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Short communication

High-performance liquid chromatographic determination of carnosic acid and carnosol in *Rosmarinus officinalis* and *Salvia officinalis*

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Abstract

A reversed-phase high-performance liquid chromatographic method for the determination of carnosic acid and carnosol, phenolic diterpenes, which showed high antioxidative effect, is established. The analysis can be accomplished within 15 min under isocratic conditions with 0.1% phosphoric acid–60% acetonitrile as the mobile phase at a flow-rate of 1.0 ml/min, with detection at 230 nm. The detection limits of carnosic acid and carnosol are 0.104 and 0.521 ng per injection, respectively. This experimental system permits a good separation and quantification of these phenolic diterpenes in the leaves of *Rosmarinus officinalis* L. and *Salvia officinalis* L.

1. Introduction

Rosemary (*Rosmarinus officinalis* L.) and sage (*Salvia officinalis* L.) have been known to have the highest antioxidant activity among herbs [1]. Compounds responsible for these antioxidant properties isolated from rosemary were mainly the phenolic diterpenes named carnosic acid [2] and carnosol [3]. In the subsequent studies, several related phenolic diterpenes, rosmanol [4] and epirosmanol with γ-lactone were identified [5]. Carnosic acid was converted into carnosol by air-oxidation. Carnosic acid has been assumed to be the main substrate for general oxidation leading to phenolic diterpene artifacts with a δ- or γ-lactone function [6]. In practice, rosmanol, epirosmanol and 7-methylepirosmanol containing a γ-lactone function were formed from carnosol [7]. So carnosic acid functions as the main component for the antioxidative activity of these herbs. Recently, a reversed-phased high-performance liquid chromatographic (HPLC) method for the determination of several phenolic diterpenes in rosemary and sage leaves [8] was carried out by the gradient elution with electrochemical detection. In order to determine the contents of carnosic acid and carnosol in rosemary and sage, we developed a simple and rapid method with isocratic elution and UV detection. In the present study, we described a method for the HPLC analysis of carnosic acid and carnosol and we determined these phenolic diterpenes in the different parts of rosemary and sage plants. Furthermore we determined the stability of carnosic acid by means of this HPLC method.

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2. Experimental

2.1. Materials

Dried leaves of rosemary were purchased from Mikuni (Osaka, Japan). A voucher specimen of *R. officinalis* and *S. officinalis* was available for inspection at the Herbal Garden, Fukuyama University. Reagent-grade chemicals and high-purity solvents were used except when specified otherwise. Aqueous solutions were prepared with deionized-distilled water. Acetonitrile was of HPLC grade and other solvents and chemicals were purchased from Wako (Osaka, Japan).

Ethyl acetate extract from dried leaves of rosemary (3.3 kg) was partitioned between 1 *M* sodium hydroxide and diethyl ether, and the sodium hydroxide layer neutralized with 2 *M* hydrochloric acid was extracted with diethyl ether. The diethyl ether extract (95 g) was chromatographed on a silica gel column (300 mm × 200 mm I.D., 75–150 μm, Wako) using benzene–acetone (10:1) to give fractions A (46.9 g) and B (13.7 g). From fraction A carnosol (2.13 g) was crystallized with benzene. The mother liquid was applied to a polyamide column (74–149 μm, Wako) and eluted with methylene chloride and methylene chloride–methanol (4:1), successively. From the methylene chloride–methanol fraction carnosic acid (1.81 g) was obtained. The crystals (4.56 g) obtained from fraction B (13.7 g) by crystallization with benzene, were chromatographed on a silica gel column followed by a Sephadex LH-20 column (25–100 μm; Pharmacia, Uppsala, Sweden) to furnish rosmanol (54 mg). The mother liquid of fraction B (5.70 g) was chromatographed on silica gel (benzene–acetone, 10:1) and Sephadex LH-20 (methanol) columns to give epirosmanol (69 mg).

2.2. Apparatus

The HPLC system consisted of two Tosoh (Tokyo, Japan) CCPD pumps equipped with a Tosoh CCP controller connected to a dynamic mixer, a 5-μl sample loop, a Tosoh SD-8012 and a Tosoh UV-8020 UV–Vis detector set at 230 nm. The data were processed by means of a SIC Chromatocorder-12 integrator to evaluate the peak areas. The purity of the chromatographic peaks was estimated using a Waters (Milford, MA, USA) Model 990J photodiode array detector.

2.3. Chromatographic conditions

The stainless-steel column (150 × 4.6 mm I.D.) was packed with a Wako Wakosil-II 5C18 HG (5 μm) and used at 25 ± 0.5°C. The separation was isocratically undertaken with a solvent consisting of 0.1% (v/v) aqueous phosphoric acid–acetonitrile (40:60) at a flow-rate of 1 ml/min.

2.4. Preparation of samples of dried and fresh herbs

Sample (10–1200 mg) in acetone (3 ml) was homogenized with a Polytron while being cooled in ice and the mixture was centrifuged (1500 *g* for 10 min) at 4°C. The acetone supernatant was then transferred to a test-tube, the residue was sonicated in a sonic cleaning bath cooling in ice for 3 min with acetone (2 ml) and centrifuged at 4°C. The residue was further extracted in the same manner. The extracts were combined and centrifuged (1500 *g* for 10 min) at 4°C, and then a portion (more than 20 μl) was injected into the HPLC column.

2.5. Recovery test

The powdered dried leaves (30 mg) of rosemary containing carnosic acid (1.20 mg) and carnosol (0.10 mg) were homogenized with a Polytron while being cooled in ice with two different amounts of the standard acetone solution (2 ml) in which 0.23 and 0.03 mg/ml or 0.115 and 0.015 mg/ml of carnosic acid and carnosol, respectively were added. After centrifugation the supernatant was transferred, the residue was further extracted with the standard acetone solution according to the above described procedure.

3. Results and discussion

3.1. Standard materials

The following compounds (Fig. 1) were identified by the comparison of ^{1}H and ^{13}C NMR spectral and the literature data [7,9]: carnosic acid, carnosol, rosmanol and epirosmanol. The purity of standard materials calculated from the peak area in the proposed method was $\geq 98\%$.

3.2. HPLC

HPLC was carried out on a Wako Wakosil-II 5C18 HG reversed-phase column, which was selected because of its large number of theoretical plates, its specific characteristics towards α-diphenolic compounds and the avoidance of poor peak shapes of these phenolic diterpenes. In order to obtain a sharp peak of carnosic acid, we adopted an elution (60% acetonitrile) to which phosphoric acid (0.1%) was added. The retention times (and capacity factors, k') were 2.78 (rosmanol, $k' = 0.91$), 2.96 (epirosmanol, $k' = 1.03$), 6.44 (carnosol, $k' = 3.43$) and 11.28 min (carnosic acid, $k' = 6.75$). The minor phenolic diterpenes such as rosmanol and epirosmanol eluted to overlap with flavonoids. Carnosic acid and carnosol, the major constituents in rosemary, were shown to be satisfactorily separated under the isocratic conditions. These peaks were identified with the retention time and the UV spectrum obtained by photodiode array detection.

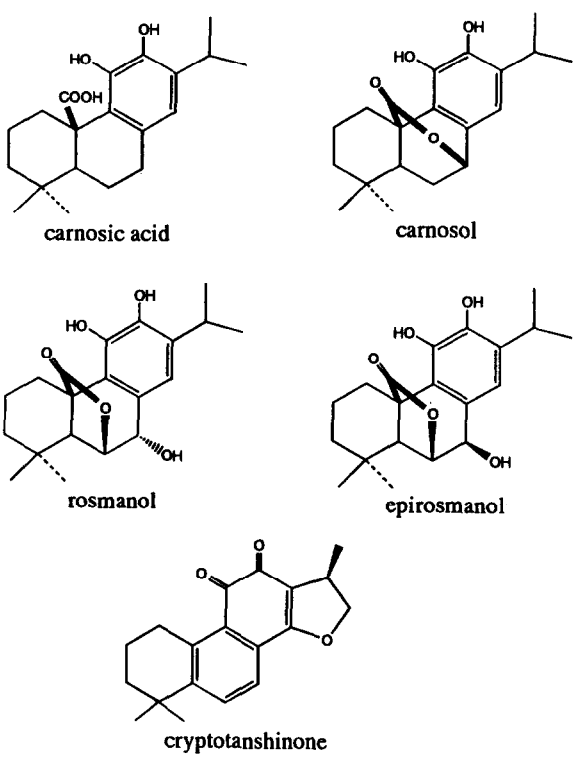

Fig. 1. Structures of phenolic diterpenes.

HPLC can be accomplished by applying acetone extract of rosemary and sage leaves without any prepurification. The determination of carnosic acid and carnosol can be achieved within 15 min without any clean-up of column. The detection limits (signal-to-noise ratio = 3) were 0.104 (carnosic acid) and 0.521 ng (carnosol) per injection. The calibration curves of carnosic acid and carnosol were linear over the ranges 0.07 μg/ml–2 mg/ml and 0.20 μg/ml–0.5 mg/ml, respectively, with correlation coefficients of 0.992–1.000. To examine the precision of this method, we injected the standard solutions of carnosic acid and carnosol at concentrations of 0.106 and 0.071 mg/ml, respectively. The relative standard deviations ($n = 10$) were 0.97 (carnosic acid) and 1.15% (carnosol). Recovery tests were performed by adding two different amounts of the standard acetone solution of carnosic acid and carnosol. The mixture was extracted and assayed according to the above procedure. The recovery of these compounds was $\geq 94.1\%$.

3.3. Stability of carnosic acid

Carnosic acid has been postulated to be the precursor of phenolic diterpene artifacts. The oxidation of carnosic acid to phenolic diterpene artifacts occurs in polar solvents, but non-polar solvents such as hexane, benzene and methylene chloride are not suited for direct injection into HPLC. As for the stability of carnosic acid in

many solvents, carnosic acid in acetone was relatively resistant to degradation at room temperature, and acetone was a suitable solvent for extraction as well as methanol or ethyl acetate.

The effect of temperature on the degradation of carnosic acid was examined in acetone solution. At −20°C in the dark, carnosic acid standard in acetone and that in rosemary acetone extract did not change at all, as shown in Figs. 2 and 3. However, when a solution of carnosic acid in acetone was left at room temperature (24°C) particularly under 12 h light condition, it decreased rapidly. The degradation of carnosic acid in the acetone extract was higher than that in acetone. Carnosic acid was transformed into carnosol followed by decomposition to form the γ-lactones, rosmanol and epirosmanol, directly [7]. Decomposition of carnosol was enhanced by light. These results indicate that raising temperature and light tend to promote the degradation of carnosic acid.

To examine whether carnosol is present in leaves of rosemary or not, fresh leaves (ca. 0.5 g) dipped in liquid nitrogen immediately after harvesting were transferred into acetone and were allowed to stand in the dark for 4 days at −20°C. Carnosol was found to be 1.7% of the content of carnosic acid. On the other hand, the ratio was

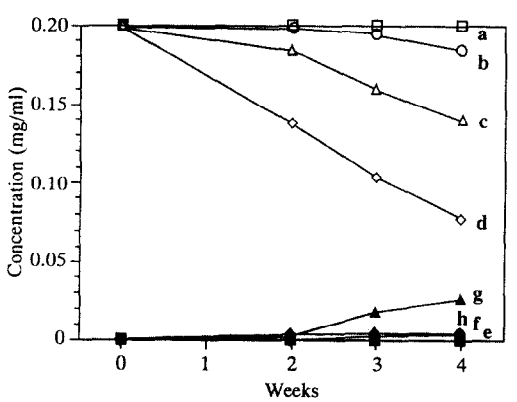

Fig. 2. Degradation of carnosic acid in acetone. Carnosic acid: a = at −20°C (dark); b = at 4°C (dark); c = at room temperature (dark); d = at room temperature (light). Carnosol: e = at −20°C (dark); f = at 4°C (dark); g = at room temperature (dark); h = at room temperature (light).

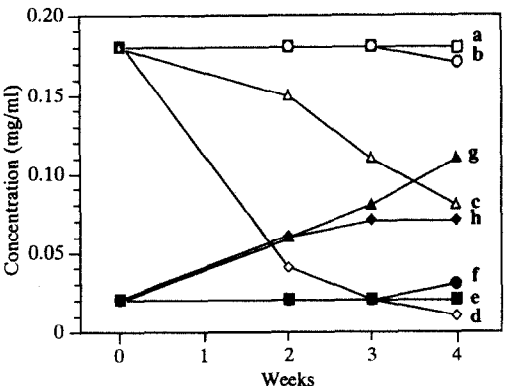

Fig. 3. Degradation of carnosic acid and carnosol in rosemary acetone solution. Carnosic acid: a = at −20°C (dark); b = at 4°C (dark); c = at room temperature (dark); d = at room temperature (light). Carnosol: e = at −20°C (dark); f = at 4°C (dark); g = at room temperature (dark); h = at room temperature (light).

5.9% in another procedure in which fresh leaves dipped in acetone immediately after harvesting were permitted to stand in the dark for 4 days at room temperature. According to the procedure described in the Experimental section, these ratios were approximately 10%. These results suggest that the content difference between carnosic acid and carnosol in these procedures comes from the conditions of the temperature

Table 1
Content of carnosic acid and carnosol in *Rosmarinus officinalis* and *Salvia officinalis*

Material	Content (mg/g)[a]	
	Carnosic acid	Carnosol
Rosmarinus officinalis		
Dried leaves	42.05 ± 0.19	3.87 ± 0.10
Fresh leaves	22.79 ± 0.40	2.38 ± 0.04
Fresh stems	0.13 ± 0.01	0.10 ± 0.01
Fresh roots	nd[b]	nd
Salvia officinalis		
Fresh leaves	12.40 ± 0.43	1.66 ± 0.21

[a] Results are means ± standard deviations from three independent experiments.
[b] Not detected.

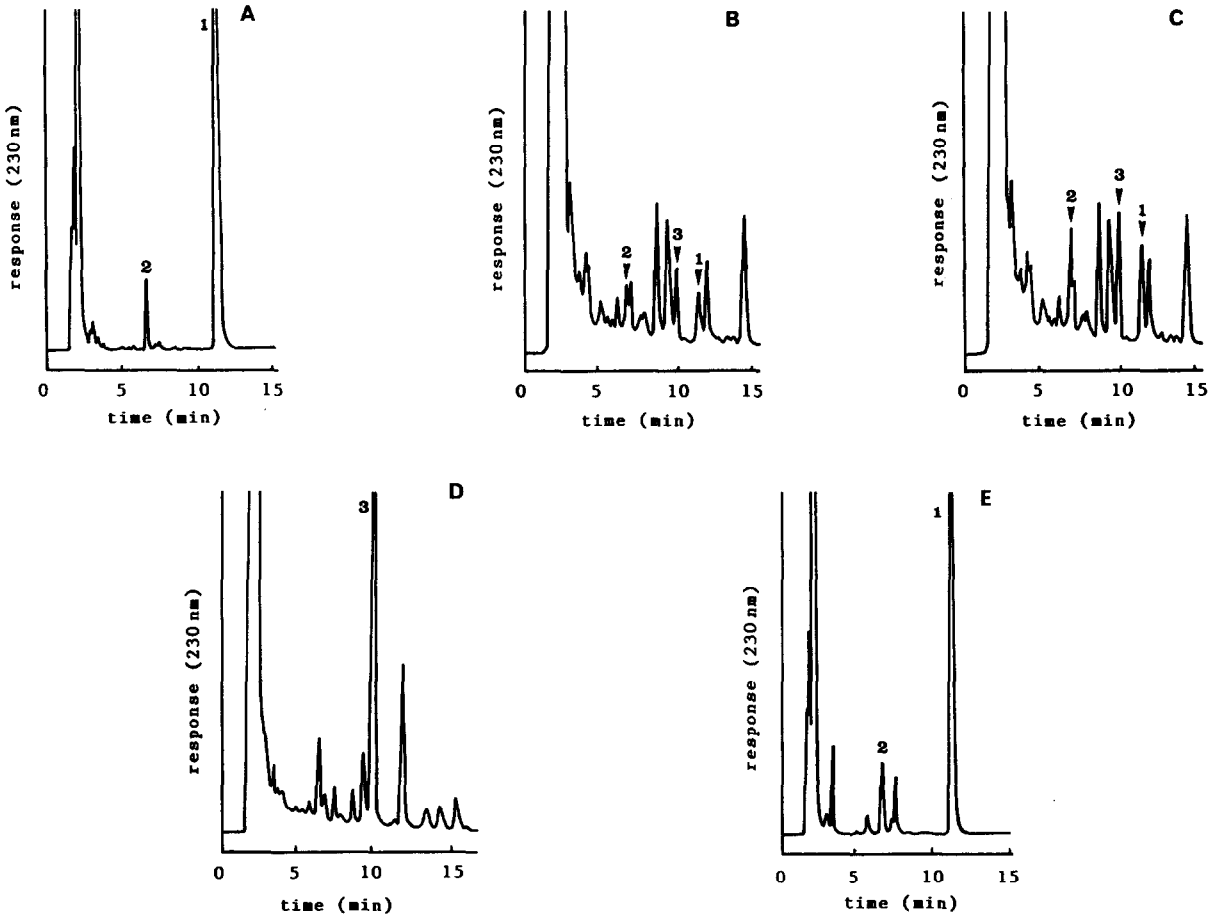

Fig. 4. Chromatograms of carnosic acid and carnosol in (A) fresh leaves, (B) fresh stems, (C) fresh stems spiked with carnosic acid, carnosol and cryptotanshinone, (D) fresh roots of rosemary and (E) fresh leaves of sage. Peaks: 1 = carnosic acid; 2 = carnosol; 3 = cryptotanshinone.

employed and air-oxidation. However, the question still remains whether carnosol is an artifact or not.

3.4. Determination of carnosic acid and carnosol

Carnosic acid and carnosol in dried and fresh leaves, fresh stems and roots of rosemary, and fresh leaves of sage were determined by the proposed method. The chromatograms are displayed in Fig. 4. The HPLC pattern of rosemary leaves was similar to that of sage, suggesting that these plants have a close botanical and biosynthetic relationship. As shown in Table 1, carnosic acid represents a main constituent (1.24–4.21%) of the leaves extract. Although carnosic acid and carnosol are not observed in the extract of roots, cryptotanshinone, an abietane-type diterpene, existed as a main constituent instead of these compounds. The roots of *Salvia miltiorrhiza* Bung are known to contain cryptotanshinone [10] which accounted for 0.36% of fresh weight as a main compound [11]. Further study is necessary to examine the bio-

synthetic relationship of carnosic acid with cryptotanshinone, an abietane-type diterpene.

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