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Chemical evidence for the synergistic effect of a cysteinyl thiol on the antioxidant activity of caffeic and dihydrocaffeic esters

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ABSTRACT

Antioxidant activity of methyl caffeate and methyl dihydrocaffeate in the presence of a cysteinyl thiol was measured in an azo-initiator-induced lipid oxidation system. The coexistence of the thiol was observed to display a synergistic effect on the antioxidant activity of both caffeates. The synergism was observed mainly with respect to the elongation of the induction period, rather than the inhibition rate for lipid oxidation. For methyl caffeate, the maximum elongation of the induction period was observed in the presence of more than two equivalents of the thiol, whereas the maximum effect on the activity of methyl dihydrocaffeate was observed in the presence of more than three equivalents of the thiol. These synergistic effects were analysed by high-performance liquid chromatography and liquid chromatography–mass spectrometry analyses of the intermediates produced during the antioxidation period. The analytical results clarified that the mono-thiol adduct of methyl caffeate and the mono- and di-thiol adducts of methyl dihydrocaffeate contributed to the synergism in the antioxidant activity of both caffeates.

1. Introduction

Polyphenols have various useful functions, with respect to not only foods but also human health (Sies, 2010). With an increased knowledge of the functions of polyphenols, various industries have incorporated these compounds into healthy foods, cosmetics and medicines. Although polyphenols have widespread applications, the most important function of polyphenols is still their potent antioxidant activity. It is well known that most of the antioxidant actions of phenolic compounds are based on radical termination reactions. The antioxidation reaction converts polyphenols to various oxidation products *via* polyphenolic radical species (Frankel, 2005). Hence, the antioxidation process should be chemically influenced by surrounding reactive molecules.

Foods are complex systems involving many types of biomolecules. Some food constituents exhibit potential reactivity towards polyphenol radicals and oxidation products of polyphenols. A number of potent antioxidative polyphenols have a catechol moiety in their structures. During the oxidation process, the catechol is converted to *ortho*-quinone *via* a semiquinone radical. Although the quinone does not exhibit any antioxidant activity, it has potential electrophilic properties and can react with surrounding nucleophilic molecules. When addition of the nucleophilic molecules occurs, the catechol structure is regenerated to exhibit antioxidant activity. The semiquinone radicals of polyphenols are also capable of reacting with other radical species, including hydrogen atoms, to regenerate the catechol structure (Masuda et al., 2008).

Niki and co-workers (1984) reported that vitamin E was regenerated with water-soluble vitamin C. Mukai and co-workers (1992) also studied the kinetics of the regeneration of vitamin E by various biological hydroquinones. This regeneration of antioxidative vitamin E is recognised as one of the antioxidant synergisms (Barclay, Locke, & MacNeil, 1983; Mahoney, 1969). Carnosic acid, a potent antioxidative diterpene widely distributed in Labiatae herbs, afforded a non-active quinone as the antioxidation product; however, it can regenerate a catechol structure by the intramolecular addition of its carboxylic acid to recover potent activity (Masuda et al., 2002). Carnosol quinone, an antioxidation product of carnosol, also regenerated a catechol moiety *via* the addition of water (Masuda, Kirikihira, & Takeda, 2005).

Typical nucleophilic molecules in food constituents are amino- or thiol-bearing compounds, such as amino acids, peptides and proteins. Saito and Kawabata (2004) reported that the radical-scavenging ability of protocatechuic acid increased with the addition of a thiol molecule. Rohn, Rawel, and Kroll (2004) demonstrated the antioxidant activity of a coupling product of quercetin, quinone and the amino groups of proteins. If thiol or amino compounds could regenerate the catechol moiety *in situ* during the oxidation reaction of polyphenols, these nucleophilic molecules should exhibit a potent synergistic effect on the antioxidant activity of polyphenols.

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Previously, we reported the reaction of a cysteinyl thiol with various polyphenols under 2,2-diphenyl-1-picrylhydrazyl (DPPH)-radical oxidation conditions (**Fujimoto & Masuda, 2012a**). The coupling reactions of polyphenols with the thiol are caused by its high nucleophilicity or radical-forming ability; therefore, some polyphenols give di- and tri-thiol adducts in addition to the initially formed mono-thiol adduct (**Awad et al., 2002**). These results prompted us to investigate the synergistic effect of the thiol on the antioxidant activity of polyphenols. For this purpose, we have focused on the esters of caffeic acid and dihydrocaffeic acid, because they are potent antioxidative polyphenols and are structurally similar to the B and C rings of a flavonoid.

In our previous investigation (**Fujimoto & Masuda, 2012a**), both caffeates afforded various thiol adducts with a recovered catechol structure. **Bassil, Makris, and Kefalas (2005)** reported that a cysteine adduct of caffeic acid possessed a stronger radical-scavenging activity but a weaker ferrous-reducing activity in comparison to caffeic acid. In this paper, we report detailed results for the investigation of the synergistic effect of a cysteinyl thiol on the antioxidant activity of both caffeates in a lipid oxidation system.

2. Materials and methods

2.1. Chemicals and instruments

Methyl caffeate (**1**) and methyl dihydrocaffeate (**2**) (each purity > 95%) were synthesised from the corresponding carboxylic acids with methanol in the presence of a catalytic amount of sulphuric acid. Mono-thiol of methyl caffeate (**6**), mono-, di-, and tri-thiol adducts of methyl dihydrocaffeate (**10**, **12**, **13**, respectively), *N*-benzoylcysteine methyl ester (**3**), and *N,N′*-dibenzoylcystine dimethyl ester (**4**) were prepared by a previously reported method (**Fujimoto & Masuda, 2012a**). 2,2′-Azobis(2,4-dimethylvaleronitrile) (AMVN) was obtained from Wako Pure Chemicals (Tokyo, Japan). Ethyl linoleate was obtained from Kanto Chemicals (Tokyo, Japan) and used after purification by silica gel 60 (Merck, Darmstadt, Germany). All solvents (extra pure grade or HPLC grade) were obtained from Nacalai Tesque (Kyoto, Japan). An LC-20AD low-pressure gradient system (Shimadzu, Kyoto, Japan) equipped with an SPD-M20A photodiode array detector and a DGU-20A3 degasser was employed for analytical HPLC. HPLC data were analysed using LC solution software (ver. 6.10, Shimadzu). A PU-9800 pump equipped with a UV-975 detector (JASCO, Tokyo, Japan) was used for the quantitative analysis of ethyl linoleate hydroperoxides. A XEVO QTOF-MS (Waters Japan, Tokyo, Japan) was used for LC–MS analysis. The sample was injected into the MS instrument through an Acuity UPLC system (Waters) and mass spectra of the observed peaks were obtained under the following conditions; mode, ESI negative; capillary voltage, 2.4 kV; cone voltage, 40 V; source temperature, 150 °C; desolvation temperature, 500 °C; cone gas flow rate, 50 L h^{-1}; desolvation gas flow rate, 1000 L h^{-1}, MS^E low collision energy, 6 V; MS^E high collision energy, from 20 to 30 V. The elemental composition of each peak compound was calculated from the high-resolution MS data of the protonated or ion-adducted molecular ion using MassLynx software (V. 4.1, Waters).

2.2. Measurement of the antioxidant activity of methyl caffeate and methyl dihydrocaffeate in the presence of a thiol derivative, N-benzoylcysteine methyl ester

To ethyl linoleate (70 μL) in a straight vial (75 mm in height, 40 mm in diameter), 200 μL of methyl caffeate or methyl dihydrocaffeate in CH_3CN (25 mM), appropriate amount of *N*-benzoylcysteine methyl ester in CH_3CN, and 500 μL of AMVN in CH_3CN (0.6 M) were added. Each solution was made up to 4 mL with the addition of CH_3CN and then incubated at 37 °C with shaking (100 times min^{-1}) in a water-bath shaker. The control experiment vial was prepared in a similar manner without the addition of methyl caffeate or *N*-benzoylcysteine methyl ester. A portion (20 μL) of the solution was taken from each vial at 1-h intervals and diluted with CH_3CN (380 μL). The diluted solution (10 μL) was injected into the HPLC to analyse ethyl linoleate hydroperoxides under the following conditions; column, YMC-Pak ODS-A (150 × 4.6 mm i.d., YMC, Tokyo, Japan); solvent, CH_3CN/H_2O = 9:1 (v/v); flow rate, 1.0 mL min^{-1}, detection, 234 nm. The antioxidant activity of appropriate amounts of the *N*-benzoylcysteine methyl ester and the activities of methyl caffeate and methyl dihydrocaffeate were also measured under the same conditions. Lipid oxidation was represented as the concentration of ethyl linoleate hydroperoxides, which was obtained using the following equation of the calibration curve of pure ethyl linoleate hydroperoxides; $y = 436{,}497x + 191$ [*y*, concentration of the hydroperoxides (mM); *x*, peak area of the hydroperoxides at 234 nm]. All data were expressed as the mean values obtained from two independent experiments.

2.3. HPLC analysis of the antioxidation products from methyl caffeate and methyl dihydrocaffeate in the presence of the thiol

An aliquot (5 μL) was taken from each antioxidant reaction vial, prepared in the above experiment, at equal intervals and injected into the HPLC column to analyse the reaction products from methyl caffeate or methyl dihydrocaffeate under the following conditions; column, Cosmosil $5C_{18}$-AR-II (250 × 4.6 mm i.d., Nacalai Tesque); solvents, A = 1% acetic acid in H_2O, B = CH_3CN; gradient conditions, linear gradient from 5% of solvent B to 100% of solvent B for 40 min; flow rate, 1.0 mL min^{-1}; detection, 245 and 280 nm. The concentrations of methyl caffeate, methyl dihydrocaffeate and their thiol adducts, which were obtained using each pure sample, were respectively calculated using the following equations: $y = 459{,}000x$ [*y*, peak area of methyl caffeate at 280 nm; *x*, methyl caffeate concentration (mM)], $y = 526{,}000x - 18{,}000$ [*y*, peak area of the mono-thiol adduct at 245 nm; *x*, mono-thiol adduct concentration (mM)], $y = 154{,}000x$ [*y*, peak area of methyl dihydrocaffeate at 280 nm; *x*, methyl dihydrocaffeate concentration (mM)], $y = 428{,}000x - 8500$ [*y*, peak area of the mono-thiol adduct at 245 nm; *x*, mono-thiol adduct concentration (mM)], $y = 721{,}000x - 45{,}000$ [*y*, peak area of the di-thiol adduct at 245 nm; *x*, di-thiol adduct concentration (mM)], $y = 889{,}000x - 51{,}000$ [*y*, peak area of the tri-thiol adduct at 245 nm; *x*, tri-thiol adduct concentration (mM)], $y = 778{,}000x$ [*y*, peak area of *N*-benzoylcysteine methyl ester at 245 nm; *x*, *N*-benzoylcysteine methyl ester concentration (mM)]. All data were expressed as the mean values obtained from two independent experiments.

2.4. Comparison of the reactivity of caffeates and their thiol adducts toward a peroxyl radical

For the comparison of the reactivity between methyl caffeate and its mono-thiol adduct, a mixture of a methyl caffeate solution (5 mM in CH_3CN, 300 μL) and the mono-thiol adduct solution (5 mM in CH_3CN, 300 μL) was prepared and then an AMVN solution (0.6 M in CH_3CN, 300 μL) was added to the solution. The solution was stirred well and then incubated at 37 °C with shaking (100 times min^{-1}) in a water-bath shaker. An aliquot (5 μL) of the solution was injected into the HPLC column to analyse the decrease in the peak area of methyl caffeate and the mono-thiol adduct at 1-h intervals under the following conditions: column, Cosmosil $5C_{18}$-AR-II (250 × 4.6 mm i.d., Nacalai Tesque); solvents, A = 1% acetic

acid in H_2O, B = CH_3CN; gradient conditions, linear gradient from 5% of solvent B to 100% of solvent B over 40 min; flow rate, 1.0 mL min^{-1}; detection, 280 nm.

For a comparison of the reactivity between methyl dihydrocaffeate, its mono-, di and tri-thiol adducts, a reaction solution was prepared by mixing the mono-thiol adduct (4.8 mM in CH_3CN, 250 μL), di-thiol adduct (4.8 mM in CH_3CN, 250 μL) and tri-thiol adduct (4.8 mM in CH_3CN, 250 μL) with methyl dihydrocaffeate (4.8 mM in CH_3CN, 250 μL). To the solution, AMVN (0.6 M in CH_3CN, 300 μL) and CH_3CN (2.7 mL) were added. An aliquot (5 μL) of the solution was injected into the HPLC column to analyse the peak area of methyl caffeate and the thiol adducts at 1-h intervals under the same conditions as described above. All data were expressed as the mean values obtained from two independent experiments.

3. Results and discussion

3.1. Antioxidant activity of methyl caffeate and methyl dihydrocaffeate in the presence of the thiol

Caffeic acid and dihydrocaffeic acid are potent radical-scavenging antioxidants (**Moon & Terao, 1998**). We employed their methyl esters to avoid the influence of their acidic functional group and also because most phenolic acids including caffeic acid exist as esters in edible plants (**Chen & Ho, 1997**; **Shahidi & Naczk, 2004**). An antioxidant assay of these methyl esters (**1** and **2**) in the presence of an equimolar amount of a peptidyl cysteine model, *N*-benzoylcysteine methyl ester (thiol in this paper, **3**), was carried out in an AMVN-induced ethyl linoleate oxidation system (**Fig. 1**). The antioxidant activity was evaluated by the inhibition of the accumulation of ethyl linoleate hydroperoxides, as measured by HPLC analysis of the hydroperoxides. Fig. 2 (panel A) shows the time course for the analytical results for hydroperoxide accumulation in the presence of methyl caffeate and *N*-benzoylcysteine methyl ester. In the same panel of Fig. 2, the results of methyl caffeate, *N*-benzoylcysteine methyl ester and the control experiment (where both methyl caffeate and *N*-benzoylcysteine methyl ester are absent) are also shown. It should be noted that the thiol exhibits very weak antioxidant activity and also shows no obvious induction period in the system employed. Although some researchers have reported that cysteine is the most potent antioxidant among the protein amino acids (**Aliaga & Lissi, 2000**; **Elias, McClements, & Decker, 2005**; **Sagrista, Garcia, Madariaga, & Mora, 2002**; **Taylor & Richardson, 1980**) and some thiols exhibit antioxidant activity (**Güngör, Özyürek, Gülçü, & Apak, 2011**), our cysteine thiol exhibited very weak activity at the initial stage of lipid

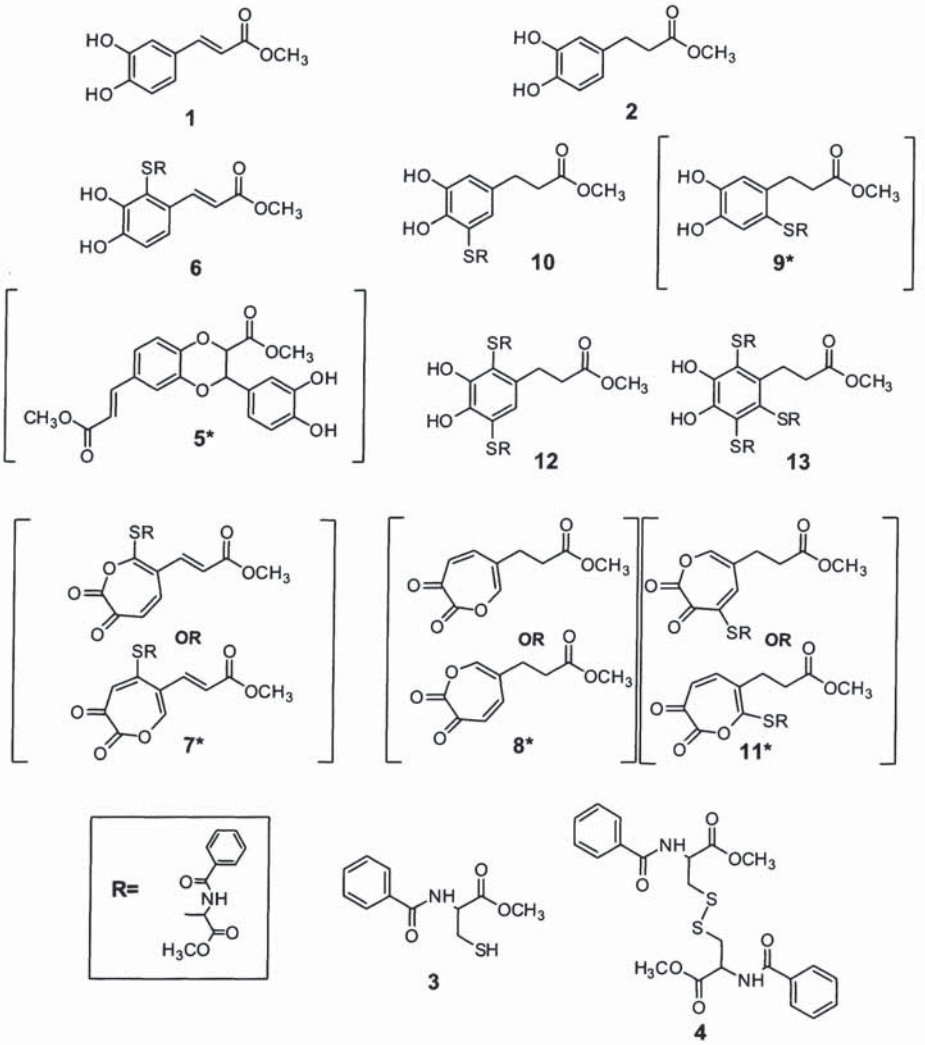

Fig. 1. Chemical structures of methyl caffeate (**1**), methyl dihydrocaffeate (**2**), cysteinyl thiol (**3**), and their products (**4–13**) in antioxidation reactions. *Indicates that the depicted structure is a presumed structure from MS and UV results.

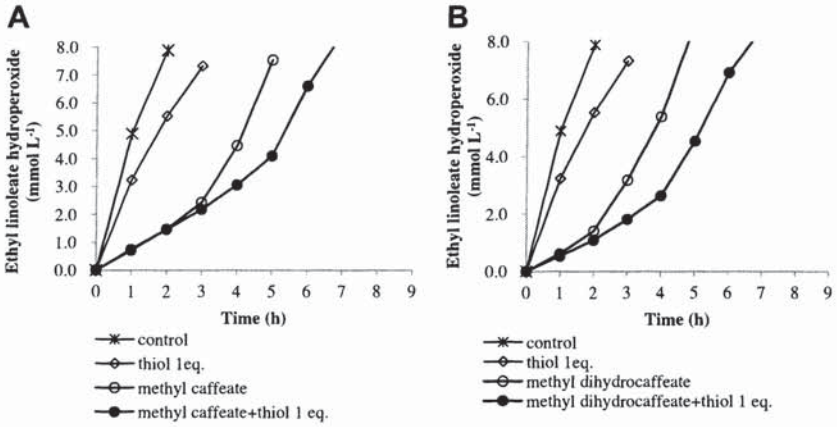

Fig. 2. Antioxidant activity of methyl caffeate (**1**, 1.25 mM) (panel A) and methyl dihydrocaffeate (**2**, 1.25 mM) (panel B) with or without equimolar amounts of *N*-benzoylcysteine methyl ester (thiol, **3**) against AMVN-induced lipid oxidation.

oxidation and no obvious induction period under the conditions employed. This negligible thiol activity simplified the analysis of the synergistic effect of the thiol on the activity of caffeates (Fujisawa & Kadoma, 2006). The panel A of Fig. 2 shows that methyl caffeate (1.25 mM) exhibits strong antioxidant activity for *ca.* 3 h. On the other hand, the coexistence of the thiol with the caffeate clearly enhanced the activity. The enhancement was particularly observed in the induction period (as represented by the inhibition time of lipid oxidation), rather than in activity efficiency (as represented by the slope of the lipid oxidation curve during the inhibition time). The induction period in the presence of the thiol was observed to be extended up to 5 h. Methyl dihydrocaffeate (1.25 mM) alone exhibited potent activity for almost 3 h, similar to methyl caffeate (Fig. 2B). The coexistence of an equimolar amount of the thiol elongated the induction period of the antioxidation of methyl dihydrocaffeate to over 4.5 h. These data indicated that the cysteinyl thiol had an efficient synergistic effect on the antioxidant capacity of both caffeate and dihydrocaffeate in the radical-initiator-induced lipid oxidation system, and an equimolar amount of thiol nearly doubled the duration of the antioxidation of both caffeates.

3.2. Concentration effect of thiol on the enhancement of the antioxidant activity of methyl caffeate and methyl dihydrocaffeate

The concentration effect of the thiol *N*-benzoylcysteine methyl ester on the activity enhancement was investigated and the data obtained are shown in Fig. 3A (methyl caffeate) and B (methyl dihydrocaffeate). Fig. 3A shows that the presence of two equivalents of the thiol enhances the antioxidant activity compared with the activity enhancement achieved by one equivalent of the thiol. It also shows that further addition of the thiol (three and four equivalents) did not cause further activity enhancement. On the contrary, in the case of dihydrocaffeate, thiol concentrations up to three equivalents contributed to the activity enhancement of the dihydrocaffeate (Fig. 3B). However, the antioxidant activity enhancement by the thiol was observed only with respect to the elongation of the induction period, and no enhancement of antioxidant efficiency was observed from the slope of the lipid oxidation curves as expressed by the caffeates with various amounts of thiol. Kadoma and Fujisawa (2008) observed a similar synergistic effect of a thiol on the antioxidant activity of phenolic acids. Okada,

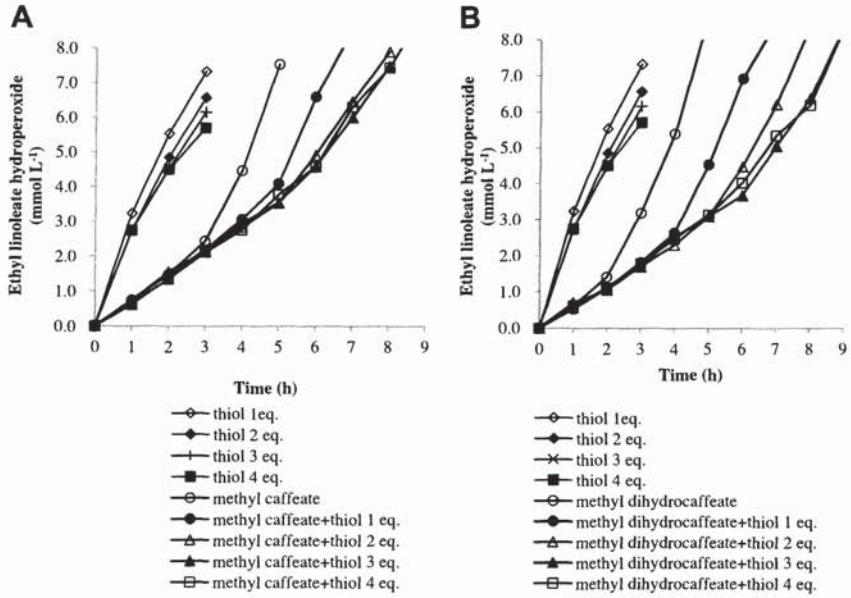

Fig. 3. Concentration effect of the thiol (**3**) on the antioxidant activity of methyl caffeate (panel **A**) and methyl dihydrocaffeate (panel **B**).

Nagai, and Bansho (1980) reported that cysteine and cysteinyl peptide had a synergistic effect on phenolic antioxidants in a lipid oxidation system. Generally, thiols have a synergistic effect on the antioxidant activity of phenolic antioxidants; therefore, we planned to obtain material evidence to explain the synergism.

3.3. HPLC and LC–MS analyses of the antioxidation products from methyl caffeate and methyl dihydrocaffeate in the presence of thiol

Radical-scavenging antioxidation of phenolic antioxidants is based on a radical-trapping reaction and the subsequent termina-

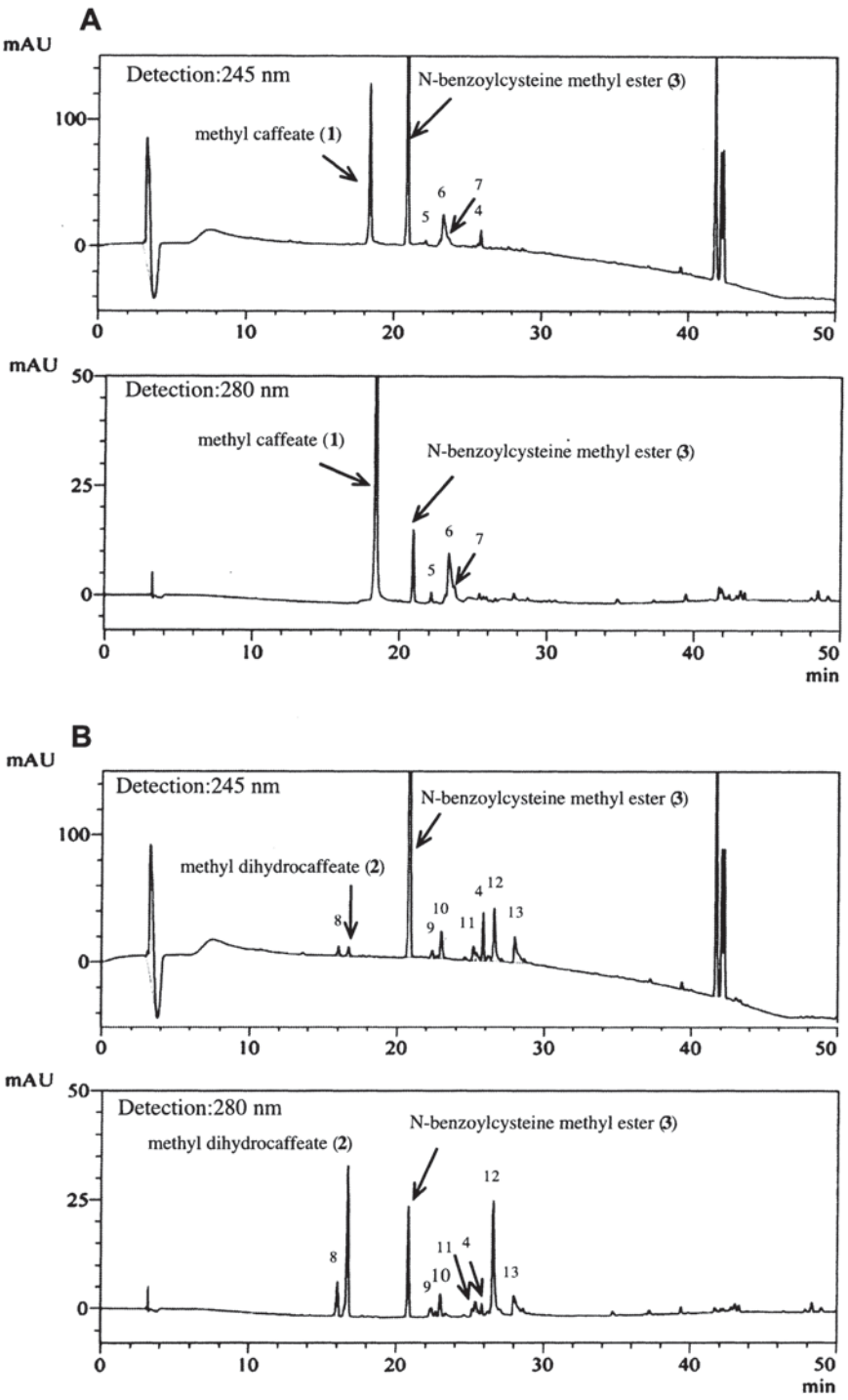

Fig. 4. HPLC analysis of the antioxidation products from methyl caffeate (**1**) in the presence of the thiol (**3**, 2 eq.) (panel A) and from methyl dihydrocaffeate (**2**) in the presence of the thiol (**3**, 3 eq.) (panel B).

tion reaction of the phenolic compounds. The termination reaction affords stable products that are detectable by HPLC analysis, and the knowledge of the structures of the products present is always useful to describe their antioxidation mechanisms (Masuda et al., 2010). We analysed the antioxidation products from methyl caffeate and methyl dihydrocaffeate in the presence of the thiol by HPLC at regular time intervals. Fig. 4 shows the HPLC profiles of the antioxidation products after a 2- or 3-h reaction of methyl caffeate and methyl dihydrocaffeate, respectively, in the presence of the thiol (two equivalents for methyl caffeate and three equivalents for methyl dihydrocaffeate). In the HPLC profile of the reaction products from methyl caffeate (Fig. 4A), a major product peak (6) is observed (retention time, 23.3 min) along with two minor peaks (5 and 7; retention times, 21.8 and 23.7 min, respectively), in addition to the methyl caffeate peak (1), the thiol peak (3) and a relatively smaller thiol dimer (a cystine derivative) peak (4) (retention times, 18.3 min, 20.8 min and 25.9 min, respectively). On the other hand, the antioxidation reaction of methyl dihydrocaffeate gave six noticeable product peaks (8–13) at retention times of 16.0, 22.3, 23.0, 25.4, 26.6 and 29.0 min, respectively, in addition to the peaks of methyl dihydrocaffeate peak (2) (retention time, 16.7 min) and peaks 3 and 4, as shown in Fig. 4B. The observed peaks were analysed by LC-TOFMS with LC-photodiode array (PDA) detection (UV spectra) and the results obtained are summarised in Table 1. The AMVN-induced radical reaction of caffeic acid gave the corresponding quinone derivative (Masuda et al., 2008), and various products were produced from the unstable quinone (Tazaki, Taguchi, Hayashida, & Nabeta, 2001) including thiol

Table 1
MS and UV analyses of typical observed peaks in the HPLC of the antioxidation reactions of methyl caffeate (**1**) and methyl dihydrocaffeate (**2**) in the presence of the thiol **3**.

Polyphenol	Retention time (min) on HPLC	λ_{max} (nm)	Observed deprotonated molecular ion (*m/z*)	Expected molecular formula	Observed typical fragment ions (*m/z*) in MS^E and their molecular formulas	Compounds
Methyl caffeate (**1**)	18.3	296,324	193.0469	$C_{10}H_9O_4$	161.0210 ($C_9H_5O_3$) 133.0269 ($C_8H_5O_2$)	Methyl caffeate (**1**)
	20.8	234	–		204.0657 ($C_{11}H_{10}NO_3$)	*N*-Benzoylcysteine methyl ester (**3**)
	21.8	321	385.0917	$C_{20}H_{17}O_8$	353.0660 ($C_{19}H_{13}O_7$) 325.0699 ($C_{18}H_{13}O_6$)	Dimer **5**
	23.3	321	430.0944	$C_{21}H_{20}NO_7S$	225.0200 ($C_{10}H_9O_4S$)	Mono-thiol adduct **6**
	23.7	310	444.0759	$C_{21}H_{18}NO_8S$	239.0005 ($C_{10}H_7O_5S$)	Oxidised compound **7**
	25.8	243	–		236.0357 ($C_{11}H_{10}NO_3S$) 204.0590 ($C_{11}H_{10}NO_3$)	*N,N′*-Dibenzoylcystine dimethyl ester (**4**)
Methyl dihydrocaffeate (**2**)	16.0	265	209.0440	$C_{10}H_9O_5$	117.0434 ($C_9H_5O_4$)	Oxidised compound **8**
	16.7	281	195.0644	$C_{10}H_{11}O_4$	121.0264 ($C_7H_5O_2$)	Methyl dihydrocaffeate (**2**)
	20.8	233	–		204.0650 ($C_{11}H_{10}NO_3$)	*N*-Benzoylcysteine methyl ester (**3**)
	22.3	241 294	448.1066	$C_{21}H_{22}NO_8S$	430.0960 ($C_{21}H_{20}NO_7S$) 195.0657 ($C_{10}H_{11}O_4$)	Mono-thiol adduct **9**
	23.0	239 294	432.1125	$C_{21}H_{22}NO_7S$	227.0326 ($C_{10}H_{11}O_4S$) 152.9981 ($C_7H_5O_2S$)	Mono-thiol adduct **10**
	24.6	243 267	446.0902	$C_{21}H_{20}NO_8S$	418.0954 ($C_{20}H_{20}NO_7S$) 241.0171 ($C_{10}H_9O_5S$) 213.0222 ($C_9H_9O_4S$)	Oxidised compound **11**
	25.8	241	–		236.0370 ($C_{11}H_{10}NO_3S$) 204.0650 ($C_{11}H_{10}NO_3$)	*N,N′*-Dibenzoylcistine dimethyl ester (**4**)
	26.6	240 273 310	669.1578	$C_{32}H_{33}N_2O_{10}S_2$	464.0813 ($C_{21}H_{22}NO_7S_2$) 259.0095 ($C_{10}H_{11}O_4S_2$)	Di-thiol adduct **12**
	28.0	244 315	906.2057	$C_{43}H_{44}N_3O_{13}S_3$	701.1315 ($C_{32}H_{33}N_2O_{10}S_3$) 496.0558 ($C_{21}H_{22}NO_7S_3$) 290.9787 ($C_{10}H_{11}O_4S_3$)	Tri-thiol adduct **13**

–, No related molecular ion was observed.

adducts (Cilliers & Singleton, 1990; Nikolantonaki & Waterhouse, 2012; Nikolantonaki et al., 2012). The quinone moiety of oxidised rosmarinic acid, which contains a sub-structure similar to that in dihydrocaffeate, is also unstable and exhibits reactivity towards thiols (Fujimoto & Masuda, 2012b).

Previously, we investigated the coupling reaction of a thiol with various polyphenols under DPPH radical oxidation conditions (Fujimoto & Masuda, 2012a). In the investigation, we found that methyl caffeate gave only the mono-thiol adduct at the 2′-position, whereas methyl dihydrocaffeate afforded mono-, di- and tri-thiol adducts. HPLC analysis revealed that the peak compound **6** in the HPLC profile in the methyl caffeate reaction was identical to the mono-thiol adduct of methyl caffeate (**6**), whose chemical structure was determined by nuclear magnetic resonance (NMR) spectroscopy in the previous investigation (Fujimoto & Masuda, 2012a). The peak compounds **10**, **12** and **13** were also identical to the mono-thiol, di-thiol and tri-thiol adduct of methyl dihydrocaffeate, respectively. The LC–MS analysis of the reaction mixtures provided molecular information, such as the molecular formula, even for several minor peak compounds. The LC–MS analysis of peaks 5 and 8 revealed that they contained no sulphur functionality. Although we could not isolate compounds **5** and **8** because of small quantities, and probably because of the inherent instability of **8**, the high-resolution MS results of **5** and **8** afforded $C_{20}H_{18}O_8$ and $C_{10}H_{10}O_5$, respectively, as the expected molecular formulae. The molecular formula derived for **5** indicated that it was a dimer of methyl caffeate. Various dimers of caffeic acid have been reported in nature and also through synthesis. Takahashi et al. reported a 1,4-benzodioxane dimer derived from caffeic acid by peroxidase oxidation (Takahashi, Matsumoto, Ueda, Miyake, & Fukuyama, 2002). The MS ion fragments observed (*m/z* 354 and 326 in the EI positive mode) of the dimethyl ester of the dimer were coincident with those of **5**, which were observed in the MS^E spectrum. Therefore, the structure of **5** is possibly the same as that of the dimer derivative. On the other hand, the molecular formula of **8** indicated that it was an oxidised compound of the quinone of methyl dihydrocaffeate. A UV absorption maximum at 264 nm of **8**, which was obtained directly by the PDA detection of HPLC, suggested that the benzene ring of **8** reacted oxidatively. Recently, we reported an oxidative ring expansion reaction of polyphenolic compounds by air oxidation (Fujimoto et al., 2010). A consideration of the reaction mechanism of the ring expansion, the molecular formula of **8** and an analysis of the UV absorption of **8** using the Woodward-Fieser rule for absorption spectra of enones (calculated λ_{max} 263–269 nm, observed λ_{max} 265 nm) (Silverstein, Bassler, & Morrill, 1992) suggested that **8** was a benzene-ring-expanded product of methyl dihydrocaffeate, as shown in Fig. 1. A higher concentration of the thiol prevented its formation, which suggests that a peroxy radical derived from the thermolysis of AMVN reacted with methyl dihydrocaffeate instead of the thiol, and thus produced **8**.

The peak compound **7**, which was observed as a shoulder of peak 6 (the mono-thiol adduct of methyl caffeate) in the HPLC of the reaction of methyl caffeate and completely separable from peak 6 in an isocratic mode (CH_3CN-1% acetic acid in H_2O = 30:70), and the peak compound **11** in the HPLC of the reaction of methyl dihydrocaffeate, as shown in Fig. **4**, displayed a relationship of molecular formulae similar to that between **8** ($C_{10}H_{10}O_5$) and methyl caffeate ($C_{10}H_{12}O_4$). The molecular formulae of **7** and **11** were found to be $C_{21}H_{19}NO_8S$ and $C_{21}H_{21}NO_8S$, respectively, both of which showed a reduction of two hydrogen atoms and an increase of one oxygen atom from the molecular formulae of the mono-thiol adduct of methyl caffeate (**6**) and that of methyl dihydrocaffeate (**10**), respectively, by comparison of their LC–MS results. Similar hypsochromic shifts were observed in the UV spectra of compounds **7** (λ_{max} 313 nm) and **11** (λ_{max} 267 nm) from absorbance maxima of **6** (λ_{max} 321 nm) and **10** (λ_{max} 294 nm) to that of **8**. These data strongly suggest that **7** and **11** might be the corresponding benzene-ring-expanded derivatives of **6** and **10**, respectively, as depicted in Fig. 1.

High-resolution MS results of the peak compound **9** indicated that this was an isomer of the mono-thiol adduct of methyl dihydrocaffeate (**10**). By NMR analysis in our previous investigation, the thiol in **10** was attached at the 5′-position. A comparison of the fragmentation patterns of **9** and **10**, which were observed in their MS^E spectra, revealed that **9** might be the 6′-thiol isomer of **10**, because **10** typically showed a strong fragment ion at *m/z* 227, whereas **9** showed almost no fragment ion at the same mass unit under the same measurement conditions. This strong fragmentation of **10** might be explained by the cleavage of the S–βC bond of the cysteinyl thiol moiety by the effect of a neighbouring hydroxyl group of the phenol moiety. Hence, the very weak fragmentation of **9** suggested that no hydroxyl group existed at the adjacent position of the thiol, and thus resulted in the 6′-thiol-substituted structure of **9** (Fig. 1).

Time-course analytical results for major peak compounds observed in the HPLC analysis of the reactions of methyl caffeate and methyl dihydrocaffeate are summarised in Fig. 5. In the series of methyl caffeate reactions, the concentration of methyl caffeate decreased linearly until 4 h; however, the addition of the thiol delayed its decrease up to 6 h as shown in panel A-a of Fig. 5. For the antioxidation period of 6 h, the peak of the mono-thiol adduct **6** increased for the first 2 h and then decreased (panel A-b in Fig. 5). These results indicate that the mono-thiol adduct **6** was formed very rapidly by reaction with the thiol, and it still exhibited potent antioxidant activity, which resulted in the enhancement of the total antioxidant activity of methyl caffeate. There is no other typical and potent antioxidative peak in the HPLC analysis of antioxidation reactions of methyl caffeate. Therefore, the longer induction period observed in the presence of two equivalents of the thiol compared with that with one equivalent of the thiol can be accounted for by the difference in the amount of the mono-thiol adduct **6** produced in both reactions (Fig. 5A-b).

In the series of methyl dihydrocaffeate reactions, the concentration of methyl dihydrocaffeate decreased linearly until 4 h, and the addition of the thiol also delayed the consumption of methyl dihydrocaffeate to up to 7 h, depending upon the concentration of the thiol, as shown in Fig. 5, panel B-a. During the antioxidation period of methyl caffeate with the thiol, the mono-, di- and tri-thiol adducts were produced (Fig. 5, panels B-b, B-c and B-d, respectively). First, the mono-thiol adduct **10** accumulated rapidly followed by the di-thiol adduct **12**. After 2–3 h, both adducts decreased gradually until 8 h. Finally, the tri-thiol adduct **13** was produced and was not consumed completely even at the end of the antioxidation period, except for the experiment using the lowest concentration of the thiol. These data indicate that the first and second additions of the thiol to the corresponding oxidation products from methyl dihydrocaffeate were very rapid, while the third addition was relatively slow. Furthermore, the antioxidant activities of mono- and di-thiol adducts are very strong and probably comparable with that of the original methyl dihydrocaffeate. However, the tri-thiol adduct might be weaker than methyl dihydrocaffeate or other thiol adducts.

3.4. Comparison of the reactivity of methyl caffeate, methyl dihydrocaffeate and their thiol adducts towards a peroxy radical

To examine the contribution of the thiol adducts to the total antioxidant activity of both caffeates in the presence of the thiol, the antioxidant ability of the original caffeates and their thiol adducts was compared according to their reactivity towards a peroxyl radical under AMVN-induced radical reaction conditions.

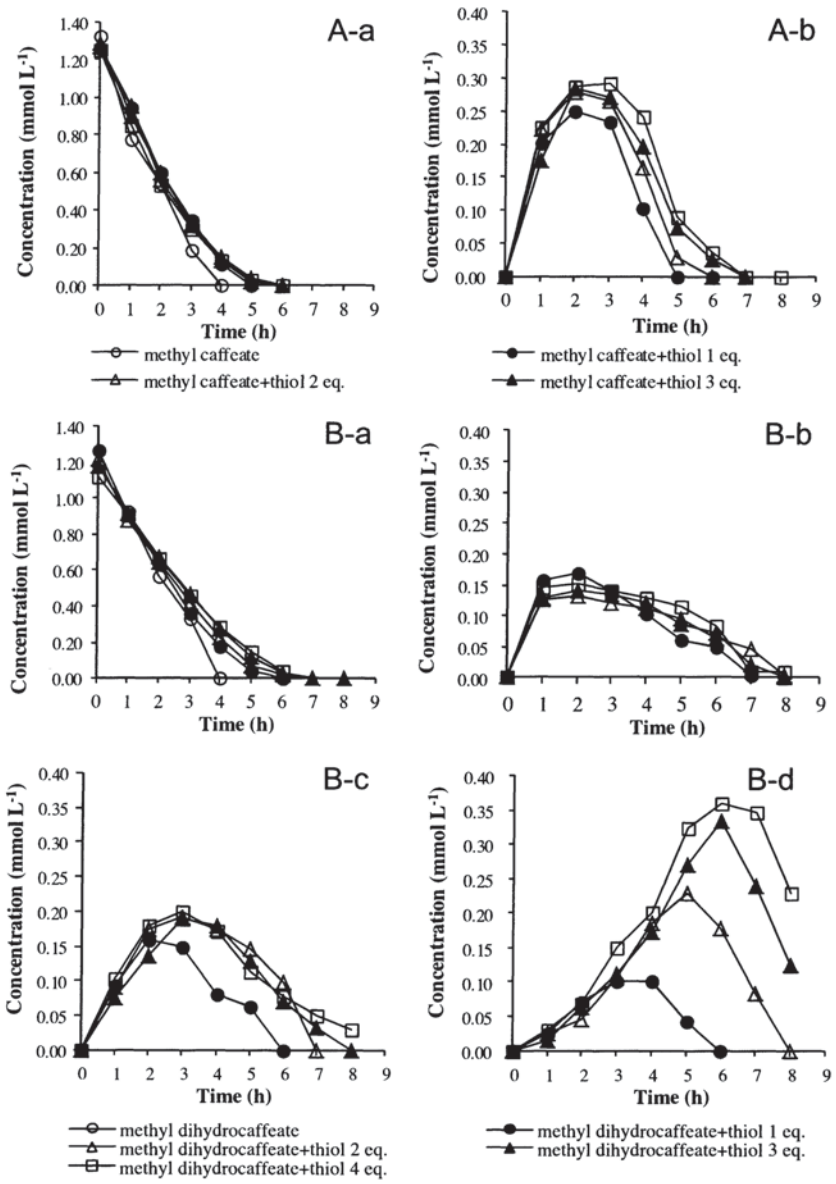

Fig. 5. Time-course analysis of the concentrations of methyl caffeate (**1**) (panel A-a), mono-thiol adduct **6** (panel A-b), methyl dihydrocaffeate (**2**) (panel B-a), its mono-thiol adduct **10** (panel B-b), di-thiol adduct **12** (panel B-c), and tri-thiol adduct **13** (panel B-d) in the antioxidation reactions with various concentrations of the thiol (**3**).

This experiment design has the advantage of being able to distinguish minute differences in the radical reactivity of the constituents (Masuda et al., 2003). Fig. 6A shows the decreases of methyl caffeate and its mono-thiol adduct **6** in equivalent solutions, and no typical differences were observed in the reactivity between methyl caffeate and the mono-thiol adduct, indicating that both compounds had the same antioxidant activity. Therefore, the addition of the thiol should double the antioxidant period of methyl caffeate – an expectation that is borne out by the antioxidant activity results. Fig. 6B shows the results of methyl dihydrocaffeate and its thiol adducts, which were from the same experimental procedure. First, the mono-thiol adduct **10** reacted rapidly with the radical, followed by methyl dihydrocaffeate and di-thiol adduct **12**. Methyl caffeate exhibited reactivity slightly higher than that of the di-thiol adduct. The tri-thiol adduct **13** was the most stable of the compounds because it was last to react with the radical. These results, in addition to the time-course analytical results obtained in the previous experiment, clearly show that the mono and di-thiol adducts fully contributed to the activity enhancement of methyl dihydrocaffeate. However, the contribution of the tri-thiol adduct was probably lower than that of the others.

The effect of the sulphur substituent on the antioxidant activity of the phenolic antioxidant has not yet been elucidated. Zahalka et al. (1988) synthesised thio-substituted tocopherols and compared their reactivity towards peroxyl radicals with that of the original tocopherols. The researchers showed that the thiotocopherols exhibited activity weaker than that of the tocopherols. On the other hand, Sato and Kise (1988) observed that the substitution of an oxygen atom by a sulphur atom in a phenol ether enhanced the inhibition time for the oxidation of a lubricant. Malmström et al. (2001) reported that a 1-thio derivative of dihydrobenzofuranol exhibited an inhibition time for the AMVN-induced linoleic acid oxidation similar to that of the 1-oxy derivative; however the inhibition rate was lower than that of the oxy derivative regardless of the very similar phenolic O–H bond-dissociation enthalpy of both compounds. Although the sulphur substit-

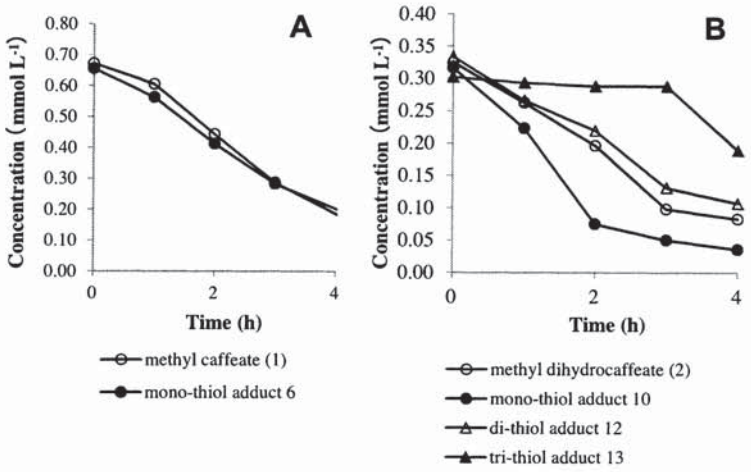

Fig. 6. Comparison of the reactivity towards the radical from AMVN between methyl caffeate (**1**) and mono-thiol adduct **6** (panel A) and between methyl dihydrocaffeate (**2**), mono-thiol adduct **10**, di-thiol adduct **12**, and tri-thiol adduct **13** (panel B).

uents are recognised to act both as electron-donating and electron-withdrawing groups, depending on the property of the surrounding structure, one radical could be stabilised by the delocalisation property of sulphur, which was derived from its low ionisation potential (Wayner & Arnold, 1984). Our results indicated that sulphur substituents possess a weak electron-withdrawing property to reduce the hydrogen-donating activity of phenolic hydroxyl groups; however, only one sulphur substituent was able to stabilise the phenolic radical. An estimation of inductive and resonance effects of functional groups has been examined (Hansch, Leo, & Taft, 1991). Yukawa and co-workers calculated slightly positive σ^0 values (+0.083) and negative $\Delta\sigma R^+$ values (−0.68) for methylsulphur substituent constants (Yukawa, Tsuno, & Sawada, 1966). Furthermore, Taft's equation employs $\sigma_I = +0.18$ and $\sigma_R = -0.173$ as the constants for the same substituent (Maruyama & Okubo, 1983). These values represent that the electron-donating property of alkyl sulphur substituents, which may influence the antioxidant efficiency of the phenolic antioxidants, was influenced negatively by the inductive effect and positively by the resonance effect. The reactivity estimation based on a linear free-energy-relationship approach is classical, but might explain our results (Van Acker, Koymans, & Bast, 1993). Both electronic properties determined the antioxidant efficiency of the thiol adducts of methyl caffeate and methyl dihydrocaffeate: the mono-thiol adduct was stronger than or comparable with the original caffeates, the di-thiol adduct was comparable with the caffeate, while the tri-thiol adduct was the weakest, owing to the presence of three electron-withdrawing groups, as shown in Fig. 6.

4. Conclusions

In summary, *N*-benzoylcysteine methyl ester, a peptidyl thiol model, has almost no antioxidant activity; however, it has remarkable potentiating property on the antioxidant capacity of caffeate and dihydrocaffeate against lipid oxidation. The potentiating effect of the thiol on the antioxidant capacity of dihydrocaffeate was larger than that of caffeate. Three equivalents of the thiol were effective on the antioxidant enhancement of dihydrocaffeate, whereas only two equivalents of the thiol were effective on that of the caffeate. This antioxidation enhancing effect of thiols, which is the positive synergism of the antioxidation of the polyphenolic antioxidants, was found for the first time. The chemical evidence of the antioxidation synergism was also revealed on the basis of the analysis for the antioxidation products that were produced in the antioxidation period. The results clearly showed that coupling of the thiol to both caffeates was fast enough to demonstrate the activity enhancement *in situ*. Time-course analysis of antioxidation products from both caffeates clarified that the mono-thiol adduct of the caffeate, and mono- and di-thiol adducts of the dihydrocaffeate contribute the synergism. The reason for the contribution of the thiol must depend on the electronic properties of sulphur substituents.

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