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Degradation Study of Carnosic Acid, Carnosol, Rosmarinic Acid, and Rosemary Extract (*Rosmarinus officinalis* L.) Assessed Using HPLC

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Supporting Information

ABSTRACT: Rosemary, whose major caffeoyl-derived and diterpenoid ingredients are rosmarinic acid, carnosol, and carnosic acid, is an important source of natural antioxidants and is being recognized increasingly as a useful preservative, protectant, and even as a potential medicinal agent. Understanding the stability of these components and their mode of interaction in mixtures is important if they are to be utilized to greatest effect. A study of the degradation of rosmarinic acid, carnosol, carnosic acid, and a mixture of the three was conducted in ethanolic solutions at different temperatures and light exposure. As expected, degradation increased with temperature. Some unique degradation products were formed with exposure to light. Several degradation products were reported for the first time. The degradation products were identified by HPLC/MS/MS, UV, and NMR. The degradation of rosemary extract in fish oil also was investigated, and much slower rates of degradation were observed for carnosic acid. In the mixture of the three antioxidants, carnosic acid serves to maintain levels of carnosol, though it does so at least in part at the cost of its own degradation.

KEYWORDS: *carnosic acid, carnosol, rosmarinic acid, rosemary extract in fish oil, degradation study, HPLC analysis*

INTRODUCTION

With increasing awareness of the health benefits of certain foods and the mounting awareness of health benefits from nutraceutical products, attention has been focused on natural antioxidants as potential replacements for artificial ones.[1,2] Rosemary is a well-known perennial which has significant antioxidative activity.[3,4] Its extracts have been added to lipids or lipid containing foods, such as fish oils,[5,6] plant seed oils,[7−9] and meats,[10] to prevent oxidation and prolong their storage time. In addition, *in vivo* rosemary or its extracts have been observed to protect biological tissues from oxidative stress.[11,12] The antioxidative activity of rosemary appears to be due in significant part to its phenolic constituents.[13,14] Carnosic acid (Figure 1-**1**) and carnosol (Figure 1-**2**) are the primary rosemary-derived phenolic diterpenes with greatest antioxidant effect.[13] Other phenolic compounds, such as rosmarinic acid (Figure 1-**3**), rosmanol (Figure 1-**4**), and epirosmanol (Figure 1-**5**), also contribute to the antioxidative properties of rosemary extract, albeit to a lesser degree.[15] Besides acting as antioxidants in food, rosemary extract and its constituents have also displayed useful physiological and medicinal properties.[16−18] For example, rosemary extract was reported to show inhibitory effects for human immunodeficiency virus (HIV) infection at very low concentrations.[18] Carnosol was recently reported as a promising anticancer and anti-inflammatory agent.[17]

Quantitation of these phenolic compounds in rosemary extract is therefore of considerable interest. Two classical methods often have been used to determine the phenolic content of rosemary extracts. The Folin-Ciocalteu assay was used to measure the total amount of phenolic compounds.[19,20] In this method the substance being tested, for example, rosemary or its components, is used to titrate the Folin-Ciocalteu reagent, and the total content of phenolic compounds is determined by the amount of the substance needed to inhibit the oxidation of the reagent. However, the Folin-Ciocalteu reagent is nonspecific. It is able to react with other reducing components that are not phenolic compounds. Thus the total content of phenolic compounds measured by this method can be inaccurate and potentially overestimated. Furthermore, the compositions of specific phenolic components of the rosemary extracts are not identified. The other approach is a chromatographic method which separates the phenolic and diterpenoid compounds. HPLC is the most frequently utilized chromatographic method for quantitative analysis of rosemary extract.[21,22] However, most reported HPLC methods require either long run times, during which the inherently unstable phenolic constituents may degrade, or baseline separation of the relevant compounds is not obtained, which compromise quantitation of the phenolic compounds in the extract. A few supercritical fluid chromatographic (SFC) methods also have been reported for the separation of antioxidative compounds in rosemary extracts. Since SFC was generally less effective analytically, it has been used primarily as a preparative method for isolating the major functional ingredients in rosemary.[23,24] Capillary electrophoresis (CE) also was used for the analyses of rosemary extract.[25,26]

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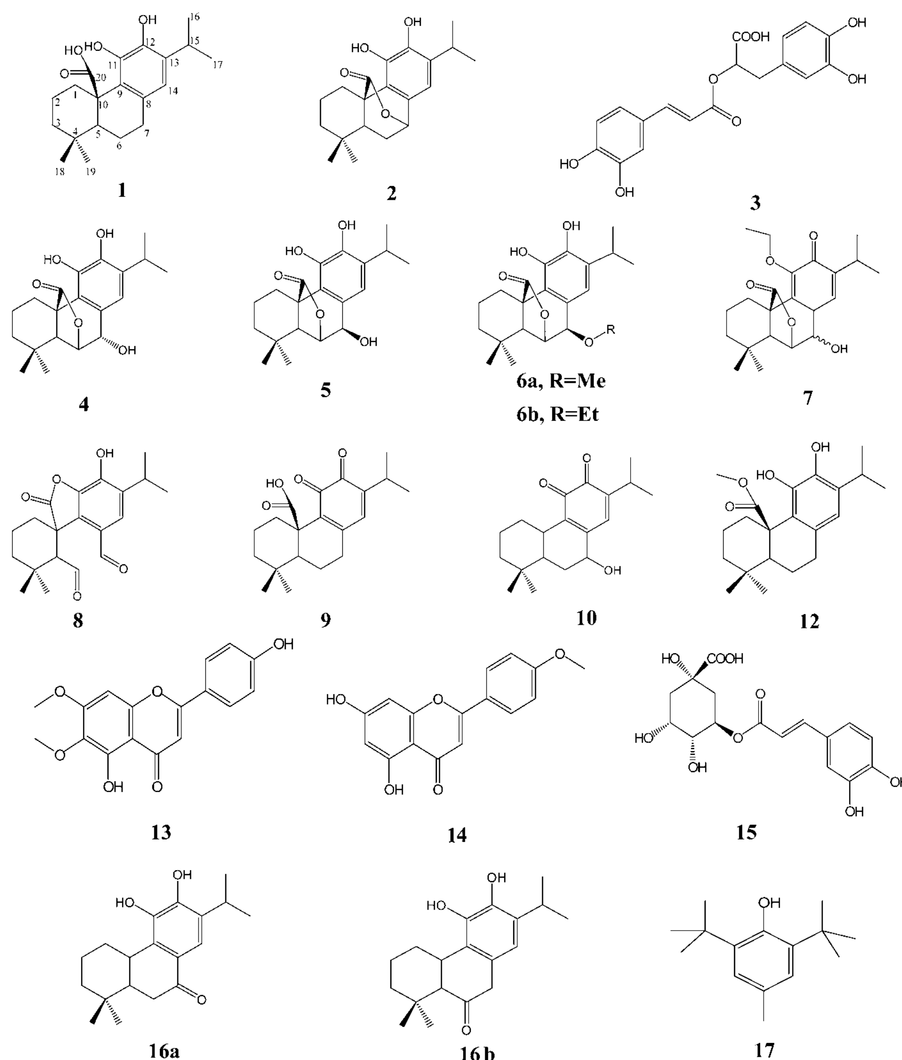


Figure 1. Structures. **1**, carnosic acid ($C_{20}H_{28}O_4$, MW 332.42); **2**, carnosol ($C_{20}H_{26}O_4$, MW 330.44); **3**, rosmarinic acid ($C_{18}H_{16}O_8$, MW 360.32); **4**, rosmanol ($C_{20}H_{26}O_5$, MW 346.42); **5**, epirosmanol ($C_{20}H_{26}O_5$, MW 346.42); **6a**, epirosmanol methyl ether ($C_{21}H_{28}O_5$, MW 360.45); **6b**, epirosmanol ethyl ether ($C_{22}H_{30}O_5$, MW 374.48); **7**, 11-ethoxyrosmanol semiquinone ($C_{22}H_{30}O_5$, MW 374.48); **8**, rosmadial ($C_{20}H_{24}O_5$, MW 344.41); **9**, carnosic acid quinone ($C_{20}H_{25}O_4$, MW 330.42); **10**, 5,6,7,10-tetrahydro-7-hydroxyrosmariquinone ($C_{19}H_{26}O_3$, MW 302.40); **11**, light induced degradation product of carnosic acid (structure unknown); **12**, methyl carnosate ($C_{21}H_{30}O_4$, MW 346.47); **13**, cirsimaritin ($C_{17}H_{14}O_6$, MW 314.29); **14**, acacetin ($C_{16}H_{12}O_5$, MW 284.27); **15**, chlorogenic acid ($C_{16}H_{18}O_9$, MW 354.31); **16a,b** ($C_{19}H_{26}O_3$, MW 302.41), structures for products of carnosic acid degradation reported in reference 44; **17**, butylated hydroxytoluene ($C_{15}H_{24}O$, MW 220.35).

However, the electropherograms often display noisy baselines which made accurate quantitation difficult.

In this investigation, we present a new HPLC method for the study of the degradation of carnosic acid, carnosol, and rosmarinic acid in ethanol solution as well as for rosemary extract in fish oil. The relationship between the degradation processes of carnosic acid and carnosol also was investigated. Finally, a new oxidative pathway of carnosic acid was proposed.

■ MATERIALS AND METHODS

Materials. Carnosol, carnosic acid, and rosmarinic acid, which are primary standards for analysis (purity $\geq$ 96%), were purchased from ChromaDex (Irvine, CA, USA). Purity designated by the manufacture's certificate was supported by the absence of detectable levels of any degradants. Rosmanol was purchased from Avachem (San Antonio, TX, USA). Formic acid was purchased from Sigma-Aldrich (Milwaukee, WI, USA). HPLC grade methanol (MeOH), ethanol (EtOH), and acetonitrile (ACN) were obtained from EMD (Gibbstown, NJ, USA). Water was purified by a Milli-Q water purification system (Millipore, Billerica, MA, USA). Highly refined omega-3 rich fish oil in which the acid functionality has been replaced by ethyl esters (DSM Nutritional Products, Heerlen, Netherlands) and the rosemary extract dissolved in fish oil (LycoRed, Orange, NJ, USA) were provided by Alcon Research, Ltd. (Fort Worth, TX, USA). The proportion of rosemary extract in fish oil was 5% (w/w).

HPLC Methods. In the HPLC method, Agilent 1200 series autosampler, pump, diode array detector (DAD), and a Cyclobond I 2000 RSP column (25 cm × 4.6 mm) were used.[27] For the degradation study of the carnosol, carnosic acid, and rosmarinic acid standards, a gradient of binary solvents was used for elution. Solvent A consisted of 70% H_2O, 30% ACN, and 0.1% formic acid. Solvent B consisted of 40% H_2O, 60% ACN, and 0.1% formic acid. At a flow rate of 1 mL/min, the eluent consisted of 100% A for the initial 4 min, and then from 4 to 17 min the composition was ramped gradually to 100% solvent B. From 17 to 20 min, the eluent composition was

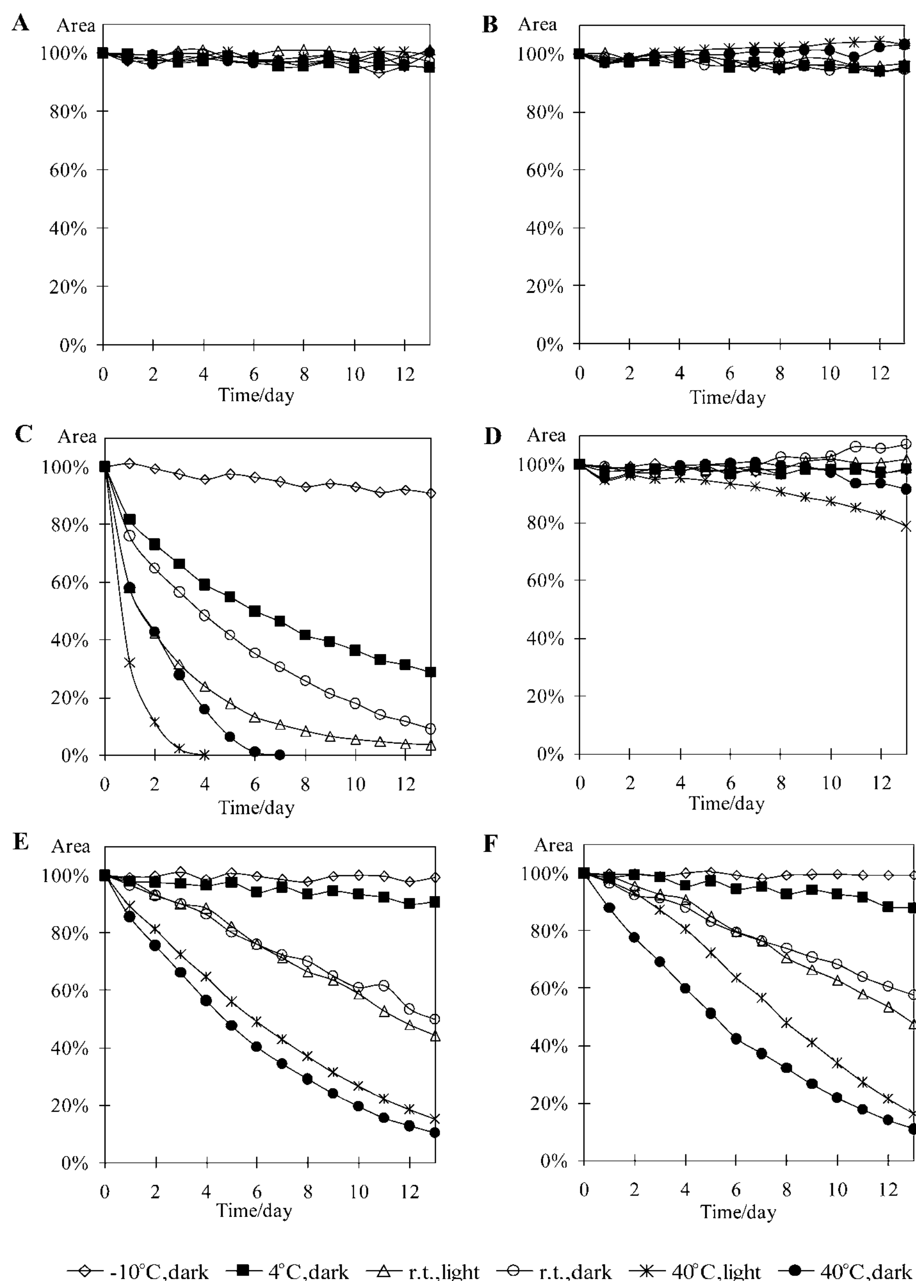


Figure 2. Degradation profiles of the ethanolic solutions of (A) rosmarinic acid by itself, (B) rosmarinic acid in the mixture, (C) carnosol by itself, (D) carnosol in the mixture, (E) carnosic acid by itself, and (F) carnosic acid in mixture under different storage conditions. See Materials and Methods for details.

returned to 100% solvent A. For the analysis of rosemary extract and the degradation study of the rosemary extract in fish oil, the procedure was modified to remove all the lipids remaining in the column after all the phenolic and diterpenoid compounds eluted by using 100% ACN as solvent C. Within the first 17 min, the procedure remained the same as described above. From 17 to 20 min, the eluent composition was ramped to 100% solvent C and then maintained at 100% solvent C from 20 to 22 min. From 22 to 25 min, the eluent composition was returned to 100% solvent A.

HPLC/MS/MS Method. In the HPLC/MS/MS method, Thermo Finnigan Surveyor autosampler, MS pump, PDA detector, and Thermo LXQ linear ion trap mass spectrometer were used. The HPLC gradients were the same as described previously. The electrospray ionization (ESI) mass spectra data were recorded in a negative ionization mode for the m/z range of 100−1000. Capillary voltage and spray voltage were set at −7 V and 4.7 kV, respectively. The normalized collision energy setting was 35 (arbitrary unit) while helium was used as the collision gas.

Calibrations. The stock solution of analyte was prepared by weighing the respective vials containing carnosol, carnosic acid, or rosmarinic acid, dissolving the contents in methanol, approximately 10 mg, and transferring them to a 10 mL volumetric flask, drying the vial in an oven at or above 110 °C and then reweighing the empty vial equilibrated to room temperature. Thus the transferred contents were accurately determined. The concentration of carnosol, carnosic acid, and rosmarinic acid in the stock solution was approximately 1000 mg/L for each of them.

Standard solutions of carnosol, carnosic acid, and rosmarinic acid were made up in the following approximate concentration ranges: 200, 400, 600, 800, and 1000 mg/L. The solvent system consisted of 150 mg/L butylated hydroxytoluene (BHT, Figure 1-**17**, European Union Code E321) in methanol. The addition of the antioxidant BHT, used only for the standard solutions for determination of the calibration curves, was purely precautionary to ensure the stability of carnosol, carnosic acid, and rosmarinic acid during the calibration study. The BHT eluted at 11.6 min and did not interfere with any of the compounds under investigation. All standards were stored at −10 °C in the dark. The linearity for the calibrations curves for carnosol, carnosic acid, and rosmarinic acid each had $R^2 \geq 0.999$, confirming the absence of any significant oxidation. The calibration curves, limit of detections (LOD), limit of quantitations (LOQ), intraday and interday precisions of the three standards are available in the Supporting Information. The DAD detector was set at four different wavelengths, 230, 254, 280, and 330 nm. At 280 nm, the chromatograms had the flattest baseline. Consequently, the peak areas measured at 280 nm were used to calculate the concentrations of the analytes. The UV spectra of carnosol, carnosic acid, and rosmarinic acid are provided in the Supporting Information.

Sample Preparation and Storage. Individual samples of carnosol, carnosic acid, and rosmarinic acid standards as well as a mixture of the three were prepared similarly to the stock solution in the calibration study except for a change in solvent to pure ethanol and exclusion of the BHT. The initial concentrations for each of these three antioxidants in either the 1-component or 3-component solutions were about 800−900 mg/L. These four standards were subjected to the following six conditions: (1) −10 °C in dark, (2) 4 °C in dark, (3) room temperature with light exposure, (4) room temperature in dark, (5) 40 °C with light exposure, (6) 40 °C in dark. The influence of temperature and light on degradation was observed for 13 days. The concentrations of the components in each sample were analyzed every 24 h.

The rosemary extract dissolved in fish oil was stored under five conditions: (1) 4 °C in dark; (2) room temperature in dark; (3) room temperature with light exposure; (4) 40 °C in dark; and (5) 40 °C with light exposure. This was monitored for 51 days. Prior to HPLC analysis, ∼100 mg of the fish oil sample was added to a 15 mL screw-cap centrifuge tube and dissolved in 5 mL of methanol.

The injection volume for each HPLC analysis was 5 μL.

RESULTS AND DISCUSSION

Analytical Method. Cyclodextrin-based columns are widely used in HPLC enantiomeric separations.[27] However, used in the reversed phase mode they are also known to separate structural isomers and other closely related compounds better than other conventional reversed phase columns.[28,29] This is due to their vastly greater shape selectivity for closely related compounds. In this study, a Cyclobond I 2000 RSP column provided better separations for the degradation products of carnosic acid and carnosol, and for other components in rosemary extract, many of which are structurally related.

Degradation of Carnosic Acid, Carnosol, Rosmarinic Acid, and Their Mixture in Ethanol Solutions. Understanding the stability of antioxidants is important for improving use and efficacy, and for establishing proper storage conditions to extend shelf life. Rosemary extract and its constituents are well-known to protect a variety of foods and food supplements from oxidative degradation.[4,13,30] However, the degradation of rosemary extract and specifically its phenolic and diterpenoid components were examined only in a few instances.[31−34] Schwarz and Ternes isolated carnosic acid and carnosol from rosemary extract and investigated their degradation in methanol over 9 days.[31] The same authors also reported the stability of the phenolic diterpenes from rosemary extract in lard under thermal stress of 170 °C.[32] Irmark et al. observed the stability of rosemary extract stored at 4 °C in the dark and at room temperature with light exposure for 14 weeks.[33] However, the effect of light and temperature on the degradation process could not be distinguished. Bano et al. investigated the oxidation of a single component, carnosic acid, in three different solvents under atmospheric conditions at 30 °C for 16 days.[34]

In the present study, the degradation of rosmarinic acid, carnosol, carnosic acid, and the mixture of these three antioxidants under ambient air exposure and in ethanolic solution was followed under a series of different storage temperatures, with or without light exposure, over a 13 day period.

Degradation Profiles of Three Primary Phenolic Antioxidants in Rosemary. Rosmarinic acid, either by itself in the ethanolic solution or presented in the ethanolic solution of the mixture, did not degrade appreciably under any of the conditions during the 13 day study. Figure 2A,B illustrated the degradation profiles of rosmarinic acid by itself and in the mixture respectively under different thermal and light exposure conditions.

On the other hand carnosol dissolved in ethanol degraded most rapidly of the three antioxidants. This was particularly noticeable at 40 °C with light exposure where the pure carnosol completely disappeared at day four (as shown in Figure 2C). The rate of degradation of carnosol increased according to the following sequence: −10 °C in dark < 4 °C in dark < room temperature in dark < room temperature with light exposure < 40 °C in dark < 40 °C with light exposure. Temperature was the major factor affecting the degradation of carnosol in solution, and light exposure further accelerated the degradation. In contrast to this, significantly slower apparent degradation was observed for carnosol in the solution of the mixture (see Figure 2D). This could be attributed to protection by the other antioxidants present in the mixture or by the compensatory conversion of carnosic acid to carnosol. (See discussion in the Degradation of the Mixture section below.)

Carnosic acid, by itself in ethanol solution and in the solution of the mixture, was fairly stable at −10 and 4 °C in dark (as shown in Figure 2E,F). At higher temperatures and under light exposure conditions it degraded, but not so rapidly as did carnosol by itself in ethanol solution. Carnosic acid exhibited similar degradation in the mixture solution and in its own solution. It was noticed that the degradation of the carnosic acid stored in dark was higher than that of the carnosic acid exposed to light at the same temperature, especially when stored at 40 °C. This was in contrast to the results observed for carnosol.

Identification of Degradation Products and Pathways of Carnosol and Carnosic Acid. Degradation Products of Carnosol. **Rosmanol, Epirosmanol and Epirosmanol Ethyl Ether.** Three major degradation products of carnosol were formed in the ethanol solutions and were labeled as compounds **4**, **5**, and **6b** in Figure 3A (structures shown in Figure 1). In

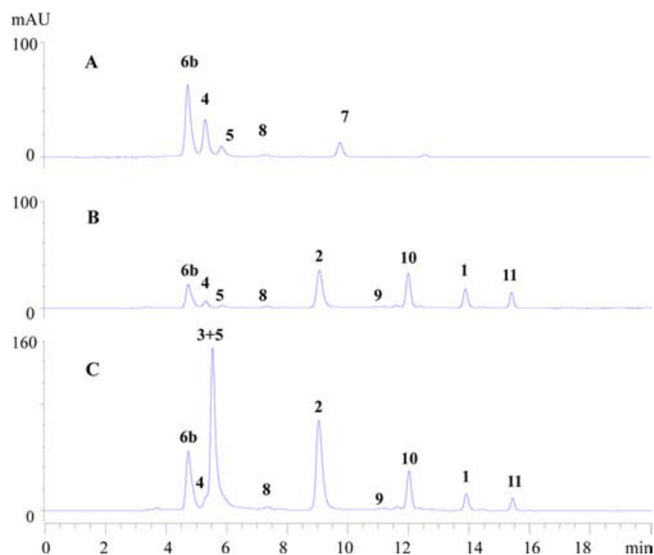


Figure 3. HPLC chromatograms of (A) carnosol, (B) carnosic acid, and (C) a mixture of carnosol, carnosic acid, and rosmarinic acid in ethanol solutions stored at 40 °C with light exposure after 13 days. **1**. carnosic acid, **2**. carnosol, **3**. rosmarinic acid **4**. rosmanol, **5**. epirosmanol, **6b**. epirosmanol ethyl ether, **7**. 11-ethoxy-rosmanol semiquinone, **8**. rosmadial, **9**. carnosic acid quinone **10**. 5,6,7,10-tetrahydro-7-hydroxy-rosmariquinone, **11**. the light induced degradation product.

negative mode of electrospray ionization (ESI), $[M - H]^-$ m/z values 345, 345, and 373 were observed for the compounds **4**, **5**, and **6b**, respectively. When collision-induced dissociation (CID) energy was applied, compounds **4** and **5** had the same fragmentation pattern which gave two fragments at m/z 301 and 283, while compound **6b** had two fragments at m/z 329 and 283. The λ_{max} values for compound **4**, **5**, and **6b** were 289, 288, and 289 nm, respectively. These data were in good agreement with the literature values of rosmanol, epirosmanol, and epirosmanol ethyl ether (Figure 1-**6b**).[35,36] The compound **4** was also confirmed by comparing it to a rosmanol standard. It had the same retention time, molecular mass, MS fragmentation, and UV spectrum as the rosmanol standard. In the tandem mass spectra, decarboxylation ($[M - H - COO]^-$) of rosmanol (**4**), epirosmanol (**5**), and epirosmanol ethyl ether (**6b**) resulted in m/z values at 301, 301, and 329, respectively. The m/z value at 283 was caused by the further loss of a H_2O from rosmanol and epirosmanol or an ethanol molecule from epirosmanol ethyl ether.

It was observed that epirosmanol ethyl ether was not formed when carnosol was dissolved in an aprotic solvent, such as acetonitrile. However, when other protic solvents, such as methanol or isopropanol, were used, the corresponding ether of epirosmanol was formed. Also, it was noticed that the formation of these three degradation products was affected by temperature. Rosmanol was most abundant among the three compounds when stored at −10 °C. However, the peak area of rosmanol became relatively smaller than that of the epirosmanol ethyl ether when the temperature increased (see SI-Figure 2 in Supporting Information). Apparently, high temperature is an important factor in the formation of epirosmanol ethyl ether, suggesting its energy barrier is higher than that for rosmanol, perhaps on the order of 1−2 kcal/mol.

11-Ethoxy-rosmanol Semiquinone. The degradation compound 7 corresponding to peak 7 in Figure 3A only appeared in the carnosol solution stored at 40 °C with light exposure and after carnosol completely degraded. As seen in Figure 4, its

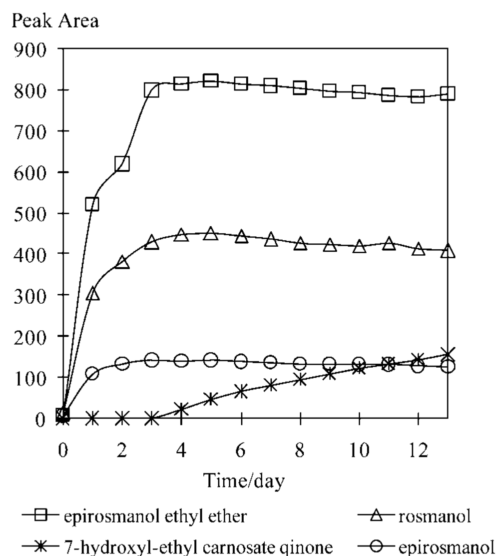


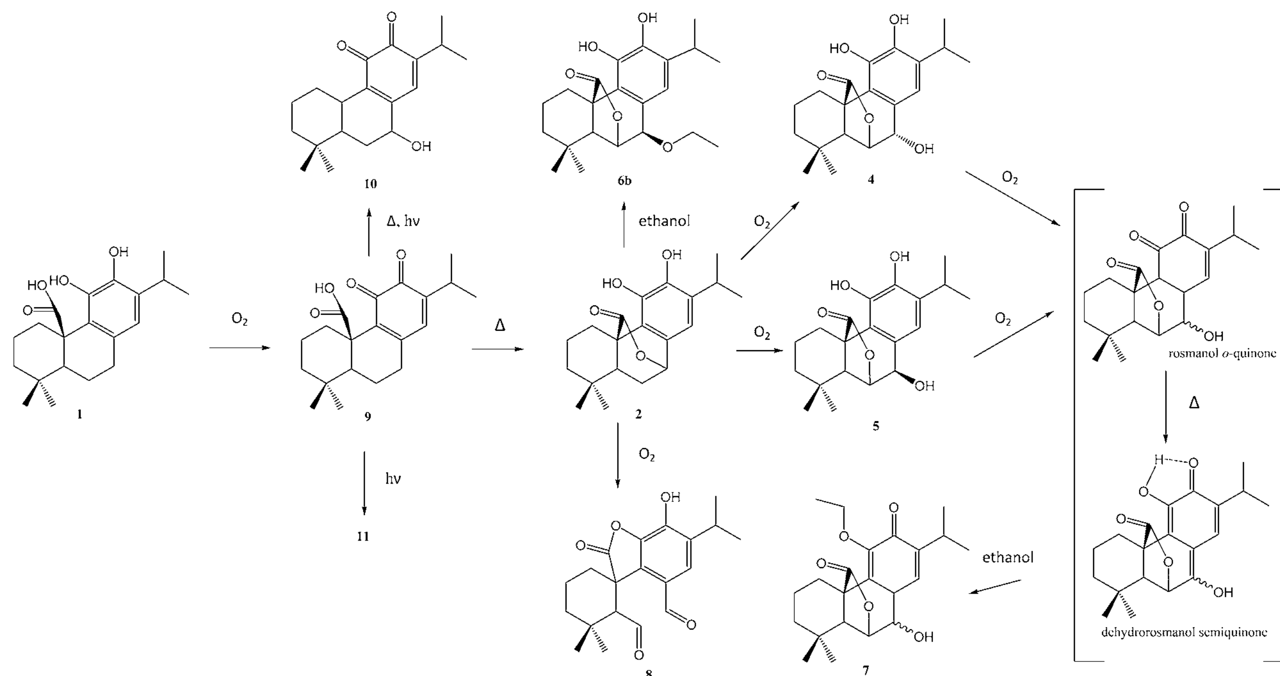
Figure 4. The peak areas of the degradation products of carnosol vs time in the carnosol solution stored at 40 °C with light exposure.

peak area increased with a slow decrease of the peak areas of epirosmanol ethyl ether, rosmanol, and epirosmanol. Thus, this degradation product was inferred to arise indirectly from carnosol via other carnosol degradation products. It was slightly ionized in ESI negative ion mode and the $[M - H]^-$ m/z value was 373. When CID energy was applied, no fragments were observed. Even though the molecular m/z value of compound 7 was the same as epirosmanol ethyl ether, the difference in the ability to ionize indicated a significant difference in their structure. It is very likely that no phenol group or carboxylic group existed in the structure of compound 7. In the UV spectrum of compound 7, a maximum absorption peak at 261 nm with a shoulder at 296 nm and a second maximum absorption peak at 322 nm with a shoulder at 335 nm also distinguished the functionalities of compound 7 from epirosmanol ethyl ether. It was reported that rosmanol and epirosmanol can be further oxidized to rosmanol *o*-quinone, which can be converted subsequently to another diterpene, galdosol, via a semiquinone intermediate when subject to heat.[37] Under the conditions investigated, this semiquinone intermediate appeared to react with ethanol and form an ethyl ether adduct instead of converting to galdosol. The structure as shown in Figure 1-7 was proposed for compound 7. The UV absorbance maximum was computed from functional group contributions and found to be consistent with the observed values.[38]

Rosmadial. The small peak that eluted at 7.34 min in chromatogram Figure 3A and was labeled as peak **8** had a molecular $[M - H]^-$ m/z at 343 and two product ions m/z at 315 and 299. The UV λ_{max} value was 286 nm. These data were in agreement with the literature values of rosmadial (Figure 1-**8**).[35,36] The m/z at 315 suggested a loss of CO and the m/z at 299 may be caused by the cleavage of CO_2 from the molecular ion.

Degradation Products of Carnosic Acid. **Carnosic Acid Quinone.** When carnosic acid was kept at −10 °C in dark for 13 days, carnosic acid quinone (Figure 1-**9**) was the only

Scheme 1. Proposed Degradation Pathway of Carnosic Acid, 1, in Ethanol Solution



degradation product observed (see SI-Figure 3 in Supporting Information). Under these conditions the degradation of carnosic acid was much slower as was the subsequent reaction of the intermediate quinone formed from it. Carnosic acid was completely converted to this compound after being stored at −10 °C in dark for 30 days. The completion of this conversion was confirmed by HPLC and NMR. The NMR spectrum of carnosic acid quinone is available in Supporting Information (SI-Figure 6). These NMR data are in agreement with the literature data for carnosic acid quinone.[39] The chemical shift of catechol hydroxyls in carnosic acid at 7.74 ppm disappeared while the shift of carboxylic group at 12.31 ppm was retained. Also, the mass spectrum showed molecular $[M - H]^-$ m/z at 329 and a fragment in the tandem mass spectrum at an m/z of 285, the result of a cleavage of a carboxylic group (CO_2) from the molecular ion. The compound had a UV maximum absorption at 428 nm which indicated the presence of an *o*-benzoquinone structure.[40]

The peak area of carnosic acid quinone increased slightly when the storage temperature increased from −10 °C to room temperature but decreased afterward (see SI-Figure 3 in Supporting Information). Carnosic acid quinone was postulated to be an intermediate in the oxidation pathway of carnosic acid to carnosol, rosmanol, etc.[34,37,41−43] It was confirmed by Masuda et al. that carnosic acid quinone can convert to carnosol, rosmanol, and 7-methylrosmanol in methanol solution when subject to 60 °C for 2 h.[43] Also, the low temperature needed for the conversion of carnosic acid to carnosic acid quinone indicated a low energy barrier for this conversion. Therefore, carnosic acid quinone was very likely to be the intermediate in the degradation pathway of carnosic acid, and its small peak area in Figure 3B at high temperatures is likely due to the conversion of carnosic acid quinone to other degradation products.

The essential role of this quinone as the primary initial degradant in the oxidation/light pathway analysis provided in Scheme 1 is also supported by its elimination when carnosic acid is stored under an inert atmosphere[31] and by the analysis of the degradation kinetics provided in the Supporting Information.

Carnosol and Its Degradation Products. At 4 °C, a degradation product was eluted at 8.98 min (labeled as peak **2** in Figure 3B). It was confirmed to be carnosol. The peak area of carnosol increased with increasing temperature (see SI-Figure 3 in Supporting Information). After carnosol was formed in the solution, degradation products **4**, **5**, and **6b** appeared in the solutions stored above 4 °C. These three degradation products were confirmed to be rosmanol, epirosmanol, and epirosmanol ethyl ether, respectively. This result indicated that these three compounds were generated from the carnosol that was produced via the degradation of carnosic acid. This agrees with the oxidation pathway of carnosic acid postulated by Wenkert et al.,[42] Bano et al.,[34] and Schwarz and Ternes.[31]

Peak **8** eluted at 7.34 min in Figure 3B had a molecular $[M - H]^-$ at m/z 343 and two product ions at m/z 315 and 299. The UV λ_{max} value of 286 nm was observed. These data are consistent with the assignment of this compound to be rosmadial,[35,36] as observed in the degradation of carnosol form which it was derived.

5,6,7,10-Tetrahydro-7-hydroxy-rosmariquinone. Another major degradation product derived from carnosic acid was eluted at 12.01 min and was labeled as peak **10** in Figure 3B. The formation of this degradation product was promoted by exposure to light. At the same storage temperature it had larger peak area in the solution exposed to light than in the one kept in the dark (as seen in SI-Figure 3 in Supporting Information).

Compound **10** was difficult to ionize, just barely ionizable in the negative mode of ESI. Therefore, the sample was concentrated by 10 times prior to LC-MS-MS analysis. A parent ion $[M - H]^-$ with m/z 301 was observed. The tandem mass spectrum gave fragments at m/z 283, 273, and 258, which suggested a loss of H_2O, CO, and an isopropyl group from the molecular ion, respectively. In the literature, two structures with m/z values of 301 were reported and are shown in Figure 1-**16a**

and **16b**.[44] However, the difficulty in ionization of compound **10** in ESI negative mode indicated the absence of phenol or carboxylic acid groups in the structure. The UV spectrum showed strong absorption at 279 and 409 nm. Thus, an *o*-benzoquinone structure was very likely to exist.[40] A structure for compound **10** was proposed as shown in Figure 1-**10**, and it was named as 5,6,7,10-tetrahydro-7-hydroxy-rosmariquinone. The common name of rosmariquinone for this base structure was proposed previously.[45]

Degradation Product Generated by Light Exposure. As shown in Figure 3B, a compound was eluted after the carnosic acid and was labeled as peak **11**. This compound only appeared in the solutions exposed to light and had strong UV absorption at 260 nm. Unfortunately, it could not be ionized in either negative or positive ion mode of ESI even at high concentrations and was labile when isolation from the solution was attempted. Insufficient amounts of this compound were collected from HPLC fractions for NMR analysis. Thus, no further information was obtained on the structure of this light induced degradation product, and it remains a topic for further investigation.

Minor Degradation Products. As seen in Figure 3B, there were three small peaks eluted at 11.52, 12.50, and 14.35 min, respectively. The first two peaks only appeared with light exposure. These compounds were not easily ionized, so given the small amounts present no further effort was made to identify their structures.

Degradation Pathway of Carnosic Acid. On the basis of the discussion above, a degradation pathway of carnosic acid was postulated and is shown in Scheme 1. Carnosic acid quinone was likely to be the intermediate in the pathway. It was confirmed that rosmanol, epirosmanol, and epirosmanol ethyl ether were generated from carnosol which is a degradation product of carnosic acid. Also, 5,6,7,10-tetrahydro-7-hydroxy-rosmariquinone and the light induced degradation product, compound **11**, were reported as degradation products of carnosic acid for the first time.

A more detailed, though incomplete, analysis of the kinetics of degradation for carnosic acid and carnosol is provided in the Supporting Information appended to this paper. There the kinetic reaction pathways are summarized and individual rate constants were derived from analysis of the stability of the initial components. The consistency of Scheme 1 is illustrated in Figure 5, where the data at 40 °C with light exposure for the gradual rate of appearance of carnosol form carnosic acid, while accommodating carnosol's degradation, are consistent with the concentrations predicted from the set of rate laws associated with the pathway.

Degradation of the Mixture. When rosmarinic acid, carnosol, and carnosic acid were dissolved in the same solution, similar degradation products were generated as in the individual standard solutions of carnosol and carnosic acid (as shown in Figure 3C). Rosmarinic acid was stable in both ethanol solutions (see Figure 2A,B). Consequently, it is not considered in the following discussion about the degradation behavior of the antioxidants presented in the mixture solution. The rosmanol and epirosmanol peaks were observed to overlap the rosmarinic acid peak in Figure 3C. Their appearance as well as that of epirosmanol ethyl ether indicated the degradation of carnosol. However, the concentrations of carnosol in all the chromatograms in SI-Figure 4 (available in Supporting Information) did not show significant change compared to their initial values.

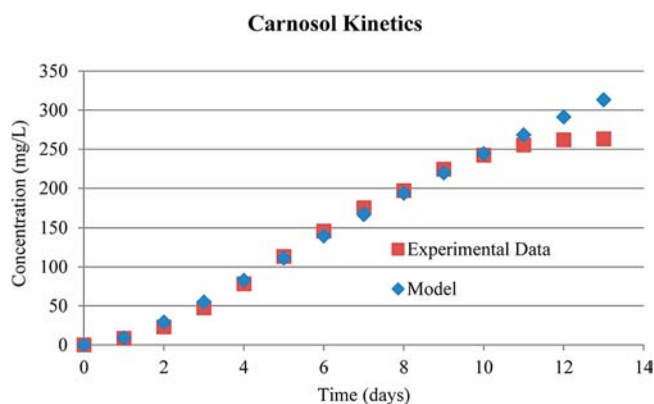


Figure 5. Kinetic profile for the carnosol concentration at 40 °C with light exposure for the degradation pathway represented in Scheme 1 (also see SI-Figures 10 and 20). These results indicate the profound consequences of including the intermediate, carnosic acid quinone, in the pathway. The effect of the degradation of carnosol as the sum of three primary pathways on the profile is slight since the total rate is small (SI-Figure 22). The values of the rate constants also indicate significant alteration in the carnosol degradation kinetics in the presence of carnosic acid. (See details in the Supporting Information.)

A study of the concentrations of carnosol and epirosmanol ethyl ether in three solutions: carnosol ethanol solution (solution 1), carnosic acid ethanol solution (solution 2), and the ethanol solution of the three mixed antioxidants rosmarinic acid, carnosol, and carnosic acid (solution 3) was carried out. Epirosmanol ethyl ether was used as an "indicator" for the rate of degradation of carnosol since it was the major degradation product of carnosol and the baseline resolution provided the most accurate quantitation. The purpose of this study was to determine if carnosic acid had the ability to "protect" carnosol, or whether the loss of carnosol was simply compensated for by that produced from carnosic acid. The results at the 13th day were used for this assignment.

The initial concentrations of carnosol, carnosic acid, and epirosmanol ethyl ether in the three solutions were shown as below:

$$C_{C,1,\text{initial}} = C_{C,3,\text{initial}} \quad (1)$$

$$C_{CA,2,\text{initial}} = C_{CA,3,\text{initial}} \quad (2)$$

$$C_{E,1,\text{initial}} = C_{E,2,\text{initial}} = C_{E,3,\text{initial}} = 0 \quad (3)$$

where $C_{C,1,\text{initial}}$ and $C_{C,3,\text{initial}}$ were the initial concentrations of carnosol in solution 1 and solution 3, respectively; $C_{CA,2,\text{initial}}$ and $C_{CA,3,\text{initial}}$ were the initial concentrations of carnosic acid in solution 2 and solution 3, respectively. $C_{E,1,\text{initial}}$, $C_{E,2,\text{initial}}$, $C_{E,3,\text{initial}}$ were the initial concentrations of epirosmanol ethyl ether in solution 1, 2 and 3, respectively.

The epirosmanol ethyl ether standard was not available and so its molar concentrations in the solutions were not calculable. Also the quantitative evaluation of this degradative pathway is unknown. However, the peak area of the epirosmanol ethyl ether in the chromatogram corresponds to its concentration since the injected volume for each HPLC analysis was the same. Thus the integrated peak area of epirosmanol ethyl ether instead of its concentration was used for this analysis/discussion. The peak area values are listed in Table 1.

If the degradation processes of carnosol and carnosic acid in the mix solution were independent of each other and occurred

Table 1. Peak Areas of Epirosmanol Ethyl Ether and Carnosol in Carnosol, Carnosic Acid, and Mixture Solutions

condition	compound	carnosol solution	carnosic acid solution	mixture solution
initial	epirosmanol ethyl ether	0	0	0
	carnosol	1562.9	0	1556.3
40 °C with light[a]	epirosmanol ethyl ether	790.7	290.3	722.1
	carnosol	0	495.0	1222.5
40 °C in dark[a]	epirosmanol ethyl ether	897.2	483.8	921.7
	carnosol	0	515.3	1423.0
room temperature with light[a]	epirosmanol ethyl ether	798.0	34.2	80.6
	carnosol	54.4	235.4	1586.2
room temperature in dark[a]	epirosmanol ethyl ether	852.0	54.2	136.1
	carnosol	140.7	316.4	1666.8
4 °C in dark[a]	epirosmanol ethyl ether	435.3	0	0
	carnosol	446.7	36.8	1532.6
−10 °C in dark[a]	epirosmanol ethyl ether	21.3	0	0
	carnosol	1418.3	0	1525.9

[a]After 13 days of storage under the conditions.

at the same rate as their degradation in the individual standard solutions, under each storage condition, then

$$P_{C,1,13th\text{-}day} + P_{C,2,13th\text{-}day} = P_{C,3,13th\text{-}day} \quad (3)$$

and

$$P_{E,1,13th\text{-}day} + P_{E,2,13th\text{-}day} = P_{E,3,13th\text{-}day} \quad (4)$$

where $P_{C,1,13th\text{-}day}$ was the peak area of remained carnosol in solution 1 after 13 days storage, $P_{C,2,13th\text{-}day}$ was the peak area of remaining carnosol (which was first generated from degradation of carnosic acid) in solution 2 after 13 days storage, $P_{C,3,13th\text{-}day}$ was the concentration of carnosol in solution 3 after 13 days storage; $P_{E,1,13th\text{-}day}$, $P_{E,2,13th\text{-}day}$, and $P_{E,3,13th\text{-}day}$ were the peak areas of epirosmanol ethyl ether in solution 1, solution 2, and solution 3 after 13 days of storage, respectively.

According to the peak area values listed in Table 1, the peak area of epirosmanol ethyl ether in solution 3 was much smaller than the sum of its peak areas in solutions 1 and 2 under the same storage conditions.

$$P_{E,1,13th\text{-}day} + P_{E,2,13th\text{-}day} > P_{E,3,13th\text{-}day} \quad (5)$$

This indicated the total amount of carnosol degraded in solution 1 and solution 2 was larger than the amount of carnosol being degraded in the mixture solution. The degradation of carnosol in this simple mixture, even in the absence of the multiple ingredients from the refined fish oil (below), was less than that found for carnosol alone in solution.

Interestingly, the peak area of carnosol in the mixture solution was significantly larger than the sum of that in found in solutions 1 and 2,

$$P_{C,1,13th\text{-}day} + P_{C,2,13th\text{-}day} < P_{C,3,13th\text{-}day} \quad (6)$$

At room temperature, the peak area of carnosol in the mixture solution was even larger than its initial value. Therefore, a portion of carnosol in the mixture solution was inferred to arise from the degradation of carnosic acid.

Thus the relatively small change in the carnosol concentration in the mixture solution with time was due in part to the protective behavior of carnosic acid toward carnosol and also to the conversion of carnosic acid to carnosol.

Degradation of Rosemary Extract in Fish Oil. Rosemary extracts containing its major antioxidants are often added to polyunsaturated fatty acids (PUFAs) to prevent oxidation during processing and rancidification during storage.[5,6] The focus of this portion of the study was to examine the stability of the two principal rosemary antioxidants, carnosic acid and carnosol, in fish oil. The specific value of the other extractables is yet to be determined.

The components in rosemary extract can be well separated by the HPLC method developed in this study (see the Materials and Methods for details). As shown in Figure 6,

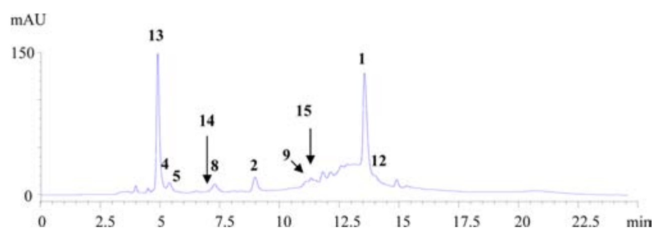


Figure 6. HPLC chromatogram of rosemary extract in fish oil diluted with methanol. The components are **1**. carnosic acid, **2**. carnosol, **4**. rosmanol, **5**. epirosmanol, **8**. rosmadial, **9**. carnosic acid quinone, **12**. methyl carnosate, **13**. cirsimaritin, **14**. acid. acacetin, **15**. chlorogenic acid.

besides carnosic acid and carnosol, cirsimaritin (Figure 1-**13**) was another major component in the rosemary extract. The minor components included acacetin (Figure 1-**14**), chlorogenic acid (Figure 1-**15**) as well as methyl carnosate (Figure 1-**12**), rosmanol, epirosmanol, rosmadial, and carnosic acid quinone, which may be derived from carnosic acid and carnosol. These compounds were confirmed by comparing the mass spectra and UV spectra (as shown in Table 2) with the values from the literature.[35,36] The structures of the three small peaks eluted between chlorogenic acid (Figure 1-**15**) and carnosic acid as well as the two small peaks eluted after methyl carnosate were not confirmed due to their low concentrations and difficulty in ionization.

When the sample of rosemary extract in fish oil was diluted with methanol and this solution was directly injected for HPLC analysis, a hump appeared at 11−15 min as shown in Figure 6. This hump was due to the UV absorbance of the fish oil matrix (see SI-Figure 5 in the Supporting Information). Consequently, the concentration of carnosic acid was quantitated by subtracting the raised baseline. The concentrations of carnosic acid and carnosol in the mixture of rosemary extract and fish oil were 4.2% and 0.60% (weight percent), respectively. Under all storage conditions, the carnosol in the processed fish oil was very stable. The fastest degradation of carnosic acid was observed at 40 °C with light exposure. After 51 days at this condition, the concentration of carnosic acid was 3.7% indicating less than 12% of carnosic acid degraded. Compared

Table 2. Mass Spectra and UV Spectra Data of Compounds

no.	compound name	t_R/min	$[M - H]^-$ m/z	major fragments (intensity) m/z	λ_{max}/nm (ε)
1	carnosic acid	13.75	331	287(100%), 244(2%)	284(1655)
2	carnosol	8.98	329	285(100%)	284(2445)
3	rosmarinic acid	5.50	359	315(15%), 223(12%), 197(27%), 179(19%), 161(100%)	330(17021)
4	rosmanol	5.36	345	301(4%), 283(100%)	288(2041)
5	epirosmanol	5.84	345	301(5%), 283(100%)	289
6	epirosmanol ethyl ether	4.70	373	329(40%), 283(100%)	289
7	11-ethoxy- rosmanol semiquinone	9.91	373	N/A	261, 296, 322, 335
8	rosmadial	7.34	343	315(34%), 299(100%)	286
9	carnosic acid quinone	11.15	329	285(100%)	428
10	5,6,7,10-tetrahydro-7-hydroxy-rosmariquinone	12.01	301	283(42%), 273(15%), 258(100%)	279, 409
11	N/A	15.46	N/A	N/A	260
12	methyl carnosate	13.97	345	301(100%), 286(6%)	289
13	cirsimaritin	4.92	313	298(100%), 283(5%)	333
14	acacetin	7.13	283	268(50%)	267, 334
15	chlorogenic acid	11.56	353	317(100%), 309(2%)	N/A

to the rate of degradation observed for carnosic acid in ethanol solution, the stability of carnosic acid in the rosemary extract was greatly enhanced when it was dissolved in the processed omega-3 rich fish oil.

ASSOCIATED CONTENT

Supporting Information

The information includes calibrations of carnosol, carnosic acid, and rosmarinic acid, HPLC chromatograms of degradation study, NMR spectrum of carnosic acid, the UV spectra of carnosol, carnosic acid, and rosmarinic acid, and the analysis of the kinetic study for degradation of carnosol and carnosic acid. This information is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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