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Revealing the Metabonomic Variation of Rosemary Extracts Using ^{1}H NMR Spectroscopy and Multivariate Data Analysis

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The molecular compositions of rosemary (*Rosmarinus officinalis* L.) extracts and their dependence on extraction solvents, seasons, and drying processes were systematically characterized using NMR spectroscopy and multivariate data analysis. The results showed that the rosemary metabonome was dominated by 33 metabolites including sugars, amino acids, organic acids, polyphenolic acids, and diterpenes, among which quinate, *cis*-4-glucosyloxycinnamic acid, and 3,4,5-trimethoxyphenylmethanol were found in rosemary for the first time. Compared with water extracts, the 50% aqueous methanol extracts contained higher levels of sucrose, succinate, fumarate, malonate, shikimate, and phenolic acids, but lower levels of fructose, glucose, citrate, and quinate. Chloroform/methanol was an excellent solvent for selective extraction of diterpenes. From February to August, the levels of rosmarinate and quinate increased, whereas the sucrose level decreased. The sun-dried samples contained higher concentrations of rosmarinate, sucrose, and some amino acids but lower concentrations of glucose, fructose, malate, succinate, lactate, and quinate than freeze-dried ones. These findings will fill the gap in the understanding of rosemary composition and its variations.

KEYWORDS: Rosemary (*Rosmarinus officinalis* L.); NMR; PCA (principal component analysis); OPLS-DA (projection to latent structure with discriminant analysis); phytomedicines

INTRODUCTION

As an evergreen shrub in the Lamiaceae family growing widely in the Mediterranean basin and part of Europe, rosemary (*Rosmarinus officinalis* L.) has been used as a herb and folk medicine for centuries around the world. In fact, it is the only commercially available herb used as a natural alternative to the synthetic antioxidants in Europe and the United States (*1*). Owing to their potent antioxidant activity, rosemary extracts were commonly added to food, cosmetic, and pharmaceutical products to inhibit lipid oxidation and to prevent off-flavor compound formation (*2*). A number of biological activities have been reported for the rosemary constituents including antimicrobial (*1*), anti-inflammatory (*3*), antiviral (*4*), and cancer prevention functions (*5*). The antioxidant and other biological activities were generally believed to be attributable to the free radical scavenging properties of rosemary secondary metabolites including polyphenolic acids, flavonoids, phenolic diterpenes, and essential oil.

Many phytochemistry studies have been conducted to identify secondary metabolites in rosemary and assess their bioactivities, primarily in vitro. For example, a systematic study (*1*) identified about 38 compounds from the rosemary essential oil using GC-MS, and antimicrobial properties of the essential oil (mixture) were evaluated against 19 microbial strains. Another comprehensive study (*6*) identified 22 antioxidant compounds from 24 commercial rosemary extracts using HPLC-UV/MS, including polyphenolic acids such as vanillic acid, caffeic acid, and rosmarinic acid; phenolic diterpenes such as carnosic acid, carnosol, and rosmadial; and flavonoids such as genkwanin and cirsimaritin. The antioxidant power was again assessed only broadly for these commercial extracts in vitro with no information related to the contributions from different metabolites. Unfortunately, some details for the studied commercial extracts were not available, especially where the plants originated, at which seasonal time point the plants were harvested, how they were processed (e.g., drying), and how the extracts were prepared. Consequently, there was no information on many of the factors that could affect the composition profile of rosemary plants.

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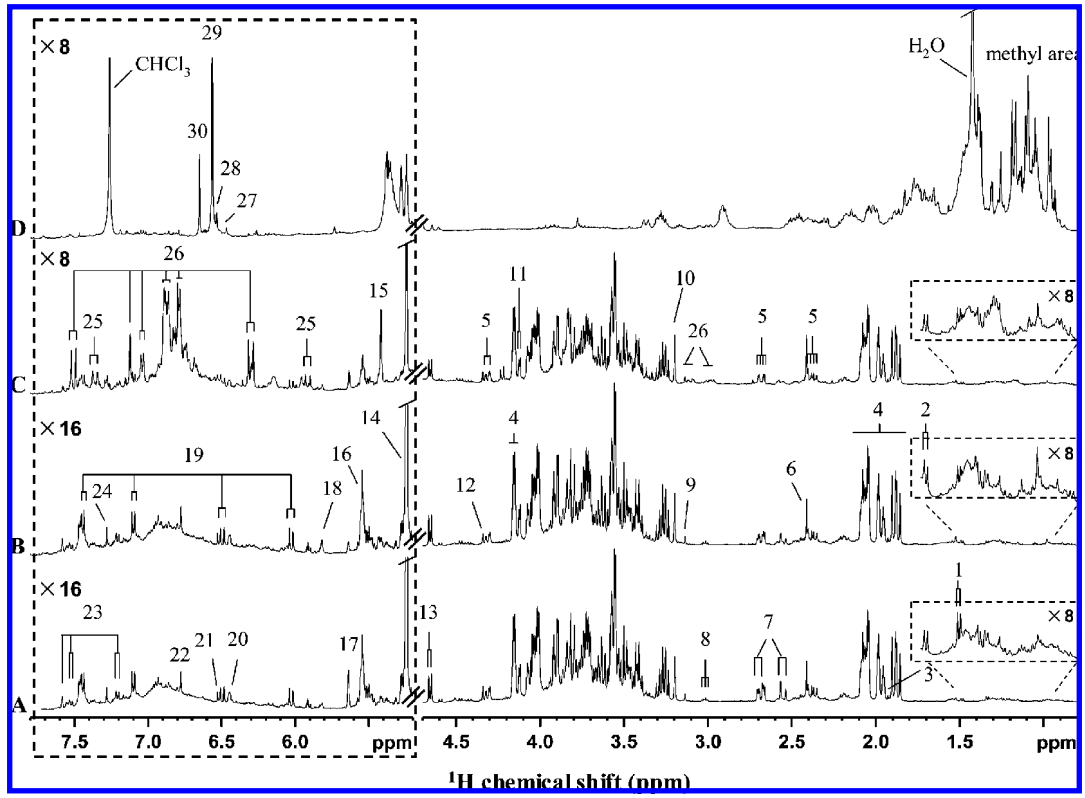

Figure 1. 500 MHz ^{1}H NMR spectra of rosemary extracts from different solvents, namely, ambient temperature water (**A**), boiling water (**B**), 50% aqueous methanol (**C**), and chloroform/methanol (v/v, 3:1) (**D**). The region δ 7.8−5.2 (see the dot box) in **A** and **B** and **C** and **D** was expanded 16 and 8 times, respectively, whereas the region δ 1.5−0.8 was expanded 8 times in comparison with the region δ 4.7−0.8.

Some studies have started to pay attention to the effects of growth environmental factors and postharvest treatments on rosemary secondary metabolite composition. However, these studies focused only on some selected secondary metabolites without considering other metabolites simultaneously. For example, it was found that the amount of rosmarinic acid was dependent on the drying treatment (*7*) and harvest seasons (*8*); the carnosic acid concentration in extracts was affected by the growth environments (*2, 9*), stresses (*10, 11*), and extraction methods (*12*). In addition, the content of flavonoids had an organ-dependent distribution during plant development (*13, 14*). For the time being, however, there was no systematic evaluation done for other metabolites such as caffeate, shikimate, and vanillate or for the total metabolite pool (i.e., metabonome) including both primary and secondary metabolites in terms of the environmental and processing effects on their contributions as a whole, which ought to be extremely important for plant metabolism and quality control of plant extracts.

Most published studies focused on secondary metabolites of rosemary; little has been done on primary metabolites such as sugars, amino acids, and carboxylic acids, which may indirectly affect the change of secondary metabolites and have important biological significance in terms of plant physiology (*15*). These primary metabolites are, in many cases, human endogenous metabolites and have, at least, some nutritional value. The lack of such comprehensive studies was probably due to the objectives of the previous studies, the complexity of the plant metabonome, and the limited analytical power of the traditional phytochemistry methods. Accompanying the birth of metabonomics and metabolomics during recent years, high-resolution NMR spectroscopy coupled with multivariate data analysis has been applied to analyze metabolite profiles and detect variations in the compositions of beverages (*16*) and phytomedicines (*17*−*19*) successfully. The metabonomics method was robust and allowed high throughput, thus being able to meet the increasing demands for analyzing complex samples such as herbs and phytomedicines without separation but with good accuracy and consistency based on the entire chemical composition of the samples.

In this work, we applied the NMR-based metabonomics strategy to investigate (a) all of the detectable metabolites in rosemary extracts (metabonome) (b) and the effects of the different extraction solvents, seasonal variations, and drying processes on the rosemary metabolite composition. The findings from the metabonomics investigation were also subjected to OPLS-DA and classical statistical analysis (one-way ANOVA) to examine the statistical significance for the concentrations of metabolites.

MATERIALS AND METHODS

Chemicals. Analytical grade methanol, chloroform, $Na_2HPO_4 \cdot 2H_2O$, and $NaH_2PO_4 \cdot 12H_2O$ were all purchased from Guoyao Chemical Co. Ltd. (Shanghai, China) and used without further treatments. Deuterated chloroform ($CDCl_3$, 99.9% D) containing tetramethylsilane (TMS, 0.03%, m/v), D_2O (99.9% D), and sodium 3-trimethlysilyl [2,2,3,3-$^2H_4$] propionate (TSP) were purchased from Sigma-Aldrich Inc. (St. Louis, MO). The buffer (Na_2HPO_4/NaH_2PO_4, 0.1 M, pH 7.4) was prepared in H_2O containing 10% D_2O and TSP (3.0 mM).

Sample Collection and Extraction. Fresh rosemary materials (5−10 cm at the top of plant shoots) including leaves and stems were collected from a herbal garden in southern London, U.K., on the 15th of each month in 2004. The plant materials were immediately sun-dried (the usual practice) or freeze-dried. For sun-drying, the samples were dried by exposure to sunlight in a ventilated place for 2 days. For freeze-drying, the samples were snap frozen in liquid nitrogen followed by lyophilization in a freeze-dryer for 48 h. Careful checking was done, and no dampening was found for all samples. They were then sealed in plastic bags, respectively, and stored dry in darkness until analysis.

The dried rosemary materials were ground with a coffee blender and sieved through a 2 mm sieve. The ground powder samples (300

Table 1. NMR Data for Rosemary Metabolites

no.	metabolite	group	δ^1H	δ^{13}C	assigned with
1	lactate	CH_3	1.33 (d, 6.9 Hz)	22.3	TOCSY, HSQC, HMBC
		CH	4.12 (q, 6.9 Hz)	71.4	
		COOH		185.3	
2	alanine	CH_3	1.48 (d, 7.3 Hz)	19.1	TOCSY, HMBC
		CH	3.78 (q, 7.3 Hz)	53.3	
		COOH		179.3	
3	acetate	CH_3	1.92 (s)	26.3	HSQC, HMBC
		COOH		184.3	
4	quinate[a]	1 C		78.3	TOCSY, HSQC, HMBC
		2 CH_2	1.88 (m), 2.09 (m)	43.6	HPLC-MS
		3 CH	4.03 (m)	70.0	
		4 CH	3.57 (m)	80.1	
		5 CH	4.16 (q, 3.4 Hz)	73.3	
		6 CH_2	1.98 (m), 2.06 (m)	40.3	
		COOH		184.3	
5	malate	α-CH	4.31 (dd, 3.1, 10.2 Hz)	73.2	TOCSY, HSQC, HMBC
		β-CH	2.68 (dd, 3.1, 15.4 Hz)	45.2	
		β'-CH	2.37 (dd, 10.2,15.4 Hz)	45.2	
		COOH		183.6	
6	succinate	CH_2	2.40 (s)	36.5	HMBC
		COOH		184.6	
7	citrate	α,α'-CH_2	2.56 (d, 15.8 Hz)	47.9	TOCSY, HMBC
		γ,γ'-CH_2	2.70 (d, 15.8 Hz)	47.9	
		β-C		78.8	
		6 COOH		184.5	
		1,5 COOH		181.7	
8	α-ketoglutarate	β-CH_2	3.01 (t, 6.9 Hz)	39.3	TOCSY, HSQC, HMBC
		γ-CH_2	2.45 (t, 6.9 Hz)	33.0	
		C=O		183.9	
		COOH		199.8	
9	malonate	CH_2	3.13 (s)		HMBC
		COOH		180.0	
10	choline	N—CH_3	3.20 (s)	56.8	HSQC, HMBC
		α-CH_2		70.4	
11	fructose	1 CH	4.11 (d, 3.7 Hz)	78.0	TOCSY, HSQC
12	tartrate	CHOH	4.34 (s)	76.9	HSQC, HMBC
		COOH		181.2	
13	β-glucose	1 CH	4.26 (d, 8.0 Hz)	99.0	TOCSY, HSQC
14	α-glucose	1 CH	5.23 (d, 3.7 Hz)	95.1	TOCSY, HSQC
15	sucrose	1 CH	5.40 (d, 3.9 Hz)	94.5	TOCSY, HSQC
		1′ CH	4.22 (d, 8.8 Hz)	79.0	
16	U1		5.54 (t, 2.0 Hz)	129.7	TOCSY, HSQC, HMBC
			2.11 (#)	57.4	
			2.36 (#)	27.6	
			2.44 (#)	41.3	
			2.49 (#)	30.1	
			4.07 (t, 6.6 Hz)	71.9	
17	U2		5.64 (t, 1.8 Hz)	110.0	TOCSY, HSQC, HMBC
			4.72 (#), 4.34 (#)	68.3	
		COOH		172.2	
18	U3		5.8 (d)	122.5	TOCSY, HSQC
			2.13 (#), 2.86 (#)	44.5	
			2.06 (#)	25.9	
			2.57 (#)	52.8	
			2.62 (#)	60.5	
19	*cis*-4-glucosyloxycinnamic acid[a]	=CH—COO	6.03 (d, 12 Hz)	128.8	COSY, TOCSY, HSQC, HMBC
		Ar—CH=	6.50 (d, 12 Hz)	132.4	HPLC-MS
		1 Ar		134.0	
		2,6 Ar—H	7.45 (d, 8.0 Hz)	119.0	
		3,5 Ar—H	7.10 (d, 8.0 Hz)	132.0	
		4 Ar—O		158.8	
		COOH		180.7	
		1′ CH	5.16 (d, 7.3 Hz)	102.9	
		2′ CH	3.58 (#)	73.2	
		3′−5′ CH	3.24−3.41 (#)	#	
20	shikimate[a]	1 C		138.9	COSY, TOCSY, HSQC, HMBC
		2 CH=	6.46 (m)	133.6	
		3 CH	4.42 (t)	75.2	
		4 CH	3.99 (m)	72.2	
		5 CH	3.72 (m)	66.3	
		6 CH	2.20 (dq)	35.6	
		6 CH′	2.78 (dd)	35.6	
		COOH		178.0	
21	fumarate	CH=CH	6.52 (s)	140.1	HSQC, HMBC,
		COOH		177.5	
22	3,4,5-trimethoxyphenylmethanol[a]	1 Ar		138.4	HSQC, HMBC
		2,6 Ar—H	6.78 (s)	107.1	HPLC-MS
		3,5 Ar		150.4	
		CH_2—O	#	76.9	
		OCH_3	3.88 (s)	#	
23	vanillate[a]	1 Ar		120.1	TOCSY, HSQC, HMBC
		2 Ar—H	7.58 (d, 1.9 Hz)	116.1	HPLC-MS
		3 Ar		150.3	
		4 Ar—O		#	
		5 Ar—H	7.20 (d, 8.0 Hz)	118.2	

Table 1. Continued

no.	metabolite	group	δ^1H	δ^{13}C	assigned with
		6 Ar—H	7.54 (dd, 1.9, 8.0 Hz)	125.6	
		OCH_3	3.93 (s)	58.9	
		COOH		177.4	
24	syringate[a]	1 Ar		138.4	HSQC, HMBC
		2,6 Ar—H	7.28 (s)	109.4	HPLC-MS
		3,5 Ar		154.7	
		COOH		176.9	
		OCH_3	3.91 (s)	59.7	
25	caffeate[a]	=CH—COOH	5.90 (d, 16.0 Hz)	118.6	TOCSY, HSQC, HMBC
		—CH=	7.37 (d, 16.0 Hz)	150.7	HPLC-MS
		COOH		171.6	
26	rosmarinate	1 Ar		129.7	TOCSY, HSQC, HMBC
		2 Ar—H	7.15 (d, 1.9 Hz)	118.1	HPLC-MS
		3 Ar		#	
		4 Ar		#	
		5 Ar—H	6.88 (d, 8.0 Hz)	117.5	
		6 Ar—H	7.00 (dd, 1.9, 8.0 Hz)	125.4	
		7 —CH=	7.51 (d, 15.9 Hz)	148.9	
		8 =CH—COO	6.31 (d, 15.9 Hz)	116.8	
		9 COO		171.9	
		1′ Ar		133.1	
		2′ Ar—H	6.89 (#)	124.6	
		3′ Ar		#	
		4′ Ar		#	
		5′ Ar—H	6.75 (#)	#	
		6′ Ar—H	6.71 (#)	120.6	
		7′ CH	2.98 (dd, 8.7, 16.7 Hz)	39.5	
		7′ CH′	3.01 (dd, 3.9, 16.7 Hz)	39.5	
		8′ CH	5.01 (dd, 3.9, 8.7 Hz)	79.1	
		9′ COOH		180.2	
27	rosmadial[a]	13 Ar		137.9	HMBC, HPLC-MS
		14 Ar—H	6.46 (s)	#	
		15 CH	3.19 (sep)	33.3	
		16, 17 CH_3		26.9	
		CHO		180.7	
28	methyl carnosate[a]	13 Ar		142.3	HMBC HPLC-MS
		14 Ar—H	6.53 (s)	#	
		15 CH	3.18 (sep)	26.5	
		16 CH_3, 17 CH_3		32.0	
29	carnosic acid[a]	13 Ar		140.5	HSQC, HMBC
		14 Ar—H	6.54 (s)	119.5	HPLC-MS
		15 CH	3.20 (sep)	27.3	
		16 CH_3, 17 CH_3	1.22 (d), 1.20 (d)	22.3	
		18 CH_3	0.98 (s)	28.1	
		19 CH_3	0.78 (s)	15.7	
30	carnosol[a]	1 CH, CH′	2.92 (m), 2.40 (m)	29.0	TOCSY, HSQC, HMBC
		2 CH, CH′	2.04 (m), 1.69 (m)	18.4	HPLC-MS
		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), 1.18 (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		132.7	
		13 Ar		141.3	
		14 Ar—H	6.65 (s)	112.0	
		15 CH	3.10 (sep)	27.0	
		16 CH_3, 17 CH_3	1.18(d), 1.20 (d)	22.3	
		18 CH_3	0.91 (s)	19.4	
		19 CH_3	0.87 (s)	31.4	
31[b]	valine	γ-CH_3	1.05 (d, 7.0 Hz)	20.6	TOCSY, HMBC
		γ'-CH_3	0.99 (d, 7.0 Hz)	19.6	
		β-CH	2.25 (m)	32.0	
32[b]	proline	α-CH	4.14 (m)	64.9	TOCSY, HSQC, HMBC
		β-CH_2	2.34 (m)	32.3	
		γ-CH_2	2.02 (m)	26.6	
		δ-CH	3.37 (m)	49.9	
		δ'-CH	3.41 (m)	49.9	
		COOH		176.3	
33[b]	asparagine	α-CH	4.01 (dd, 4.6, 7.6 Hz)	54.2	TOCSY, HSQC, HMBC
		β-CH	2.86 (dd, 7.6, 16.1 Hz)	37.7	
		β'-CH	2.96 (dd, 4.6, 16.1 Hz)	37.7	
		γ-$CONH_2$		177.6	
		COOH		176.8	

[a] Metabolite structure and numbering are in the Supporting Information. [b] The signals were found in sun-drying extracts. [c] Multiplicity: s, singlet; d, doublet; t, triplet; q, quartet; dd, doublet of doublets; sep, septet; m, multiplet. U, unidentified signal; #, signals or multiplicities were not determined; Ar, aromatic ring.

mg) were extracted in a flask with four different solvent systems (5 mL), respectively, by vortexing for 30 s followed with the discontinuous ultrasonication for 30 min (i.e., 1 min sonication with a 1 min break). The temperature increase induced by sonication was found to be <5 °C. Four solvent systems were employed, namely, the ambient temperature water (designated A), boiling water (designated B), 50%

Table 2. HPLC-MS Data for Some Rosemary Metabolites[a]

metabolite	t_R[b] (min)	UV λ_{max}[c] (nm)	mass ions (ESI)[d] $[M-H]^-$	mass ions (ESI)[d] major fragments
quinic acid[e]	3.0	201	191	
vanillic acid[e]	8.6	254, 291	167	
syringic acid[e]	9.3	280	197	167
3,4,5-trimethoxyphenylmethanol[e]	9.8	264	197	153
caffeic acid[e]	13.3	242, 296	179	
cis-4-glucosyloxycinnamic acid[e]	13.6	288, 321	325	163
6-hydroxyluteolin-7-glycoside[e]	17.3	277, 341	463	301
eriocitrin[e]	19.4	285, 328	597	
rosmarinate[e]	23.3	292, 328	359	197, 161
cirsimaritin[e]	44.6	274, 334	313	
carnosic acid[f]	22.1	284	331	287
carnosol[f]	28.9	284	339	285
rosmadial[f]	33.4	234, 290	345	
methylcarnosate[f]	38.2	282	345	

[a] HPLC-MS analysis was done in acidic (formic acid) condition; thus, metabolites were supposed to be in the acid form. [b] Retention time in HPLC. [c] Maximum ultraviolet absorbance. [d] MS data from an electrospray ionization source under negative ionization mode. [e] Metabolites from the aqueous solvent extracts. [f] Metabolites from chloroform/methanol solvent extract.

aqueous methanol (designated C), and chloroform/methanol (3:1, v/v) (designated D). For the boiling water extraction, the water temperature was allowed to decrease naturally without interference. The samples that were harvested in February, April, June, and August were extracted with solvent C to investigate the seasonal variations in their metabolite compositions. The seasonal variation of metabolite concentrations was calculated as the difference against the concentration in February, that is, $[C_m - C_F]/C_F$ (where C_m and C_F stand for the concentrations in the given month and in February, respectively). The freeze-dried or sun-dried rosemary samples collected in June were extracted with solvent C to evaluate the effects of two drying processes.

In all cases, the same pool of raw materials was divided into five portions, and each portion was extracted three times sequentially. The resultant three stock solutions were combined, respectively, and centrifuged at 10000 rpm for 10 min. The supernatants from solvents A and B were lyophilized directly, whereas the supernatants from solvents C and D were condensed at 30 °C with a rotary evaporator to remove the organic solvents before lyophilization.

NMR and HPLC-DAD-ESI-MS Measurements. The extracts (10 mg) in the form of dried powder from solvents A, B, and C were dissolved in 600 μL of sodium phosphate buffer (0.1 M, pH 7.4) containing 10% D_2O (v/v) and TSP (3 mM), whereas the powder (~10 mg) from solvent D was dissolved in 600 μL of $CDCl_3$ containing 0.03% TMS. The supernatants (500 μL) were transferred into 5 mm NMR tubes, respectively, after agitation and centrifugation (10000 rpm, 6 min). ^{1}H NMR spectra were recorded at 298 K on a Varian Inova 500 MHz NMR spectrometer operating at 500.123 MHz equipped with an inverse detection probe with a shielded Z-gradient. A standard one-dimensional pulse sequence (RD − 90° − t_1 − 90° − t_m − 90° − acquisition) was employed with the irradiations at the water frequency during the recycle delay (RD, 2 s) and the mixing time (t_m, 100 ms) to suppress the water signal. t_1 was set to 6 μs, and a 90° pulse length was adjusted to about 10 μs for each sample. Sixty-four transients were collected into 32 K data points for each spectrum with a spectral width of 20 ppm. All free induction decays (FID) were multiplied by an exponential function with a 1 Hz line broadening factor prior to Fourier transformation (FT).

For assignment purposes, ^{1}H−^{1}H COSY, ^{1}H−^{1}H total correlation spectroscopy (TOCSY), ^{1}H−^{13}C HSQC, and ^{1}H−^{13}C HMBC NMR 2D spectra were acquired for selected samples on a Bruker Avance III 600 MHz spectrometer equipped with an inverse detection cryogenic probe. For COSY and TOCSY experiments, 48 transients per increment and 256 increments were collected into 2048 data points with the spectral width of 8 ppm for both dimensions. TOCSY spectra were acquired using MLEV-17 as a spin−lock scheme with the mixing time of 80 ms. Both HSQC and HMBC spectra were acquired using the gradient-selected sequences. In HSQC, composite pulse broadband decoupling (globally alternating optimized rectangular pulses, GARP) was employed on ^{13}C during the acquisition period, and typically 2048 data points with 256 scans per increment and 200 increments were acquired with spectral widths of 6300 Hz in the ^{1}H dimension and 25641 Hz in the ^{13}C dimension. In HMBC, the spectral width was 6786 Hz in the ^{1}H dimension and 30000 Hz in the ^{13}C dimension, and 400 transients were collected into 2048 data points for each of 100 increments. The data were zero filled to 2048, and a sine or a shifted sinebell-squared function was applied to the FID, in both dimensions, prior to FT.

Some secondary metabolites detected in NMR were further confirmed using the HPLC-DAD-ESI-MS method. The HPLC system (Agilent series 1200, Waldbronm, Germany) consisted of a diode array detector, a quaternary pump, an autosampler, and a column oven. MS measurements were performed on a Bruker MicroTOFQ mass spectrometer with an electrospray ionization source (Bruker Daltonics, Bremen, Germany). Five percent of the eluent was directed to MS using a BNMI unit (Bruker BioSpin). The chromatographic separation was carried out on an Ace 5 C18-HL column (250 mm × 4.6 mm; ACT, Scotland) at 25 °C with the injection volume of 20 μL (2 mg/mL). The elution was performed using water (containing 0.1% v/v formic acid) and acetonitrile with a step gradient lasting 60 min at the flow rate of 1 mL/min. For the extracts of the aqueous solvents, the elution gradient was from 5 to 70% acetonitrile, whereas it was from 10 to 90% acetonitrile for the chloroform/methanol extract. The ESI source was operated with a nebulizer pressure of 0.8 bar, whereas the drying gas was delivered at the flow rate of 10 L/min at 180 °C. The capillary voltage was 4000 V, and the collision energy level was −10.0 eV/z. MS data were acquired in a scan range between 50 and 1000 Da under negative ionization.

Data Analysis. The ^{1}H NMR spectra (δ 0.5−8.5) were divided into regions with equal width of 0.008 ppm (4 Hz) using AMIX (v. 3.8, Bruker Biospin) after phase and baseline corrections. The region δ 4.67−5.20 in the spectra from extracts with solvents A, B, and C was excluded to eliminate the effects of imperfect water suppression. For solvent D extract, the spectral regions δ 1.20−1.30 and 7.20−7.40 were also removed to eliminate the residual water and $CHCl_3$ signals, respectively. The bucketed regions were normalized to the total sum of the spectral integral to compensate for the overall concentration difference. Multivariate data analysis was carried out on the normalized NMR data sets with the software package SIMCA-P$^+$ (version 11.0, Umetrics, Sweden). Principal component analysis (PCA) was conducted using mean-centered scaling, and the results are presented as the scores and loadings plots; each point in the former represented each sample, whereas the latter showed the magnitude and manners of the NMR signals (thus metabolites) to classification. Further analysis on NMR data was carried out using the orthogonal projection to latent structure with discriminant analysis (OPLS-DA) method (*20*) with unit variance scaling (UV), and the loadings in the coefficient plots were calculated back from the coefficients incorporating the weight of the variables contributing to the sample classification in the model (*21*). The OPLS-DA model was constructed using NMR data as the X matrix and the class information identifier for the extract category as the Y variables. One orthogonal component was calculated for the model to remove the systematic variation in the NMR spectra unrelated to the information between the different groups. The quality of the model was described by the parameters R^2X, representing the total explained variation for X, and Q^2, indicating the predictability of the model related to its statistical validity. The validation of the model was conducted using a 5-fold cross-validation method. The coefficient plots were generated with MATLAB scripts (http://www.mathworks.com/) with some modifications and were color-coded with absolute value of coefficients (r). The coefficient plot showed the variables (resonances) contributed to classification and the significance of such contribution. In this study, a correlation coefficient of $|r| = 0.81$ was used as the cutoff value for the statistical significance based on the discrimination significance at the level of $p = 0.05$, which was determined according to the test for the significance of the Pearson's product-moment correlation coefficient.

For analysis of variance (ANOVA), metabolite concentration was calculated from the integrals of selected metabolite NMR signals (least

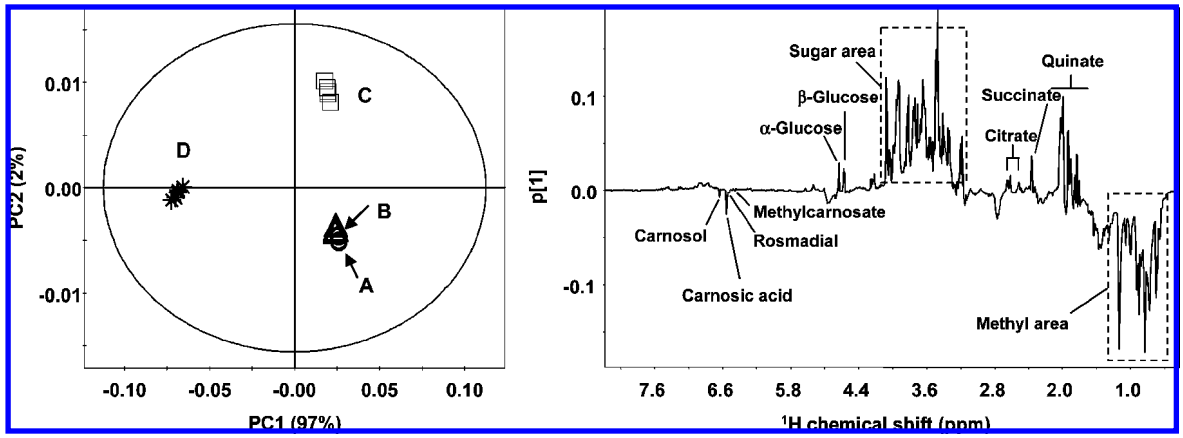

Figure 2. PCA scores plot (left) and corresponding loadings plot (right) of rosemary extracts from the four different solvents [(A) ambient temperature water (○); (B) boiling water (△); (C) 50% aqueous methanol (□); and (D) chloroform/methanol (v/v, 3:1) (∗)].

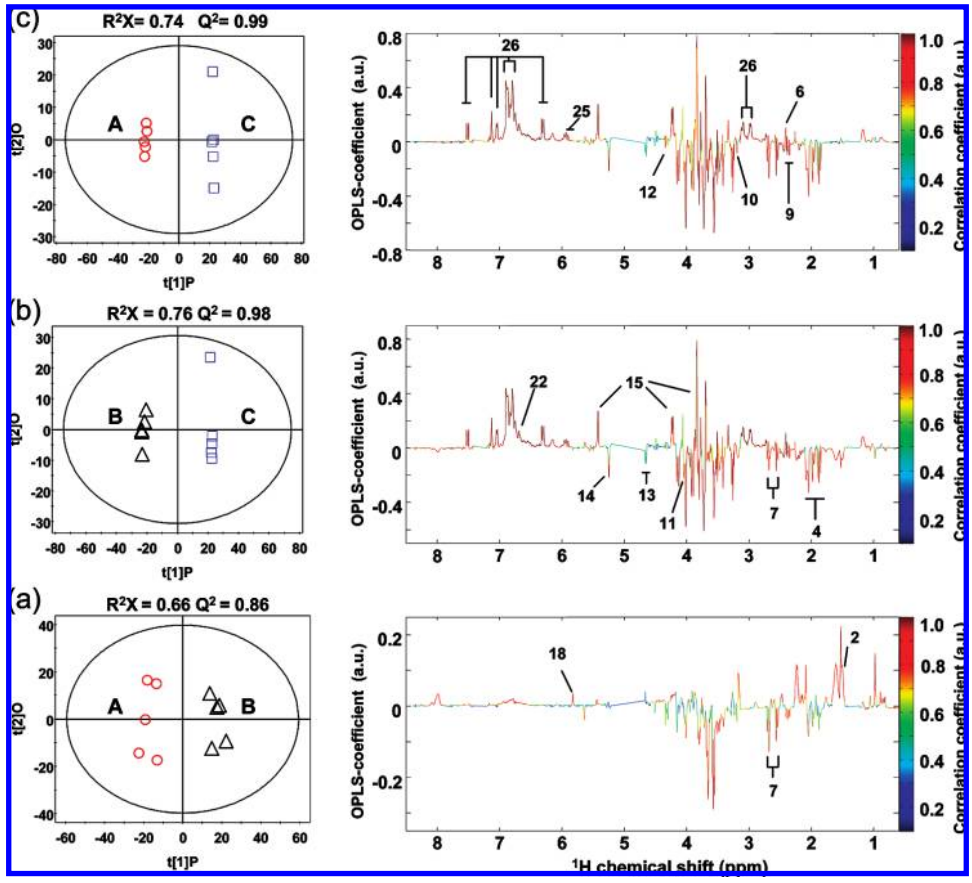

Figure 3. OPLS-DA scores (left) and coefficient plots (right) for rosemary extracts from the three aqueous solvents [(A) ambient temperature water (○); (B) boiling water (△), and (C) 50% aqueous methanol (□) (the metabolite numbers are listed in **Table 1**)]. The color scale shows the significance of metabolite variations between the two classes.

overlapping ones) relative to that of internal reference (TSP or TMS) with known concentration. Although the values of relaxation time, T_1, for metabolites and references were different and the concentrations measured here were semiquantitative, our treatments were still valid when the concentration changes between samples were compared because the intersample T_1 variations were small for the same metabolite (or reference). The obtained metabolite concentration was subjected to classical statistical analysis (one way-ANOVA) using SPSS 13.0 software with a Tukey post-test.

RESULTS AND DISCUSSION

NMR and HPLC-MS Analysis of Rosemary Extracts. **Figure 1** shows the typical ^{1}H NMR spectra of rosemary extracts obtained from ambient temperature water (A), boiling water (B), 50% aqueous methanol (C), and chloroform/methanol (v/v, 3:1) (D). The resonances in these spectra were assigned to individual metabolites according to the literature (*22*−*24*) and extensive analysis of the ^{1}H−^{1}H COSY, ^{1}H−^{1}H TOCSY, ^{1}H−^{13}C HSQC, and ^{1}H−^{13}C HMBC 2D NMR spectra (**Table 1**). To our best knowledge, this is the first comprehensive study on both primary and secondary metabolites for rosemary extracts together with their ^{13}C NMR data, even though a few ^{13}C signals were not determined in such complex mixtures. Apart from those compounds already reported in the literature (*6, 24, 25*), more metabolites were observed in this study, for the first time,

Table 3. OPLS-DA Coefficients and Metabolite Content of Rosemary Extracts with Different Solvents

metabolite	coefficient (*r*)[a]			mean ± SD[b] (mg/g)			
	B /A[c]	C/A	C/B	A	B	C	D
rosmarinate	0.60	0.99	0.99	4.32 ± 0.56	4.86 ± 0.14	21.72 ± 1.91[d]	–
caffeate	0.32	0.97	0.99	1.62 ± 0.10	1.78 ± 0.22	4.50 ± 0.65[d]	–
syringate	0.58	0.91	0.90	0.75 ± 0.04	0.89 ± 0.06	1.24 ± 0.01[d]	–
vanillate	0.36	0.23	0.45	0.91 ± 0.06	0.92 ± 0.01	0.98 ± 0.13	–
cis-4-glucosyloxycinnamic acid	0.58	0.88	0.78	5.24 ± 0.41	5.56 ± 0.35	8.56 ± 0.19[d]	–
3,4,5-trimethoxyphenylmethanol	0.70	0.99	0.99	1.06 ± 0.06	1.29 ± 0.05	4.73 ± 0.21[d]	–
carnosic acid	–	–	–	–	–	–	40.72 ± 12.13
carnosol	–	–	–	–	–	–	14.01 ± 2.07
methylcarnosate	–	–	–	–	–	–	7.54 ± 2.89
rosmadial	–	–	–	–	–	–	4.58 ± 2.14
sucrose	0.68	0.99	0.99	3.46 ± 0.22	4.16 ± 0.17	19.74 ± 1.27[d]	–
glucose[f]	0.29	−0.99	−0.99	45.68 ± 3.25	48.08 ± 4.72	37.04 ± 3.09[d]	–
fructose	0.14	−0.99	−0.99	32.28 ± 0.56	33.02 ± 2.47	24.12 ± 0.02[d]	–
quinate	−0.45	−0.96	−0.95	49.84 ± 0.76	50.22 ± 3.61	43.78 ± 2.02[d]	–
shikimate	0.71	0.99	0.99	2.18 ± 0.14	2.32 ± 0.16	3.00 ± 0.06[d]	–
citrate	−0.88	−0.98	−0.98	10.16 ± 0.18	8.80 ± 1.35[e]	6.46 ± 1.19[d]	–
succinate	−0.45	0.85	0.87	2.40 ± 0.05	2.44 ± 0.16	2.72 ± 0.01[d]	–
malate	−0.44	0.56	0.76	14.54 ± 1.04	14.32 ± 2.34	14.82 ± 0.27	–
fumarate	0.65	0.99	0.99	0.32 ± 0.03	0.34 ± 0.03	0.68 ± 0.01[d]	–
tartrate	0.57	−0.76	0.63	2.48 ± 0.23	2.36 ± 0.46	2.44 ± 0.09	–
malonate	−0.64	0.97	0.97	1.09 ± 0.03	1.08 ± 0.06	1.24 ± 0.04[d]	–
lactate	−0.40	−0.61	−0.59	1.36 ± 0.21	1.16 ± 0.15	1.02 ± 0.04	–
acetate	0.68	0.21	−0.12	0.62 ± 0.03	0.70 ± 0.12	0.66 ± 0.21	–
alanine	0.84	−0.67	−0.88	0.70 ± 0.01	1.08 ± 0.26[e]	0.65 ± 0.03	–
choline	0.35	0.79	0.82	1.40 ± 0.01	1.52 ± 0.10	1.38 ± 0.08	–

[a] The coefficients from OPLS-DA results; positive and negative signs indicate positive and negative correlation in the concentrations, respectively. The coefficient of 0.81 was used as the cutoff value for the significant difference evaluation ($p < 0.05$). –, no resonances were present in the corresponding extracts. [b] The average concentration and standard deviation (mean ± SD, mg/g of dried rosemary material) were obtained from five parallel samples. [c] The extracts were from ambient temperature water (A), boiling water (B), 50% aqueous methanol (C), and chloroform/methanol (D). [d] Significant difference compared with B by one-way ANOVA analysis ($p < 0.05$). [e] Significant difference compared with A by one-way ANOVA analysis ($p < 0.05$). [f] The sum of α-glucose and β-glucose concentrations.

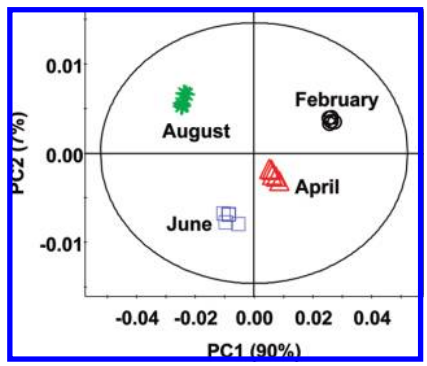

Figure 4. PCA scores plot for the samples harvested in February (○), April (△), June (□), and August (∗).

including 18 primary metabolites and 3 secondary ones such as quinate, *cis*-4-glucosyloxycinnamic acid, and 3,4,5-trimethoxyphenylmethanol. To confirm the metabolite assignments from NMR, HPLC-MS analysis was also conducted, and 14 secondary metabolites were characterized with molecular ions, major fragment ions, and UV absorbance (see **Table 2** for details).

The newly found secondary metabolites were carefully identified using 2D NMR and confirmed with LC-MS data. The ring structure of quinate was established by the extensive couplings between two methylene groups (2-H, δ 1.88, 2.09; 6-H, δ 1.98, 2.06) and three hydroxylated methine groups (3-H, δ 4.03; 4-H, δ 3.57; 5-H, δ 4.16) in the $^1H-^1H$ TOCSY spectrum. After determination of the direct H−C bonding patterns using $^1H-^{13}C$ HSQC NMR, long-range correlations were found between methylene groups and a hydroxylated quaternary carbon and carboxylic group in the $^1H-^{13}C$ HMBC spectrum, whereas the methine protons were correlated with the quaternary carbon but not with carbonyl carbon. All of the observations allowed establishment of the structural connectivity of quinate. Because this compound was detected in rosemary for the first time, an HPLC-MS study was carried out to obtain further confirmation with its parent ion $[M - H]^-$ (*m/z* 191), hydrophilicity (retention time = 3.0 min on C18), and maximum UV absorbance at 201 nm (lack of aromatic rings). For *cis*-4-glucosyloxycinnamic acid, the $^1H-^1H$ TOCSY spectrum showed two coupled aromatic doublets (2,6-H, δ 7.45; 3,5-H, δ 7.10; J = 8 Hz) corresponding to a para-substituted benzene ring and two coupled doublets (8-H, δ 6.03; 7-H, δ 6.50; J=12 Hz) corresponding to two *cis*-olefinic protons. Following determination of the direct H−C coupling using $^1H-^{13}C$ HSQC, the linkages between the *cis*-olefinic moiety and a carboxylic group and the aromatic ring were established using $^1H-^{13}C$ HMBC. Furthermore, the coupling between a doublet (δ 5.16, J = 7.3 Hz) corresponding to anomeric proton of a β-glycoside and a hydroxylated aromatic carbon (4-C, δ 158.8) in HMBC established the proposed structure. MS results showed a parent ion $[M - H]^-$ at *m/z* 325 and an aglycone fragment ion $[M - H - 162]^-$ at *m/z* 163, indicating the presence of a glucose or galactose residue. Because the galactosides of phenolic acids have rarely been observed in plant extracts, this compound was tentatively identified as *cis*-4-glucosyloxycinnamic acid. 3,4,5-Trimethoxylphenylmethanol was also identified using the above approaches. The HSQC spectrum showed that a singlet (2-H, δ 6.78) was attached to an aromatic carbon (2-C, δ 107.1). In HMBC the singlet had the correlations with three aromatic carbons (1-C, δ 136.0; 2-C, δ 107.1; 3-C, δ 150.4) and a hydroxylated carbon signal at δ 76.9, indicating the presence of a symmetric 3,4,5-substituted phenylmethanol moiety. With these data and the long-range correlation between methyoxyl protons (δ 3.93) and an aromatic carbon (3-C, δ 150.4) in HMBC, the atomic connectivity of 3,4,5-trimethoxylphenylmethanol was established. A parent ion $[M - H]^-$ at *m/z* 197

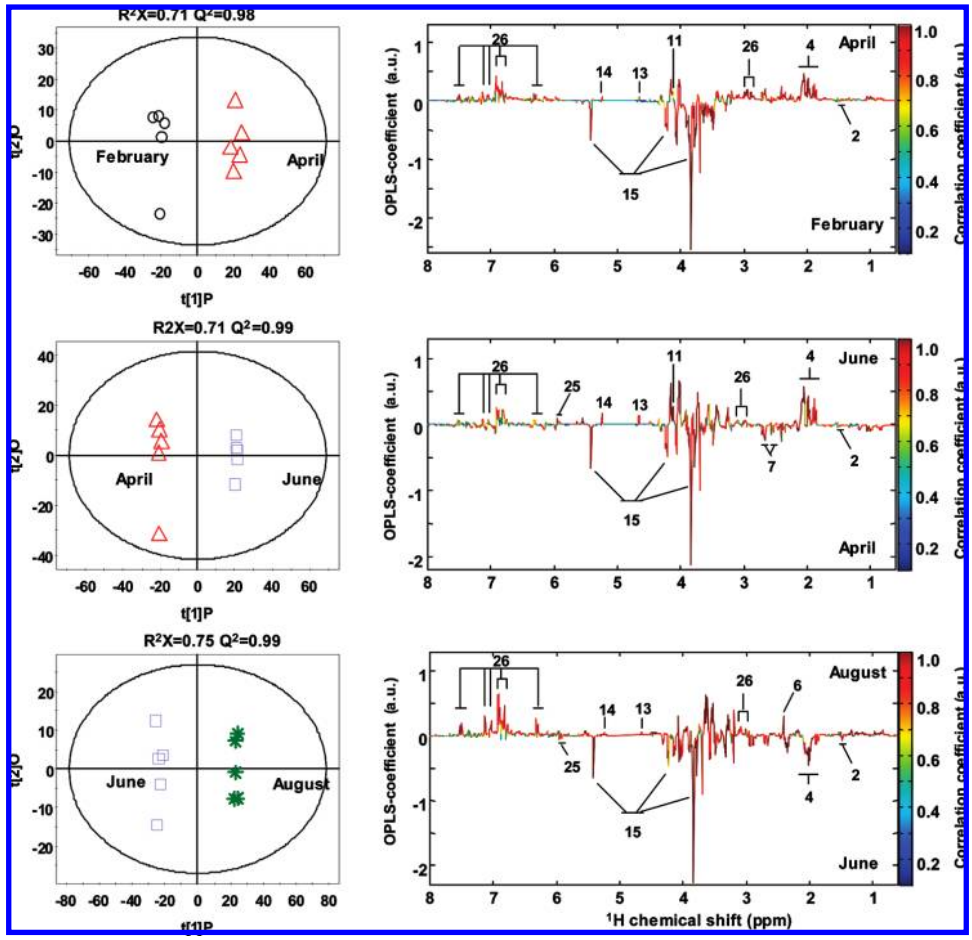

Figure 5. OPLS-DA scores (left) and coefficient plots (right) for the samples harvested in February (○), April (△), June (□), and August (∗) (the metabolite numbers are listed in **Table 1**). The color scale shows the significance of metabolite variations between the two classes.

Table 4. OPLS-DA Coefficients and Metabolite Content of Rosemary Extracts from Harvesting Months

	coefficient (r)[a]			mean ± SD[b] (mg/g)			
metabolite	April/Feb	June/April	Aug/June	Feb	April	June	Aug
rosmarinate	0.95	0.81	0.98	4.84 ± 0.28	7.48 ± 0.81[c]	8.64 ± 0.35[d]	13.64 ± 0.58[e]
caffeate	0.54	0.94	−0.65	1.06 ± 0.04	1.28 ± 0.19	2.08 ± 0.06[d]	1.76 ± 0.01
syingate	0.91	−0.83	0.91	0.42 ± 0.01	0.76 ± 0.15	0.51 ± 0.02[d]	0.68 ± 0.01[e]
sucrose	−0.98	−0.97	−0.98	41.06 ± 0.19	28.1 ± 1.77[c]	15.56 ± 0.35[d]	2.60 ± 0.93[e]
glucose[f]	0.93	0.96	0.77	2.96 ± 0.32	4.32 ± 1.07[c]	7.06 ± 0 0.53[d]	7.94 ± 1.12
fructose	0.96	0.98	−0.95	5.34 ± 0.23	7.24 ± 0.82[c]	9.76 ± 0.26[d]	6.58 ± 0.71[e]
citrate	0.79	−0.95	0.77	3.84 ± 0.02	4.22 ± 0.13	2.94 ± 0.01[d]	3.36 ± 0.25
succinate	0.98	−0.99	0.99	0.68 ± 0.02	0.88 ± 0.06[c]	0.66 ± 0.03[d]	0.90 ± 0.07[e]
quinate	0.96	0.98	−0.99	8.84 ± 0.19	12.3 ± 0.60[c]	15.42 ± 0.7[d]	13.98 ± 0.90[e]
alanine	−0.51	−0.84	−0.99	0.58 ± 0.01	0.56 ± 0.01	0.48 ± 0.01[d]	0.34 ± 0.03[e]

[a] The coefficients from OPLS-DA results, positive and negative signs indicate positive and negative correlation in the concentrations, respectively. The coefficient of 0.81 was used as the cutoff value for the significant difference evaluation. [b] The average concentration and standard deviation (mean ± SD, mg/g of dried rosemary material) were obtained from five parallel samples. [c] The significant difference by one-way ANOVA analysis ($p < 0.05$): April vs February. [d] The significant difference by one-way ANOVA analysis ($p < 0.05$): June vs April. [e] The significant difference by one-way ANOVA analysis ($p < 0.05$): August vs June. [f] The sum of α-glucose and β-glucose concentrations.

and a fragment ion at m/z 153 (loss of CH_2O and CH_3) in HPLC-MS confirmed its molecular mass and the presence of the methyl group.

In addition, some flavonoids were also detected in our HPLC-MS study such as cirsimaritin, eriocitrin, and 6-hydroxyluteolin-7-glycoside, which were observed in rosemary previously (*8, 15*). This was further confirmed by the fact that cirsimaritin was detected in our preliminary LC-NMR study (data not shown). These flavonoids were, however, not clearly observed in our NMR spectra, probably because of their lower concentrations in the extracts and the resonance overlapping in the aromatic region. The more detailed characterization of rosemary flavonoids is in progress using the sophisticated LC-SPE-NMR-MS hyphenated technology. Nevertheless, these metabolite assignments are sufficient for the purposes of understanding the metabonomic variations in this study.

To compare the metabolite composition of different extracts, ^{1}H NMR spectra were acquired for the extracts from four different solvent systems. It was apparent that the spectra of rosemary extracts from three polar solvents (**Figure 1A−C**) were dominated by sugars such as sucrose, fructose, α-glucose, and β-glucose; carboxylic acids such as lactate, acetate, malate,

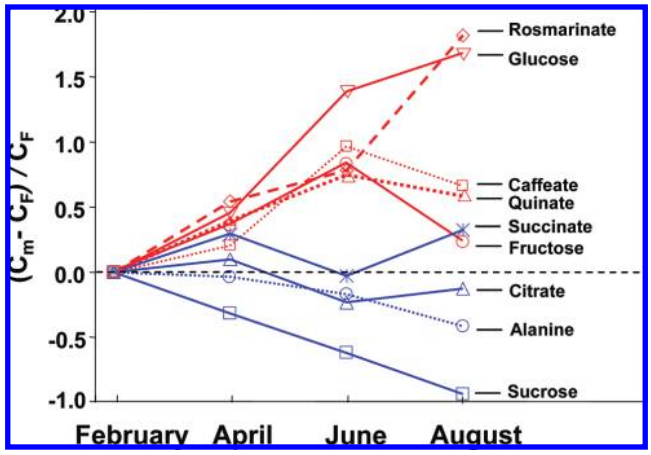

Figure 6. Metabolite concentration changes relative to that in February (see Materials and Methods for details) for samples collected from different time points.

succinate, citrate, α-ketoglutarate, malonate, tartrate, fumarate, and quinate; and amino acids such as alanine, asparagine, proline, and valine (although some were visible only in the sun-dried samples). Choline was also observable. Although the signal intensity of these metabolites appeared in the aliphatic region varied to a certain degree among the three extracts, marked differences were more evident in the aromatic region. For example, the resonances from rosmarinate, caffeate, and syringate had much higher intensities for the aqueous methanol extract than the water extracts. The spectrum of the chloroform/methanol extract (**Figure 1D**) was drastically different from the other three extracts, in which the major components were phenolic diterpenoid compounds such as carnosic acid, carnosol, methylcarnosate, and rosmadial. Only small amounts of carboxylic acids, sugars, amino acids, and polyphenolic acids were detectable in this extract, although they were abundant in the other three ones. This is not surprising because these metabolites are much more hydrophilic and not favored in chloroform-based solvent extraction. Even from the above qualitative analysis, nevertheless, it is clear that the metabolite profile of the plant extracts is critically dependent on the solvents employed. However, the complexity of rosemary extracts and multiple spectral data, which are necessary for statistical purposes, make it prohibitively difficult for the spectra to be analyzed with the naked eye; multivariate data analysis is more appropriate for mining such complex data.

Multivariate Data Analysis for Rosemary Extracts. Initially, PCA was conducted on the spectral data, and two principal components were calculated for the extracts obtained from four different solvents with a total of 97.0 and 1.8% of variables being explained by PC1 and PC2, respectively. The scores plot (**Figure 2**) showed that, in the first principal component PC1, obvious differences were present for the samples obtained from the polar solvents (A−C) and from the less polar solvent D. It is also interesting to note that samples from the individual solvents were clustered together closely, indicating the excellent reproducibility in the extraction procedures and NMR measurements. The corresponding loadings plot showed that, compared with the extracts from the three aqueous solvents, the chloroform/methanol extract contained a higher level of diterpenes such as carnosic acid and its derivatives together with much lower levels of sugars and carboxylic acids.

To understand the significance of variables (i.e., metabolites) contributing to classification, the spectral data were further subjected to OPLS-DA. The coefficient plots showed the metabolites having contributions to the class difference, and the correlation coefficients (with color-coded scale) for NMR signals indicated the significance of the metabolites' contribution. Because there were no established criteria to assess the collective significances when all variables were considered in the case of multivariate data analysis, here each variable (i.e., metabolite) was assessed only individually and respectively using the criteria for univariate analysis. The coefficient of 0.81 was used as the cutoff value that was calculated on the basis of discrimination significance at the level of 0.05 ($p = 0.05$). By doing so, nevertheless, our results will be conserved; thus, the statistical significance is underestimated to some extent.

Figure 3 showed the OPLS-DA scores plots and corresponding coefficient plots for the aqueous solvent extracts. Clear separations were observed for two water extracts (**Figure 3a**), for extracts from methanol/water (50%) and boiling water (**Figure 3b**), and samples from methanol/water (50%) and ambient temperature water (**Figure 3c**), respectively. The values of R^2X and Q^2 listed on the scores plots indicate that these models were of reasonable quality. The coefficient plots were color-coded with the absolute value of correlation coefficients, where a hot-colored signal (red) indicates more significant contribution to the class separation than a cold-colored one (blue); the positive and negative signs indicate the direction of the changes (i.e., positive and negative correlation) for the metabolites. The coefficients for some metabolites, indicating the importance of their contributions, are summarized in **Table 3**. Between two water extracts, most of the metabolites showed no significant difference except for citrate and alanine judged by the coefficients ($|r| > 0.81$). However, compared with the boiling water extracts, the aqueous methanol extracts contained significantly lower levels of glucose, fructose, quinate, citrate, and alanine ($r < -0.81$) but significantly higher levels of rosmarinate, caffeate, *cis*-4-glucosyloxycinnamic acid, syringate, 3,4,5-trimethoxyphenylmethanol, sucrose, shikimate, succinate, fumarate, and malonate ($r > 0.81$). The difference between the chloroform/methanol extract and the extracts from aqueous solvents was so huge that we did not perform OPLS-DA further.

The concentrations of most metabolites were also calculated using the integration areas of the selected NMR signals (least overlapping ones) relative to the internal standard TSP (for aqueous extracts) or TMS (for chloroform extracts). All data are expressed as mean ± standard deviation (mean ± SD mg/g of dried rosemary material) from the five parallel samples (**Table 3**). The statistical significances between them was analyzed by one-way ANOVA analysis ($p < 0.05$). Compared with ambient water extracts, most constituents appeared to have higher levels in boiling water extracts except for citrate, tartrate, malonate, quinate, and lactate. However, significant difference occurred only for citrate and alanine, which is in good agreement with the OPLS-DA results. Such a minor compositional difference between two extracts probably resulted mainly from metabolite solubility rather than from enzyme change because boiling water was expected to purge most enzymic activities, but ambient temperature water was not. Using the same approach, the significant differences were assessed for aqueous methanol extracts in comparison with the boiling water extracts. The results were highlighted by significantly higher levels of rosmarinate, caffeate, syringate, *cis*-4-glucosyloxycinnamic acid, 3,4,5-trimethoxyphenylmethanol, sucrose, shikimate, succinate, fumarate, and malonate together with lower levels of glucose, fructose, quinate, and citrate, which is also consistent with the OPLS-DA results. Because aqueous methanol (1:1) was generally accepted as a solvent to purge enzymic activity by precipitate proteins (including enzymes) and boiling water was

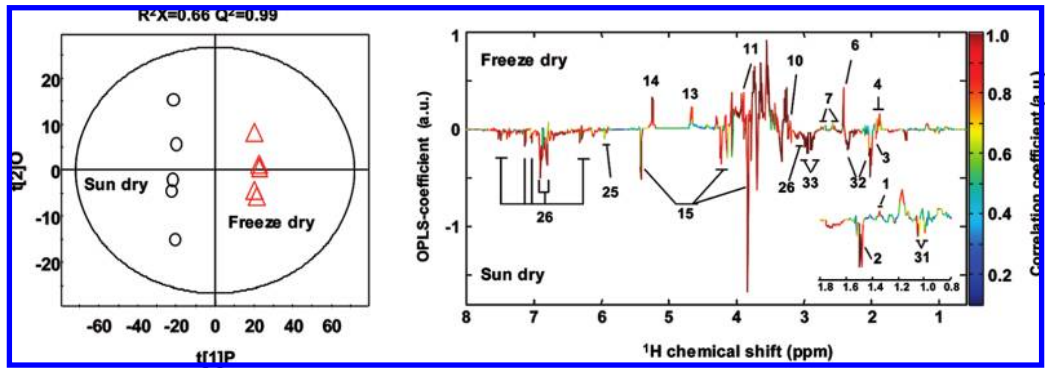

Figure 7. OPLS-DA scores (left) and coefficient plots (right) for the samples from sun-dried (○) and freeze-dried (□) rosemary materials (the metabolite numbers are listed in **Table 1**). The color scale shows the significance of metabolite variations between the two classes.

Table 5. OPLS-DA Coefficients and Metabolite Content of the Rosemary Extracts from Different Drying Processes

	coefficient (r)[a]	mean ± SD[b] (mg/g)	
metabolite	freeze/sun-dry	freeze-dry	sun-dry
rosmarinate	−0.96	6.32 ± 0.10	8.45 ± 0.54[c]
caffeate	−0.23	1.94 ± 0.29	2.08 ± 0.08
syringate	−0.47	0.46 ± 0.01	0.44 ± 0.04
cis-4-glucosyloxycinnamic acid	0.81	3.69 ± 0.08	4.32 ± 0.01[c]
3,4,5-trimethoxyphenylmethanol	−0.51	2.04 ± 0.09	1.75 ± 0.03
sucrose	−0.96	7.40 ± 0.37	15.36 ± 2.00[c]
glucose[d]	0.99	15.1 ± 2.38	7.00 ± 0.44[c]
fructose	0.94	10.42 ± 0.01	9.64 ± 0.42[c]
tartrate	0.23	1.06 ± 0.05	0.88 ± 0.06[c]
malate	0.86	5.76 ± 0.10	4.52 ± 0.18[c]
choline	0.97	0.60 ± 0.03	0.52 ± 0.16[c]
manolate	−0.85	0.54 ± 0.01	0.56 ± 0.01
citrate	0.58	3.82 ± 0.58	2.94 ± 0.01
succinate	0.99	1.18 ± 0.01	0.66 ± 0.03[c]
acetate	−0.77	0.16 ± 0.08	0.20 ± 0.01
quinate	0.81	18.9 ± 0.89	15.42 ± 0.7[c]
alanine	−0.99	0.30 ± 0.02	0.48 ± 0.01[c]
latate	0.86	0.42 ± 0.01	0.34 ± 0.01[c]
asparagine	−0.99	2.46 ± 0.03	5.46 ± 0.01[c]
valine	−0.96	0.32 ± 0.01	0.38 ± 0.01[c]
proline	−0.99	0.96 ± 0.05	4.69 ± 0.19[c]

[a] The coefficients from OPLS-DA results; positive and negative signs indicate positive and negative correlation in the concentrations, respectively. The coefficient of 0.81 was used as the cutoff value for the significant difference evaluation. [b] The average concentration and standard deviation (mean ± SD, mg/g of dried rosemary material) were obtained from five parallel samples. [c] Significant difference compared with freeze-dried samples ($p < 0.05$). [d] The sum of α-glucose and β-glucose concentrations.

expected to denature enzymes, the major compositional differences between extracts from boiling water and methanol/water (1:1, v/v) were probably also from extraction efficiency or solubility differences rather than enzymic changes.

The chloroform/methanol extract contained almost exclusively carnosic acid and its derivatives (the concentration of carnosic acid was in the range of 40.72 ± 12.13 mg/g of dried rosemary material) with few metabolites observed in common with the other three extracts. With relatively thorough analysis of phenolic diterpenes in the literature using the targeted analysis approach, further analysis was no longer considered here for this group of metabolites. Previous studies reported that methanol and DMSO solvents effectively extracted carnosic acid and carnosol as well polyphenolic acids (*14*). Our study has shown that chloroform/methanol is an excellent solvent system for the selective extraction of some phenolic diterpenes, which are lipophilic antioxidants. In addition, aqueous methanol was more efficient for extracting hydrophilic antioxidants such as rosmarinate, caffeate, and syringate than water.

Effects of Seasonal Variation on the Rosemary Metabolite Composition. The samples collected in February, April, June, and August were extracted with 50% aqueous methanol to investigate the seasonal effects on the rosemary metabolite composition. The ^{1}H NMR spectra showed that the extracts of rosemary harvested in different months had similar chemical constituents (data not shown), although with differences highlighted in their concentrations. A PCA model constructed with two principal components (PC1, 90%; and PC2, 7%) showed clear separation for the extracts obtained from four different months and tight clustering for each group (**Figure 4**), indicating the sensitivity and powerfulness of such classification methods. Further analysis using OPLS-DA (**Figure 5**) showed the clear monthly differentiation for the plant extracts with the values of R^2X/Q^2 indicating the model validity and the metabolites having significant contributions to the monthly classification ($r > 0.81$ or $r < -0.81$) (**Table 4**). The concentrations of some metabolites were also calculated (see **Table 4**, mean ± SD, mg/g of dried rosemary material).

Compared with the February samples, the extracts from April showed significantly higher levels of romarinate, syringate, glucose, fructose, succinate, and quinate and lower levels of sucrose. In addition, extracts obtained in June showed statistically higher concentrations of rosmarinate, caffeate, glucose, fructose, and quinate and lower concentrations of syringate, sucrose, citrate, succinate, and alanine compared with the samples from April. Furthermore, the samples collected in August had statistically higher quantities of rosmarinate, syringate, and succinate and lower amounts of sucrose, fructose, quinate, and alanine than the samples harvested in June. The metabolite concentrations calculated from the integral of the metabolite resonances were also subjected to ANOVA ($p < 0.05$); the results were broadly consistent with those of OPLS-DA.

The seasonal concentration variations of some rosemary metabolites are shown in **Figure 6** relative to that in February. Compared with in February, the concentration of succinate rose in April and August, whereas the concentration of citrate showed a slight increase in April but some decrease in June and August. Overall, the levels of sucrose and alanine showed a steady decrease from February to August, whereas the concentrations were increased for rosmarinate, caffeate, quinate, glucose, and fructose. Specifically, from February to August, the mean concentration of rosmarinate showed an almost 2-fold increase (from 4.84 ± 0.28 to 13.64 ± 0.58 mg/g of dried rosemary material), which broadly agreed with the previous observations about the seasonal variations, probably owing to the weather conditions (*8, 14*). In contrast, the level of sucrose decreased more than an order of

magnitude (from 41.06 ± 0.19 to 2.60 ± 0.93 mg/g of dried rosemary material) in the same period. The opposite trend for the levels of rosmarinate and sucrose was observed previously in the cultured cell systems, which suggested that sucrose affected the biosynthesis of rosmarinate (*26*). Such inverse correlation was also evident here between sucrose level and the levels of glucose and fructose, probably owing to sucrose breakdown as observed in suspension cultures of *Coleus* cells (*26*). It has been reported that the biosynthesis of quinate can result from D-glucose (*27*); thus, the same trend of changes for the quinate and glucose levels was understandable. This further suggested that the changes of secondary metabolites were influenced by primary metabolites. However, the metabolic pathways associated with such changes are well beyond the scope of this study.

Effects of Drying Processes on Rosemary Metabolite Composition. To investigate the dependence of rosemary metabolite composition on the drying processes, the aqueous methanol extracts from freeze-dried and sun-dried rosemary samples were analyzed with ^{1}H NMR followed by multivariate data analysis. The NMR spectra of two different extracts showed some clear differences in their signal intensities (data not shown). The scores plot from OPLS-DA (**Figure 7**) shows that the freeze-dried samples were clearly discriminated from the sun-dried ones in terms of their metabolite composition, and the correlation coefficients and quantitative information about the relevant metabolites are listed in **Table 5**. Compared with those from the sun-dried samples, the extracts from freeze-dried ones contained significantly lower amounts of rosmarinate, sucrose, alanine, asparagine, valine, and proline ($r < -0.81$) together with significantly higher levels of glucose, fructose, malate, choline, succinate, quinate, and lactate ($r > 0.81$). No significant differences were observed for some metabolites such as caffeate, syringate, 3,4,5-trimethoxyphenylmethanol, tartrate, citrate, and acetate. The levels of these metabolites were also subjected to ANOVA, and similar conclusions were obtained.

These metabolite compositional differences were probably attributable to the different effects of two drying processes on the cellular enzyme activities. During the freeze-drying process enzymes cause little metabolite changes due to low temperature and lack of water availability. In contrast, during the sun-drying process, gradual water depletion will cause drought stress to the plant cells, inducing metabolic changes to various degrees. In fact, elevations of proline in the crop plants (*28*) and sucrose in the leaves of *Lupinus albus* L. (*29*) were also reported when they were subjected to drought stress. Although the exact mode of action remains to be fully understood for such drought-induced metabolic responses, this study indicates that NMR-based metabonomic analysis is probably an effective way to investigate such stress-induced biochemical processes for plants. This also strongly suggests that the effects of drying processes have to be taken into consideration when plant metabolite compositions are studied in terms of phytomedicines and food.

In summary, the rosemary metabolite composition is complex and largely dominated by plant primary metabolites such as sugars, organic acids, and amino acids and secondary metabolites such as phenolic diterpenes and polyphenolic acids. The concentrations of different metabolites in rosemary extracts vary with extraction solvents, harvesting seasonal time points, and drying processes. From the levels of chemical constituents, the extraction solvent appeared to be the major factor resulting in the qualitative and quantitative differences. The extraction efficiency for different metabolites differs remarkably according to the solvent system employed. This was also discussed in a recent work that gave a detailed protocol for extraction of plant tissues using perchloric acid (*30*). It is therefore essential to take these factors into consideration and make appropriate choices for the metabolites concerned. Being consistent with the ANOVA results, the present study confirms the validity of statistical significances derived from the much more efficient OPLS-DA approach. For the composition-based quality control of herbs and phytomedicines, therefore, NMR-based metabonomics offers an excellent holistic method to monitor variations resulting from various environmental and postharvest processing factors.

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Supporting Information Available: Structures of metabolites. This material is available free of charge via the Internet at http://pubs.acs.org.

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