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Products of the reaction between α- or γ-tocopherol and nitrogen oxides analyzed by high-performance liquid chromatography with UV-visible and atmospheric pressure chemical ionization mass spectrometric detection☆

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Abstract

The reaction products of α- or γ-tocopherol with nitric oxide in the presence of molecular oxygen were isolated and characterized. The consumption of tocopherols and the formation of the major products were monitored by high-performance liquid chromatography (HPLC) by a gradient elution method. The quantitative analysis of these compounds with UV-Vis detectors, however, was interfered by several minor products having similar UV spectra and retention times as those of the major ones. In order to establish a quantitative analytical method for the products, we investigated other detection methods, and found that atmospheric pressure chemical ionization (APCI), LC–MS was a more selective and better analytical method for these compounds.

Keywords: Tocopherols; Nitrogen oxides

1. Introduction

Vitamin E (tocopherol) derivatives are antioxidants that exist ubiquitously in biological systems. Some of them are incorporated in the biological membrane, and play a crucial role as scavengers of radicals, which are formed by chain reactions initiated by active oxygen species, resulting in lipid peroxidation [1–5]. There are four Vitamin E derivatives, which are named as α, β, γ, and δ-tocopherols, respectively. They are different in the numbers and positions of methyl group(s) on the chroman rings. Among them, α- and γ-tocopherol (α- and γ-Toc) are major antioxidants in vivo [6–11].

Nitric oxide (NO) is a molecule that has a variety of physiological activities, and is essential for organisms to be survived. An excessive amount of NO, however, is harmful and causes various types of organ damage because NO is a radical molecule [12–15]. Nitrogen dioxide (NO_2), which causes damage toward proteins or peptides, is also a radical molecule formed by the reaction of NO with molecular oxygen [6,16–18].

Therefore, it is important to study the interaction between NO or other nitrogen oxides and tocopherols, which are leading antioxidants in biological systems, and to clarify the defense mechanism against lipid peroxidation [19,20].

In the present study, α-Toc and/or γ-Toc were reacted with certain amounts of NO and O_2, and the reactivities of these two compounds were investigated. The consumption of the starting material and, the formation of products were monitored continuously by using high-performance liquid chromatography (HPLC) with a gradient elution method.

The main oxidation products from α-Toc were identified as, being α-tocopheryl quinone (α-TQ) [21], tocored (TR) [22–24], and 2,3-dihydro-7a-(3-hydroxy-3,7,11,15-tetramethyl-hexadeca-6,10,14-trienyl)-3,5,6,9,10,11a-hexamethyl-3-3-(4,8,12-trimethyl-3,7,11-tridecatrienyl)-1*H*-pyrano[2,3a]xanthene-8(7a*H*),11(11a*H*)-dione (dimer) [25–30]. The products from the reaction of γ-Toc were separated and characterized as TR and 5-nitro-γ-tocopherol (5-NGT) [6–8,23,24,31].

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Although these products had been characterized with two UV-Vis detectors connected in series and a multi-wave UV-Vis detector, the separations and quantification of the products were interfered in some cases due to the formation of several minor products that have similar UV spectra to those of the major ones. Thus, other detection methods were investigated, and it was found that the application of atmospheric pressure chemical ionization (APCI)–MS to the usual HPLC afforded a partial improvement to the problem [32]. Furthermore, the use of a shorter column (150 mm × 4.6 mm i.d.) and a change of the mobile phase from acetonitrile and diethyl ether to methanol and ethyl acetate highly improved the sensitivity, and a quantitative analytical method for these products was established. This paper describes these results.

2. Experimental

2.1. Chemicals

α- and γ-Toc were kindly supplied from Eisai (Tokyo, Japan). α-TQ was purchased from Wako (Osaka, Japan). The oxidation products of α- and γ-Toc (TR, dimer, 5-NGT) were isolated from the reaction mixtures, and purified by preparative TLC and preparative HPLC before being used as the standard samples. NO gas was purchased from Takachiho (Miyazaki, Japan). Acetonitrile and methanol were of HPLC grade, and diethyl ether was the first grade obtained from Wako. Anhydrous acetonitrile (99.8%) was purchased from Sigma Aldrich.

2.2. HPLC conditions

The HPLC system consisted of a Hitachi L-6200 intelligent pump, a Rheodyne 7125 injection valve, Hitachi L-4200, and L-7400 UV detectors (268 and 290 nm; because α-Toc, γ-Toc, and their oxidation products had various absorption maxima in the UV region, the two wavelengths were used for the simultaneous determination of those compounds) and a Hitachi L-4500 diode array detector (200–500 nm) connected in series, a Hitachi D-7000 chromato-integrator (Hitachi, Tokyo, Japan), Capcell Pak C_{18} UG120 S-5 (250 mm × 4.6 mm i.d., Shiseido, Tokyo, Japan) as an analytical column, and Capcell Pak C_{18} UG120 S-5 (10 mm × 4.0 mm i.d.) as a pre-column. The system was operated at room temperature. Acetonitrile–water (95:5) was used as mobile phase A, and mobile phase B was acetonitrile–diethyl ether (2:3). The gradient ran from 0% B up to 31% B over the first 25 min; from 31% B up to 100% B over 25 to 42 min; and 100% B isocratic over 42 to 50 min. Thereafter, the gradient was returned to the mobile phase A to prepare for the next run. A 12.5 μl aliquot of the extract of the reaction solution in acetonitrile–diethyl ether (2:3) was injected. The flow rate was 1.0 ml/min.

The analytical column used for LC–APCI–MS was Capcell Pak C_{18} UG120 S-5 (150 mm × 4.6 mm i.d., Shiseido) with a pre-column, Capcell Pak C_{18} UG120 (10 mm × 4.0 mm i.d.). The stepwise elution was carried out as follows: 0–7 min methanol, 7.1–20 min methanol–ethyl acetate (4:1). Thereafter, the stepwise elution was returned to methanol of the mobile phase to prepare for the next run. A 10.0 μl aliquot of the extract of the reaction solution in methanol–ethyl acetate (4:1) was injected. The flow rate was set at 1.0 ml/min.

2.3. LC–MS conditions

The assay was carried out using the Model M-1200 LC–MS system (Hitachi). The detection of analytes was attained by negative-ion with an APCI interface using the selected-ion monitoring (SIM) technique. The absorbance was monitored at 268 nm. The drift voltage and needle electrode voltage were 45 and 3000 V, respectively. The nebulizer and desolvation temperature were set at 200 and 400 °C, respectively.

2.4. Sample preparation

α-Toc or γ-Toc was dissolved in anhydrous acetonitrile at a concentration of 2×10^{-3} M. The solution was placed in a three-necked flask equipped with a septum rubber, two-way stopcock, one way of which was attached to an Ar balloon. The flask was degassed and filled with Ar by bubbling with dry Ar for 30 min, then sealed. NO gas was passed through a column of soda lime and 10 M NaOH solution. Then, the required amount of NO (1 equivalent, 448 μl) was measured using a Hamilton gas-tight syringe, and added to the reaction vassel. After 30 min, 1 equivalent of O_2 was added to the mixture using the same syringe. At intervals, 0.2 ml samples of the reaction mixture were withdrawn using a Hamilton gas-tight syringe, purged with Ar gas to remove the solvent and nitrogen oxides in samples, and stored at −30 °C under Ar gas. The residue of the samples, which included the oxidation products obtained from the reaction, were dissolved in eluent B [acetonitrile–diethyl ether (2:3) or methanol–ethyl acetate (4:1)] and analyzed by the HPLC and LC–MS.

3. Result and discussion

3.1. Oxidation of tocopherols

Fig. 1A and B show HPLC chromatograms of a mixture of 1 mM α-Toc and 1 mM γ-Toc in anhydrous acetonitrile solvent. The peaks with retention times of 21.9, and 24.4 min (290 nm) correspond to α-Toc(2), and γ-Toc(1), respectively.

Since there are reports which claim that α- and γ-Toc interact with each other under the oxidative conditions, reactions using both α- and γ-Toc as substrates in the presence of NO and O_2 were performed to investigate the interaction

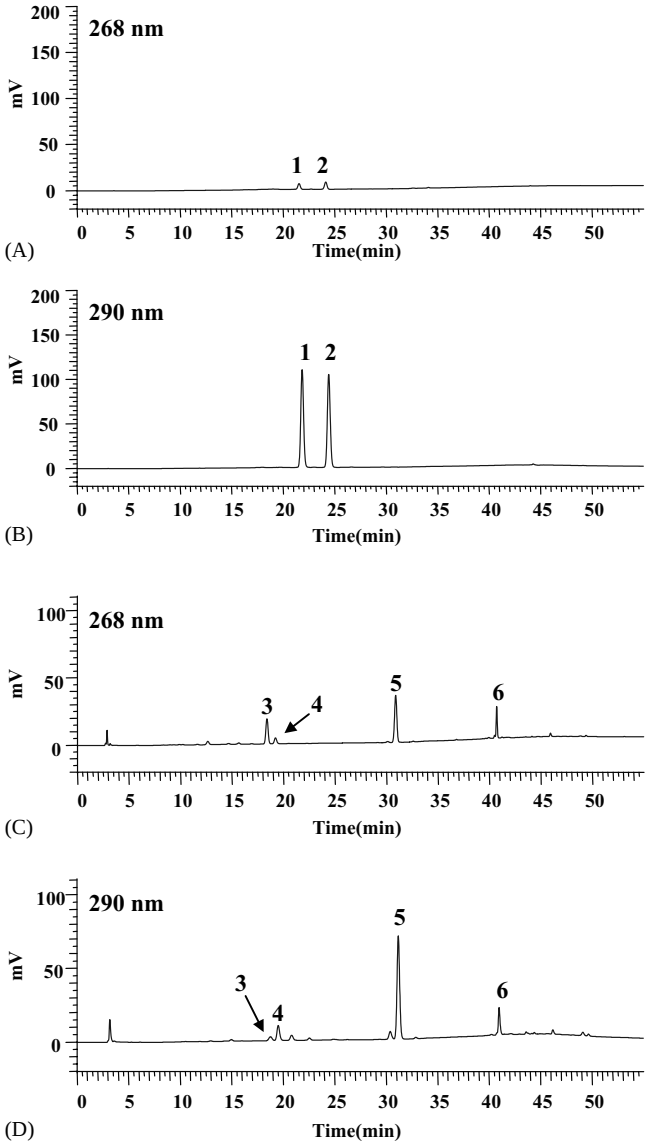

Fig. 1. (A and B) Chromatograms of a mixture of 1 mM α-Toc and 1 mM γ-Toc in anhydrous acetonitrile solvent. (C, D) Chromatograms of the reaction solution after a 2 h reaction of a mixture of 1 mM α-Toc and 1 mM γ-Toc with 2 mM NO in the presence of 2 mM O_2 in anhydrous acetonitrile. The reaction products were monitored at 268 nm (A, C) and 290 nm (B, D). Column length, 250 mm; eluent, from acetonitrile–water (95:5) to acetonitrile–diethyl ether (2:3), linear gradient (1 = γ-Toc, 2 = α-Toc, 3 = α-TQ, 4 = TR, 5 = 5-NGT, 6 = dimer).

of two tocopherols in our reaction system. Fig. 1C and D show HPLC chromatograms of the reaction solutions after reacting for 2 h a mixture of 1 mM α-Toc and 1 mM γ-Toc with 2 mM NO in the presence of 2 mM O_2 in anhydrous acetonitrile.

The peaks with retention times of 18.4, 19.2, 30.9, and 40.7 min at 268 nm (19.0, 19.5, 31.2, and 41.0 min at 290 nm) correspond to α-TQ, TR, 5-NGT, and dimer, respectively. Three major products, namely α-TQ [21], TR [6,22], and dimer [25–30], were identified in the reaction of α-tocopherol by HPLC chromatograms of the reaction solutions under similar reaction conditions, In the case of γ-Toc, TR, and 5-nitro-γ-tocopherol (5-NGT) [5,6,22] were obtained. These structures were determined by comparing the various spectral data with those reported in previous papers.

When two tocopherols coexisted, the reaction rate was changed compared to those of each Toc as a substrate. However, it was difficult to identify and quantify the products, since the peaks of the minor products overlap with those of the major products. Therefore, it was necessary to change the detection method, and it was found that LC–APCI–MS was an appropriate one.

3.2. Optimization of the analysis conditions for LC–APCI–MS

Various oxidation products were detected and characterized with LC–APCI–MS under the same conditions as those of UV detection. By using a high concentration of diethyl ether in the eluent and/or a change of the eluent composition according to the gradient method, however, the noises of the chromatograms were enhanced and detection of the dimer was interfered. In order to detect a lipophilic substance, such as the dimer, more efficiently, a column length of 250 mm (Capcell Pak C_{18} UG120 S-5) was converted into 150 mm. In addition, ethanol and ethyl acetate were used instead of acetonitrile–diethyl ether for the eluate in order to increase the ionization efficiency of the samples analyzed with LC–APCI–MS.

Fig. 2A and B show HPLC chromatograms of a mixture of 1 mM α-Toc and 1 mM γ-Toc in anhydrous acetonitrile. The peaks with retention times of 5.4, and 6.1 min (290 nm) correspond to α-Toc(2), and γ-Toc(1), respectively.

Fig. 2C and D show HPLC chromatograms of the reaction mixtures after a 2 h reaction of 1 mM α-Toc and 1 mM γ-Toc with 2 mM NO in the presence of 2 mM O_2 in anhydrous acetonitrile. The peaks with retention times of 4.3, 9.2, and 17.2 min at 268 nm, correspond to α-TQ, 5-NGT, and dimer, at 290 nm, the peaks with retention times of 4.7, 9.5, and 17.5 min correspond to TR, 5-NGT, and dimer, respectively. Although the analysis time was shortened compared to the experiments shown in Fig. 1, it was impossible to separate the peaks of α-TQ and TR.

3.3. Calibration curves and precision

3.3.1. APCI–MS detection [column length, 150 mm; A, methanol; B, methanol–ethyl acetate (4:1)]

The peak areas were calculated under negative conditions on selected-ion chromatograms of γ-Toc, α-Toc, α-TQ, TR, and 5-NGT at *m/z* 415, 429, 446, 430, and 460, respectively. The linear relationships between the amounts of the compound and the peak areas in the APCI–MS chromatogram were obtained between 0.024 and 1.2 mmol/l (γ-Toc), 0.02 and 1.2 mmol/l (α-Toc), 0.002 and 1.12 mmol/l (α-TQ), and 0.022 and 1.09 mmol/l (5-NGT), respectively, these results were reproducible (five assays) in all cases. Linear

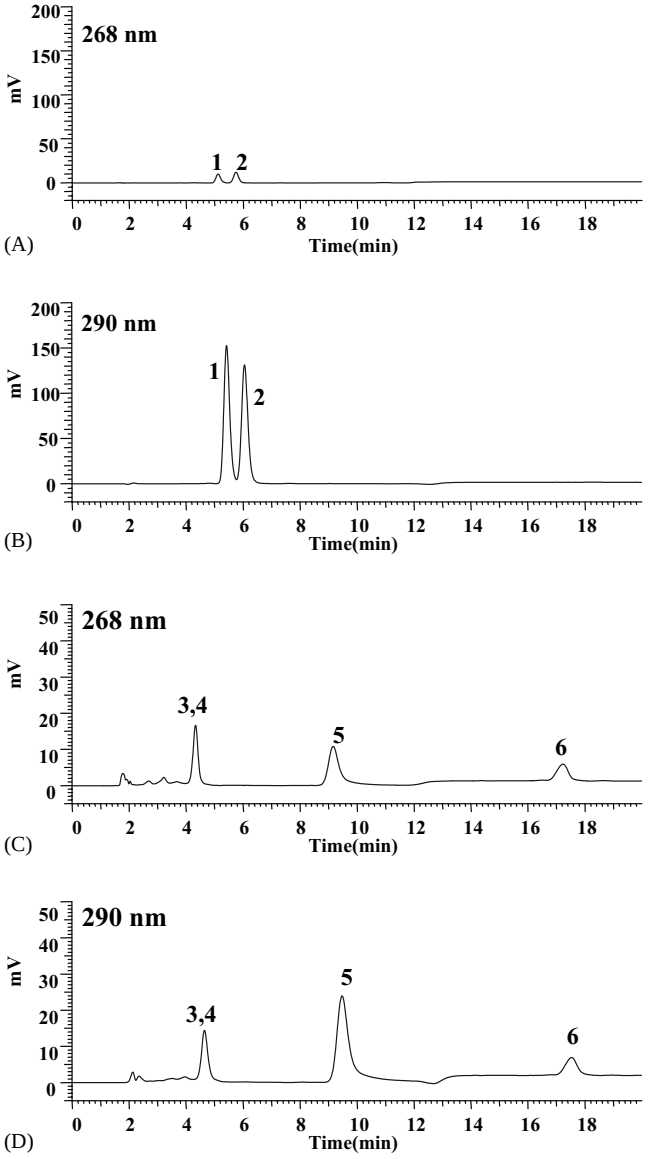

Fig. 2. (A, B) Chromatograms of a mixture of 1 mM α-Toc and 1 mM γ-Toc in anhydrous acetonitrile solvent. (C, D) Chromatograms of the reaction solution after a 2 h reaction of a mixture of 1 mM α-Toc and 1 mM γ-Toc with 2 mM NO in the presence of 2 mM O_2 in anhydrous acetonitrile. The reaction products were monitored at 268 nm (A, C) and 290 nm (B, D). Column length, 150 mm; eluent, 0–7 min methanol, 7.1–20 min methanol–ethyl acetate (4:1) stepwise elution (1 = γ Toc, 2 = α-Toc, 3 = α-TQ, 4 = TR, 5 = 5-NGT, 6 = dimer).

relationships were obtained from calculations of the peak area (R) and the concentration (x, mol/l) of each product (γ-Toc, α-Toc, α-TQ, 5-NGT). The results are as follows:

γ-Toc; $R = 1419904.2x + 105571$,
α-Toc; $R = 1831097.8x + 117868$,
α-TQ; $R = 1367114.0x + 80739$,
5-NGT; $R = 1984361.5x - 91497$,

The R.S.D. values of the retention times were <1%, and those of the peak areas on the total-ion chromatograms (TICs) were <5%.

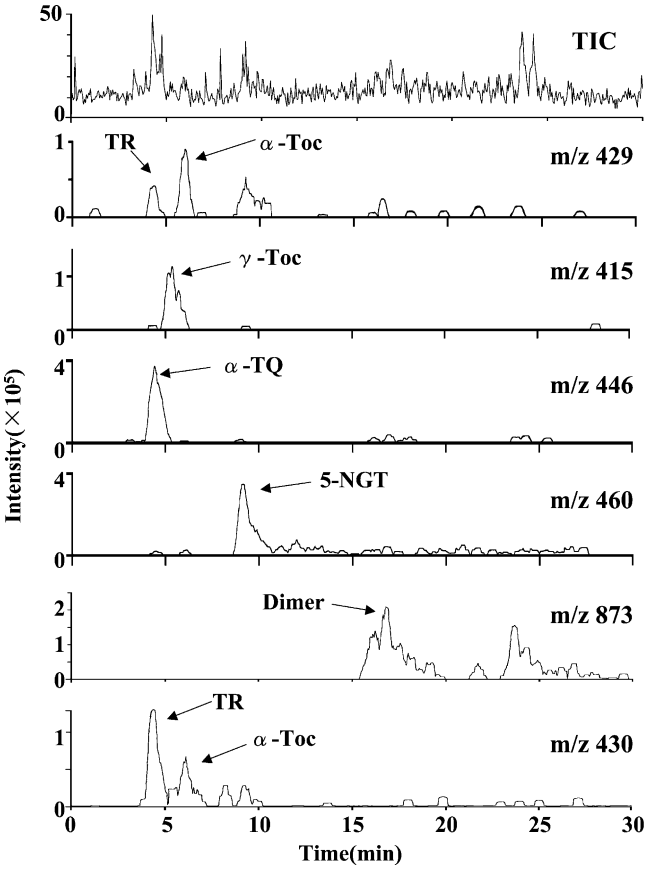

Fig. 3. Total ion chromatogram (TIC) and mass chromatograms of the reaction solution after a 2 h reaction of a mixture of 1 mM α-Toc and 1 mM γ-Toc with 2 mM NO in the presence of 2 mM O_2 in anhydrous acetonitrile under negative-ion conditions. The chromatographic conditoins are described in Section 2.

3.4. Identification and quantification with LC–APCI–MS

Fig. 3 shows the TIC and mass chromatograms under the negative-ion conditions using LC–APCI–MS, and of the oxidation products of α- and γ-Toc in 2 h reaction of α- and γ-Toc with NO in the presence of O_2 (using the same sample as in Fig. 2). The peaks of α-Toc(2), γ-Toc(1), α-TQ(3), 5-NGT(5), dimer(6), and TR(4) were detected on the mass chromatograms of m/z 429, 415, 446, 460, 873, and 430, respectively. By using APCI–MS, the recovery of α-Toc became measurable along with the complete separation of α-TQ(3) and TR(4), which could not be accomplished by a UV detector. Moreover, the sensitivity became 50 times better than those of UV detection. The molecular mass of α-Toc(2) differs from that of TR by only 1 U; thus, two peaks of α-Toc(2) and TR(4) were detected at m/z 429 and 430. The intensity of the molecular ion $[M - H]^-$ of α-Toc(2) was higher than that of the isotope ion of TR(4) at m/z 429. On the contrary, the molecular ion $[M - H]^-$ of TR(4) was higher than the isotope ion of α-Toc(2) at m/z 430.

The retention times of the peaks were supposed to be the same as those in UV detection, this assumption was

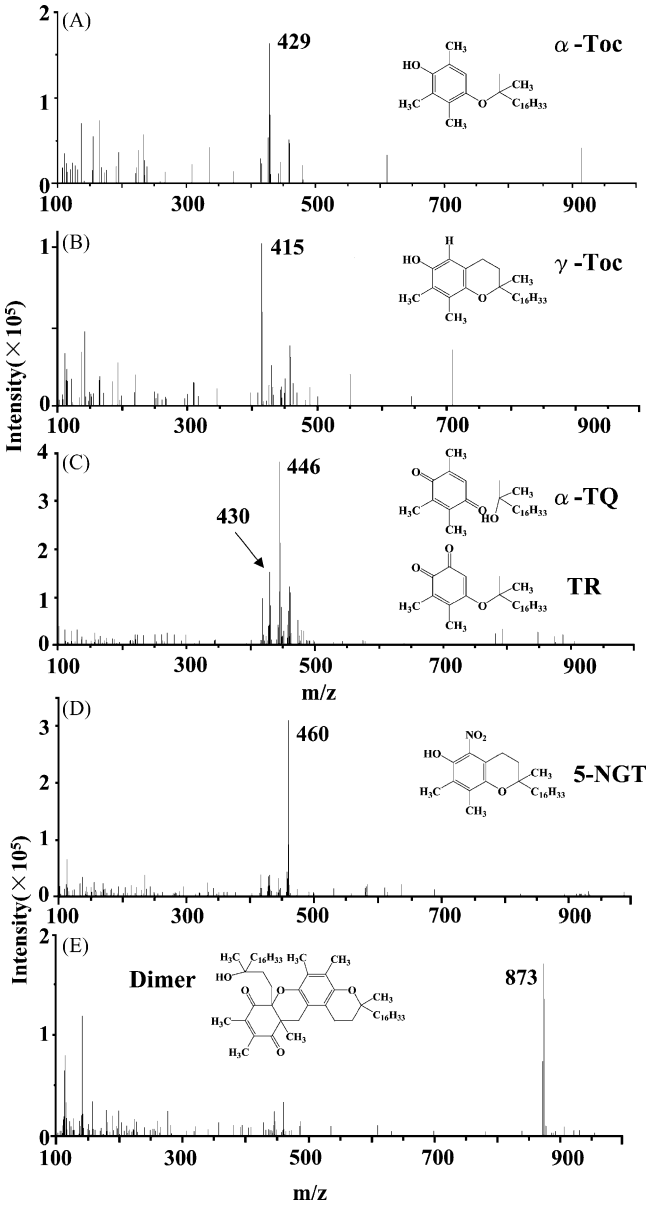

Fig. 4. Mass spectra (APCI) of the products obtained after a 2 h reaction of a mixture of 1 mM α-Toc and 1 mM γ-Toc with 2 mM NO in the presence of 2 mM O_2 in anhydrous acetonitrile: (A) α-Toc; (B) γ-Toc; (C) α-TQ and TR; (D) 5-NGT; (E) dimer.

confirmed by the data given below. Fig. 4 shows that the peak at a retention time of 6.1 min afforded the base peak of *m/z* 429, which corresponded to α-Toc (molecular ion peak *m/z* 429). The peaks at retention times of 5.4, 4.4, 9.2, 16.8 and 4.3 min afforded *m/z* 415, 446, 460, 873, and 430, which suggested that they were γ-Toc, α-TQ, 5-NGT, dimer, and TR, respectively. Fig. 4 shows the mass spectra of peaks (1–6) in the mass chromatogram (Fig. 3). The molecular ions $[M-H]^-$ of α-Toc(2), γ-Toc(1), 5-NGT(5), and dimer(6) were observed at *m/z* 429, 415, 460, and 873, respectively, as base peaks. Although there was no separation between the peak of α-TQ(3) and that of TR(4), it became possible to identify these compounds because of the appearances of molecular ions $[M-H]^-$ of *m/z* 446 and 430 in the mass spectrum (Fig. 4C). The concentrations of α-Toc(2), γ-Toc(1), α-TQ(3), and 5-NGT(5) in the case of Fig. 3 were calculated as 0.02, 0.06, 0.14, and 0.20 mM, respectively, using the calibrations shown in Section 3.3.

4. Conclusion

In this study, the major oxidation products of α- and γ-Toc were identified using the LC–APCI–MS system. By this method, it became possible to measure the mass spectra of these compounds in the presence of other minor compounds, which showed similar UV spectra to those of the major ones. In addition, the sensitivity became much better than those of UV detection. Therefore, an analysis of the reaction mixtures by LC–MS enabled us to characterize various major products even in the presence of many analogous minor ones. This is the first example where the LC–APCI–MS system was applied to analyze Toc reaction. Further, since the oxidation of Toc derivatives always gives many products, our method should be useful for the identification and quantification of the reaction of these compounds.

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