; $\delta$ 1.55, 1.29, m), and between 2 methine protons ($\delta$ 1.73, dd; $\delta$ 5.41, dd) and the methylene protons ($\delta$ 2.21, 1.18, m). Long range correlations in $^1$H-$^{13}$C HMBC spectrum (Fig. 2(e)) were found between the proton ($\delta$ 5.41, s) and a methine carbon ($\delta$ 45.2), 2 aromatic carbons ($\delta$ 121.3, $\delta$ 112.0) and a carbonyl group ($\delta$ 175.6), other correlations were also observed between the aromatic proton ($\delta$ 6.65, s) and the isopropyl carbon ($\delta$ 27.0), a methine carbon ($\delta$ 77.5) and 2 aromatic carbons ($\delta$ 121.3, $\delta$ 141.3). All these data confirmed the structure of carnosol. ESI-MS analysis gave the pseudomolecular ion $[M-H]^-$ at $m/z$ 329 and the fragment ion $[M-H-44]^-$ at $m/z$ 285 in negative ion mode, respectively, suggesting the molecular mass of 330 and the presence of a carboxyl group. This and the maximum absorbance in UV spectrum at 284 nm agreed well with the MS and UV characteristics of carnosol reported previously[18,24].

Peak 2 ($T_R$ 23.3 min, Fig. 1(b)) was the most intense one in the HPLC chromatogram of rosemary extract from 50% aqueous methanol, which had the UV maximum absorbance at 292 and 328 nm suggesting the presence of the typical aromatic structure. ESI-MS data showed the pseudomolecluar ion $[M+H]^+$ at $m/z$ 361 with two major fragments at $m/z$ 195 and 163 in the positive ion mode, coinciding with the literature reported MS data[18] for rosmarinic acid. It had been found to be one of the most abundant metabolites in rosemary extracts. The $^1$H NMR spectrum (Fig. 3(a)) showed 2 doublets at $\delta$ 7.51 and 6.30 with the large proton-proton coupling (15.9 Hz) corresponding to a pair of *trans*-olefinic protons. In the aromatic region of the $^1$H-$^1$H TOCSY spectrum (Fig. 3(c)), 2 *ABX*-spin systems were clearly visible for 3 protons $\delta$ 7.06 (d, 1.9 Hz), 6.78 (d, 8.0 Hz) and 6.97 (dd, 1.9, 8.0 Hz) and for other 3 protons $\delta$ 6.76 (d, 1.8 Hz), 6.70 (d, 8.0 Hz) and 6.63 (dd, 1.8, 8.0 Hz) (see Table 1), suggesting the presence of two 1, 3, 4-subsitutied benzene moieties. Another *ABX*-spin system (-CHOH-$CH_2$-) was found in the aliphatic region consisting of 1 doublet of doublets at $\delta$ 5.19 (dd, 3.9, 8.7 Hz) corresponding to an oxygen-bearing proton, and 2 further sets of doublet of doublets at $\delta$ 3.01 (dd, 8.7, 16.7 Hz) and $\delta$ 3.11 (dd, 3.9, 16.7 Hz) corresponding to 2 geminal protons. All the chemical shift data and coupling patterns agreed well with those of rosmarinic acid reported previously[25].

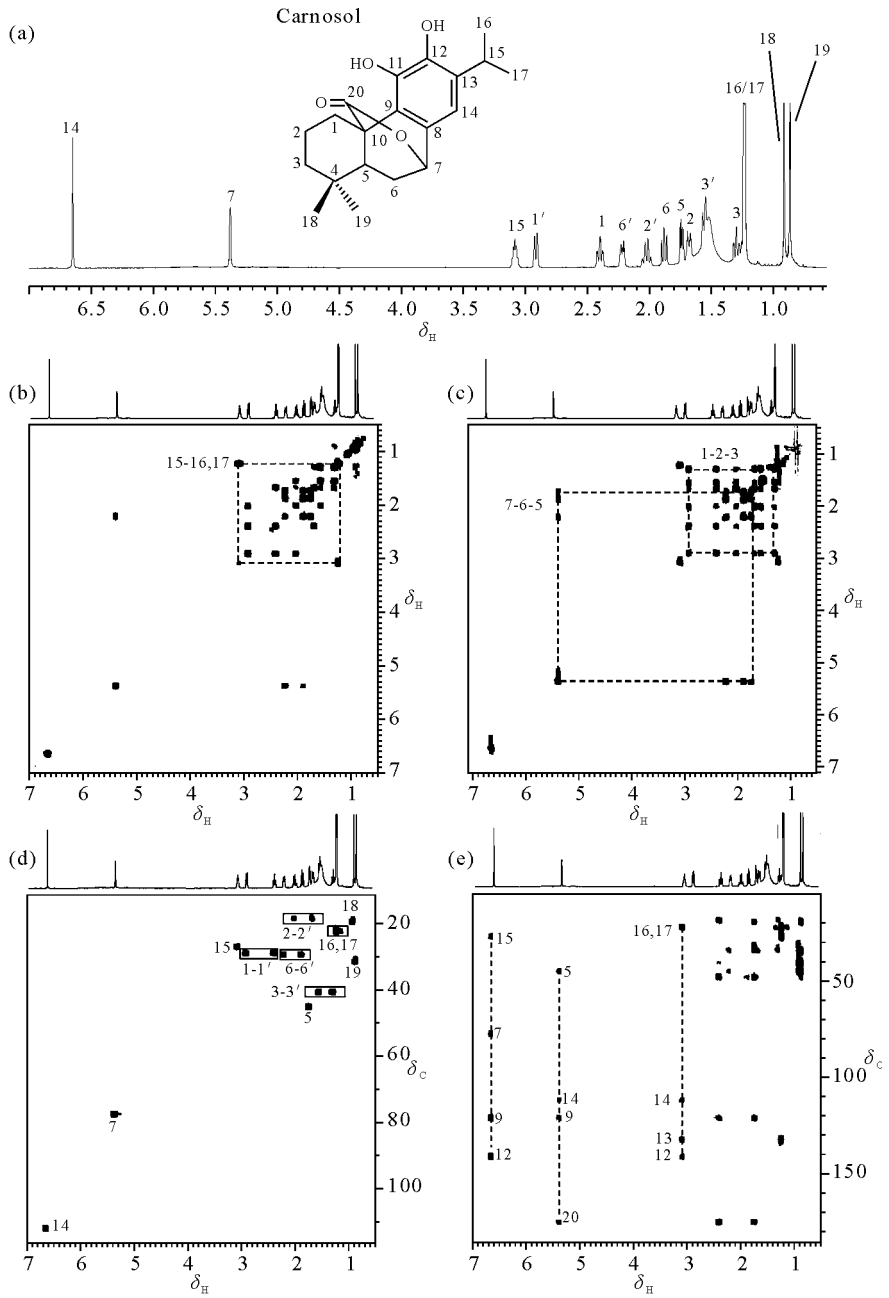


Fig. 2　600 MHz NMR spectra obtained with HPLC-DAD-SPE-CryoNMR-MS system for peak 1 from the chloroform-methanol extract (single trapping), (a) 1D $^{1}$H NMR; (b) $^{1}$H-$^{1}$H COSY 2D NMR; (c) $^{1}$H-$^{1}$H TOCSY 2D NMR; (d) $^{1}$H-$^{13}$C HSQC 2D NMR; (e) $^{1}$H-$^{13}$C HMBC 2D NMR

Peak 3 ($T_R$ 23.1 min, Fig. 1(b)) had the UV maximum absorbance at 337 and 268 nm implying the features of flavonoid structure. Although peak 3 was trapped in the same cartridge with peak 2 because of their close $T_R$ values, its $^{1}$H NMR spectrum (Fig. 3(b)) showed clearly 2 singlets at $\delta$ 6.68 and 6.54 corresponding to aromatic or

olefinic protons, respectively, and the aromatic proton peaks at $\delta$ 6.96 (d, 8.8 Hz) and $\delta$ 7.93 (d, 8.8 Hz) suggested the structure of a *para*-substituted benzene ring. Furthermore, the observations of a doublet at $\delta$ 5.15 (d, 7.8 Hz) and a series of protons at $\delta$ 3.43～3.59 (m) implied the presence of a glycoside residue with $\beta$-configuration; a

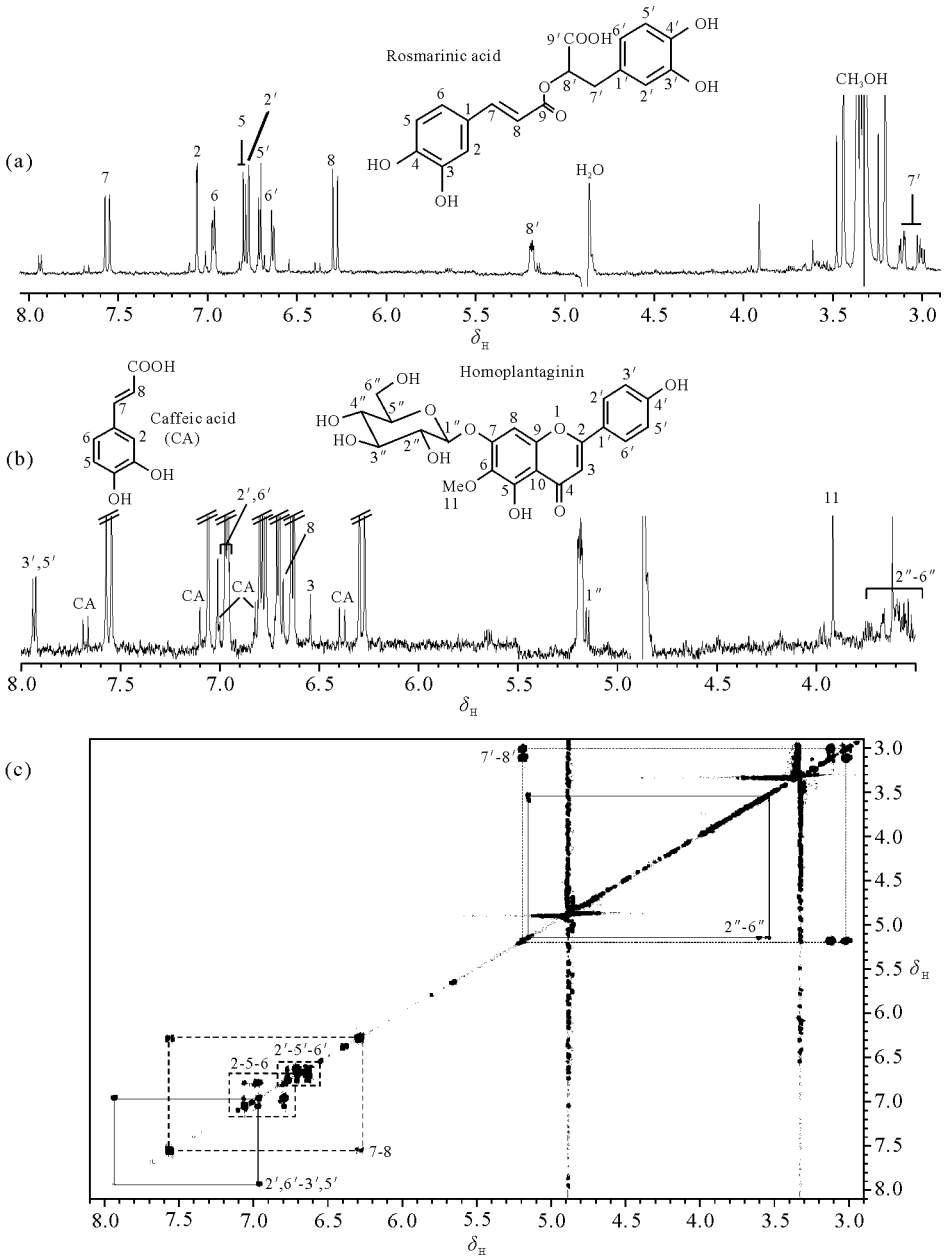


Fig. 3 600 MHz NMR spectra obtained with HPLC-DAD-SPE-CryoNMR-MS system for peak 2 and peak 3 from the 50% aqueous methanol extract (single trapping), (a) $^1$H NMR for peak 2; (b) $^1$H NMR for peak 3, CA: caffeic acid; (c) $^1$H-$^1$H TOCSY 2D NMR, the coupling correlations were represented by the dot and solid lines for peak 2 and peak 3, respectively

singlet at $\delta$ 3.91 was assignable to a methoxyl group. Its $^1$H-$^1$H TOCSY spectrum (Fig. 3(c)) confirmed the presence of vicinal proton couplings in benzene ring and a glucosidic coupling system (Table 1). Based on these observations, the peak 3 can be assignable to homoplantaginin. Its ESI-MS data showed a prominent pseudomolecluar ion at $[M+H]^+$ $m/z$ 463 confirming its molecular mass and a fragment ion $[M+H-162]^+$ at $m/z$ 301 confirming the presence of the glucoside residue, which were in good agreement with those of homoplantaginin reported in rosemary extract previously[18]. In addition, Fig. 3(b) also showed some smaller proton resonances, including the *ABX*-spin coupling protons at $\delta$ 7.10 (d, 1.8 Hz), $\delta$ 6.81 (d, 8.1 Hz) and $\delta$ 7.00 (dd, 1.8, 8.1 Hz), and a pair of trans-olefinic protons at $\delta$ 7.69 (d, 16.0 Hz) and $\delta$ 6.38 (d, 16.0 Hz), suggesting the structure of caffeic acid (CA). In fact, caffeic acid has been reported in the previous studies of rosemary extracts[18,26], and our NMR data, together with ESI-MS data (a pseudomolecluar ion at $[M+H]^+$ $m/z$ 181), confirmed the presence of caffeic acid further. The quantitative analysis showed that the concentration of rosmarinic acid was about 8 and 12 times as high as that of homoplantaginin and caffeic acid, respectively. The above discussion indicated that, even when 3 metabolites with the large concentration differences (up to an order of magnitude) were trapped on the same cartridge, their structures could still readily be elucidated.

**Table 1　NMR data for 6 constitutes of rosemary extracts**

| No. | Compounds | Groups | $\delta_H$ | $\delta_C$ | Assigned with |
|---|---|---|---|---|---|
| Peak 1 | carnosol | 1 CH, CH′ | 2.92 (m), 2.40 (m) | 29.0 | COSY, TOCSY, |
| | | 2 CH, CH′ | 2.04 (m), 1.69(m) | 18.4 | HSQC, HMBC |
| | | 3 CH, CH′ | 1.55 (m), 1.29(m) | 40.7 | |
| | | 4 C | | 34.4 | |
| | | 5 CH | 1.73 (dd) | 45.2 | |
| | | 6 CH, CH′ | 2.21 (m), 1.88(m) | 29.4 | |
| | | 7 CH | 5.41 (dd) | 77.5 | |
| | | 8 Ar | | 131.9 | |
| | | 9 Ar | | 121.3 | |
| | | 10 C | | 48.2 | |
| | | 11 Ar-OH | | — | |
| | | 12 Ar-OH | | 141.3 | |
| | | 13 Ar | | 132.7 | |
| | | 14 Ar-H | 6.65 (s) | 112.0 | |
| | | 15 CH | 3.10 (sep) | 27.0 | |
| | | 16 $CH_3$, 17 $CH_3$ | 1.88 (d), 1.20(d) | 22.3 | |

**Continuation of the Table 1**

| No. | Compounds | Groups | $\delta_H$ | $\delta_C$ | Assigned with |
|---|---|---|---|---|---|
| | | 18 $CH_3$ | 0.91 (s) | 19.4 | |
| | | 19 $CH_3$ | 0.87 (s) | 31.4 | |
| | | 20 COO | | 175.6 | |
| Peak 2 | rosmarinic acid* | 1 Ar | | | TOCSY |
| | | 2 Ar-H | 7.06 (d,1.9 Hz) | | |
| | | 5 Ar-H | 6.78 (d,8.0 Hz) | | |
| | | 3 Ar | | | |
| | | 4 Ar | | | |
| | | 6 Ar-H | 6.97 (dd,1.9, 8.0 Hz) | | |
| | | 7 -CH= | 7.55 (d,15.9 Hz) | | |
| | | 8 =CH-COO | 6.29 (d,15.9 Hz) | | |
| | | 9 COOH | | | |
| | | 2′ Ar-H | 6.76 (d,1.8 Hz) | | |
| | | 3′ Ar | | | |
| | | 4′ Ar | | | |
| | | 5′ Ar-H | 6.70 (d,8.0 Hz) | | |
| | | 6′ Ar-H | 6.63 (dd,1.8, 8.0 Hz) | | |
| | | 7′ CH | 3.01 (dd,8.7,16.7 Hz) | | |
| | | 7′ CH′ | 3.11 (dd,3.9,16.7 Hz) | | |
| | | 8′ CH | 5.19 (dd,3.9, 8.7Hz) | | |
| | | 9′ C=O | | | |
| Peak 3 | homoplantaginin | 8 CH | 6.68 (s) | | TOCSY |
| | | 3 CH | 6.54 (s) | | |
| | | 2′ 6′Ar-H | 6.96 (d,8.8 Hz) | | |
| | | 3′ 4′Ar-H | 7.93 (d,8.8 Hz) | | |
| | | 11 $CH_3O$ | 3.91 (s) | | |
| | | 1″ CH-OH | 5.15 (d,7.8 Hz) | | |
| | | (2″,3″,4″,5″) | (3.45,3.53,3.55,3.59,m) | | |
| Peak 3 | caffeic acid* | 7 -CH= | 7.67 (d,16.0 Hz) | | TOCSY |
| | | 8 =CH-COO | 6.38 (d,16.0 Hz) | | |
| | | 2 Ar-H | 7.10 (d,1.8 Hz) | | |
| | | 5 Ar-H | 6.81 (d,8.0 Hz) | | |
| | | 6 Ar-H | 7.00 (dd,1.8, 8.0 Hz) | | |

**Continuation of the Table 1**

| No. | Compounds | Groups | $\delta_H$ | $\delta_C$ | Assigned with |
|---|---|---|---|---|---|
| Peak 4 | 6-methoxyluteolin | 8 CH | 6.98 (s) | | TOCSY |
| | -7-glycoside | 3 CH | 6.64 (s) | | |
| | | 2′Ar-H | 7.43 (d,2.2 Hz) | | |
| | | 5′ Ar-H | 6.93 (d,8.4 Hz) | | |
| | | 6′ Ar-H | 7.46 (d,2.2, 8.4 Hz) | | |
| | | 11 $CH_3O$ | 3.91 (s) | | |
| | | 1″ CH-OH | 5.14 (d,7.7 Hz) | | |
| | | (2″,3″,4″,5″) | (3.43,3.53,3.59,3.73,m) | | |
| | | 6″ CH-OH | 3.96 (m) | | |
| Peak 5 | *cis*-4-glucosyloxy | 2, 6 Ar-H | 7.62 (d, 8.0 Hz) | | TOCSY |
| | cinnamic acid* | 3, 5 Ar-H | 6.99 (d,8.0 Hz) | | |
| | | 7 Ar-CH= | 6.88 (d,12.0 Hz) | | |
| | | 8 =CH-COO | 5.83 (d,12.0 Hz) | | |
| | | 1′ -CH | 4.92 (d,7.3 Hz) | | |
| | | (2′-6′ CH-OH) | (3.35,3.41,3.56, m) | | |

* The $^{13}C$ resonances were obtained in Ref [22]; — The $^{13}C$ resonances were not determined

Peak 4 ($T_R$ 20.2 min, Fig. 1(b)) represents a small chromatographic peak thus not an abundant metabolite. It had 2 maximum absorbance values at 277 and 341 nm in its UV-visible data suggesting a probable flavonoid structure. Its $^1H$ NMR spectrum (Fig. 4(a)) showed 2 singlets at $\delta$ 6.98 and 6.64 corresponding to aromatic or olefinic protons, respectively. Furthermore, an *ABX*-spin system was observable for the aromatic protons at $\delta$ 7.43 (d, 2.2 Hz), $\delta$ 6.93 (d, 8.4 Hz) and $\delta$ 7.46 (dd, 2.2, 8.4 Hz) suggested the presence of an 1,3,4-subsitutied benzene moiety. Moreover, observation of a doublet at $\delta$ 5.14 (d, 7.4 Hz) and a series of protons at $\delta$ 3.43～3.96 (m) implied the presence of a glycoside residue with $\beta$-configuration; a singlet at $\delta$ 3.91 was assignable to a methoxyl group. Its $^1H$-$^1H$ TOCSY spectrum (Fig. 4(b)) confirmed the *ABX*-spin system mentioned above and the presence of a glucosidic coupling system (Table 1). These NMR data were in good agreement with those for 6-methoxyluteolin-7-glucoside which was previously reported in the plant *Salvia Tomentosa*[27] and its similar compound of 6-hydroxyluteolin-7-glucoside had been found in rosemary[18]. ESI-MS data showed a prominent pseudomolecluar ion at $[M+H]^+$ at $m/z$ 479 confirming its molecular mass, together with two fragment ions $[M+H-162]^+$ at $m/z$ 317 and $[M+H-30]^+$ at $m/z$ 449 confirming the presence of the glucoside residue and a methoxyl group.

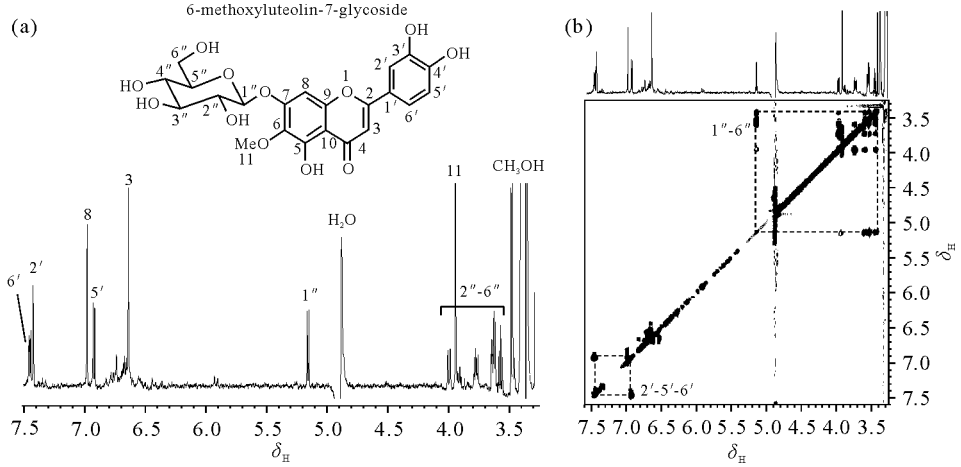


Fig. 4 600 MHz NMR spectra obtained with HPLC-DAD-SPE-CryoNMR-MS mode for peak 4 from the 50% aqueous methanol extract (single trapping), (a) $^1$H NMR; (b) $^1$H-$^1$H TOCSY 2D NMR

Peak 5 had a $T_R$ of 13.6 min (Fig. 1(b)) indicating its weak hydrophobicity and this small peak was selected to represent a metabolite in the rosemary extracts having much lower concentration. Multiple trappings (of 10 chromatographic runs) were conducted to enable its structure determination using the HPLC-DAD-SPE-CryoNMR-MS method. Its ESI-MS data (Fig. 5(a)) showed the pseudomolecluar ion $[M-H]^-$ at $m/z$ 325, a dimer ion $[2M-H]^-$ at $m/z$ 651 implying its molecular mass of 326 and an aglycone fragment ion $[M-H-162]^-$ at $m/z$ 163 indicating the presence of a hexose residue. Its NMR spectra (Fig. 5(b) and (c)) showed the aromatic proton peaks at $\delta$ 6.99 (d, 8.0 Hz) and $\delta$ 7.62 (d, 8.0 Hz), indicating the presence of a *para*-substituted benzene ring. 2 coupled doublets with the coupling constant of 12 Hz for signals at $\delta$ 5.83 and $\delta$ 6.88 suggested the presence of 2 *cis*-olefinic protons. The extensive coupling of proton signals at $\delta$ 4.92 (d, 7.13 Hz), 3.56 (m), 3.41 (m) and 3.35 (m) was also observable in $^1$H-$^1$H TOCSY spectrum (Fig. 5(c)) suggesting the presence of a β-glucose residue. Furthermore, the $^1$H-$^{13}$C HMBC spectrum in our previous study[23] showed a long-range correlation between the proton ($\delta$ 4.92) and a carbon signal at $\delta$ 158.8 for this metabolite, suggesting a β-glucoside linkage between the anomeric proton and a hydroxylated aromatic carbon. Therefore, the structure of this peak was assigned as *cis*-4-glucosyloxycinnamic acid, which has been also observed in other plant extracts[28,29]. It is particularly interesting to note that, the structure of this metabolite can still be elucidated when mixed with high concentration dibutyl phthalate (about 10 times as high as the analyte). Such observation again confirms that the current method is efficient in the case of background impurities in the same chromatographic peak.

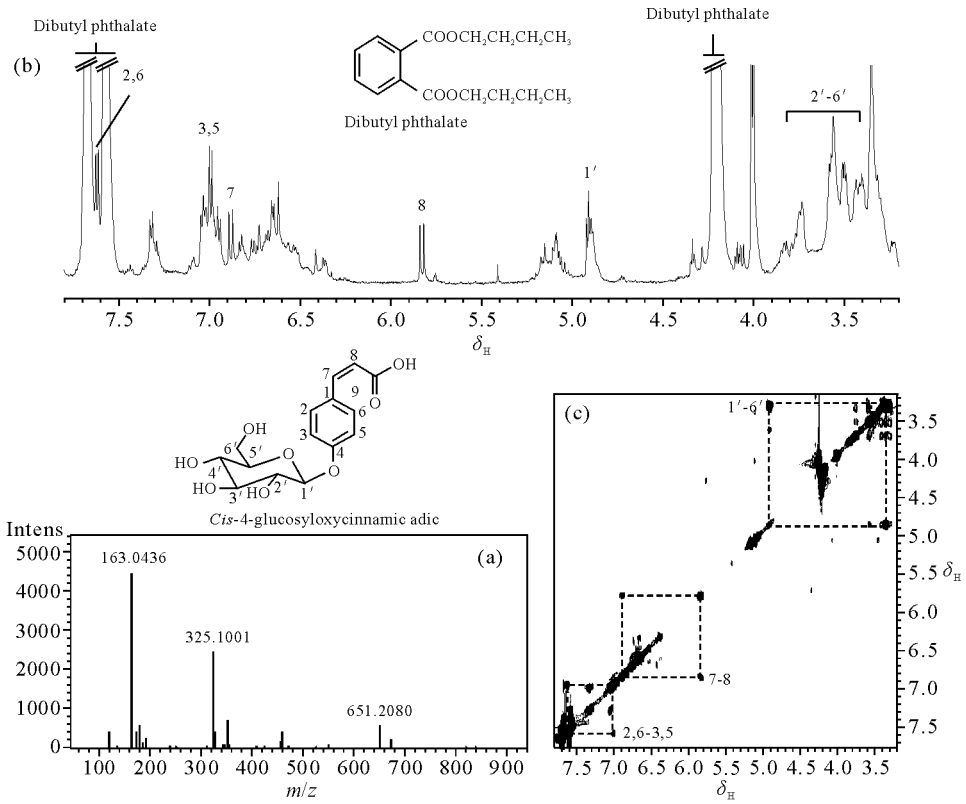


Fig. 5　600 MHz NMR spectra obtained with HPLC-DAD-SPE-CryoNMR-MS mode for peak 5 from the 50% aqueous methanol extract (ten trappings), (a) ESI-MS data in negative ion mode; (b) $^1$H NMR; (c) $^1$H-$^1$H TOCSY 2D NMR. The background signals were originated from dibutyl phthalate as impuirity

## 3　Conclusions

With 6 metabolites as representatives in complex rosemary extracts from 2 different solvents, examples for a phenolic diterpene (carnosol), 2 phenolic acids (rosmarinic acid and caffeic acid), 2 flavonoids (6-methoxyluteolin-7-glucoside and homoplantaginin) and 1 coumaric acid (*cis*-4-glucosyloxycinnamic acid) were successfully identified and elucidated by using the hyphenated HPLC-DAD-SPE-CryoNMR-MS method. It was concluded that this technique was well suited for structural elucidation of natural compounds having a variety of structural features and concentrations. This study also indicated that complete assignments of $^1$H and $^{13}$C resonances for metabolites were possible even based on the analytical-scale HPLC separation. Although more detailed structural elucidation for other components of rosemary remains to be complete and in progress, it is already clear that the hyphenated HPLC-DAD-SPE-CryoNMR-MS method is extremely powerful and efficient for rapid structural determination of the components in the complex metabolite mixtures even when multiple components are present in the same chromatographic peak, which may have many potential applications in metabonomics studies.

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# 基于 HPLC-DAD-SPE-CryoNMR-MS 技术的代谢物快速定性和结构确定

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**摘 要**：快速定性并有效地确定代谢物结构是代谢组学、植物化学和天然药物发现等研究的重要内容. 本文以迷迭香的两种溶剂提取物(氯仿/甲醇＝3/1 和 50％含水甲醇)作为模型样品，通过分析其中的一些化学成分，展现了 HPLC-DAD-SPE-CryoNMR-MS 无缝联仪技术在确定代谢物结构方面的应用潜力. 基于高效液相色谱、紫外吸收、核磁共振波谱及质谱数据，具有代表性的 6 个迷迭香代谢物的结构得到了确定，它们分别为 1 个酚类二萜(鼠尾草酚)、2 个酚酸(迷迭香酸和咖啡酸)、2 个黄酮(6-甲氧基木樨草素-7-葡萄糖苷和高车前甙)和 1 个香豆酸 (顺-4-香豆酸葡萄糖苷). 该技术使分析型 HPLC 柱所分离的组分可以获得高质量同核及异核二维核磁共振波谱，从而能够更加便捷地确定微量成分的结构. 这些研究结果不仅提供了上述 6 种代谢物详细的谱学数据，而且证实 HPLC-DAD-SPE-CryoNMR-MS 技术是天然产物以及其他复杂体系结构分析的重要工具，即使同一色谱峰中含有浓度相差一个数量级的多个代谢物时，该方法依然有效.

**关键词**：核磁共振；结构鉴定；HPLC-DAD-SPE-CryoNMR-MS；迷迭香

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