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Phenolic content and antioxidant activity of olive extracts

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Abstract

The phenolic component of freeze-dried olive fruit was fractionated by high-performance liquid chromatography using ultraviolet, atmospheric pressure chemical ionisation (APCI) and electrospray ionisation (ESI) detection. The fractions together with several standards were tested for antioxidant activity in an aqueous and a lipid system. The negative ion mode of APCI and ESI showed less fragmentation than positive ion mode. The latter was generally more useful in obtaining fragmentation data and hence structural information. Some olive phenolics notably tyrosol exhibited a low ionisation efficiency in both APCI and ESI. There was no simple relationship between antioxidant activity and chemical structure. The ranking of antioxidant activity was strongly dependent on both the test system and on the substrate demonstrating the need to examine activity in both aqueous and lipid systems. Significant antioxidant activity was seen in most olive fractions and this was related to phenolic content. The kinetics of the oxidation process are complex and suggest that multiple pathways may be involved at different antioxidant concentrations.

Keywords: Phenolic compounds; Antioxidant; Olive; Mass spectrometry

1. Introduction

Oxidation reactions are of major significance in human physiology as well as the food industry. In the former, oxidative stress has been linked to diseases such as atherosclerosis, cancer, and tissue damage in rheumatoid arthritis (Halliwell, 1994; Basu, Temple, & Garg, 1999) while the food industry has long been concerned with issues such as rancidity and oxidative spoilage of fruits, vegetables and beverages (Shahidi & Wanasundara, 1992; Martinez & Whitaker, 1995). Lately, there has been a convergence of interest among researchers in these fields (Robards, Prenzler, Tucker, Swatsitang, & Glover, 1999) as the role of antioxidants in the diet (and their impact on human health) has come under attention. For example, diets rich in fruits and vegetables have been associated with lower risk of coronary heart disease and cancer (Keys, 1995). The notion that the deleterious effects of oxidative metabolism can be ameliorated through a diet rich in antioxidants has gained credibility and the term ''functional foods'' (Strain & Benzie, 1998) has been applied to foods with these nutrients. Among the components of the so-called ''Mediterranean Diet'' phenolic compounds are receiving increased attention as epidemiological studies have shown that consumption of foods and beverages rich in phenolics is correlated with reduced incidence of heart disease (Cook & Samman, 1996).

An important aspect of the study of antioxidants has been the measurement of antioxidant activity. Various tests are available (Robards et al., 1999) depending on the area of interest. Studies involving the Rancimat test are used to compare ''natural'' versus synthetic antioxidants in commercial fats and oils in an effort to find suitable replacements for compounds such as butylatedhydroxyanisole (BHA) and butylatedhydroxytoluene (BHT; Dziedzic & Hudson, 1983; Hudson & Lewis, 1983). In aqueous systems, the Trolox Equivalent Antioxidant Activity (Miller & Rice-Evans, 1997) of several juices and individual phenolic compounds was measured by radical scavenging of the 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radical cation. Various systems have been studied to determine the effect of antioxidants on low density lipoprotein (LDL) oxidation. Meyer, Donovan, Pearson, Waterhouse, and Frankel (1998b) monitored hexanal production with cinnamic acid antioxidants; and Scaccini, Nardini, Daquino, Gentili, Di Felice, and Tomassi (1992) measured thiobarbituric acid reactive substances (TBARS) and diene formation when olive oil was used

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as an antioxidant. The possibility of synergism between aqueous and lipid antioxidants in the protection of LDL is also being recognised (Ghiselli, Damicis, & Giacosa, 1997; Harats, Chevion, Nahir, Norman, Sagee, & Berry, 1998). Fruit, vegetable, and beverage extracts contain antioxidants with a wide variety of polarities and fractionation prior to antioxidant testing would provide valuable information on the relative contribution of each fraction to overall activity. Such an approach has been used (e.g. Litridou et al., 1997) but has not been widely adopted.

Olives are a fruit that are a particularly rich source of phenolic antioxidants (Ryan & Robards, 1998), including distinctive compounds such as verbascoside, ligstroside and oleuropein (Ryan, Robards, Prenzler, Jardine, Herlt, & Antolovich, 1999). Extraction of compounds from fruit is commonly achieved with methanol or aqueous methanol (Amiot, Fleuriet, & Macheix, 1986; Antolovich, Prenzler, Robards, & Ryan, 2000). Studies on the antioxidant activity of olive phenolics have tended to look at either single components (Baldioli, Servili, Perretti, & Montedoro, 1996) or fruit/oil extracts (Litridou et al., 1997). Given the wide variety of phenolics (structures, solubilities) and the fact that despite extensive study, not all have been identified, we have measured the antioxidant activity of fruit extracts that have been fractionated on the basis of polarity. Such an approach has several advantages: lipophilic and hydrophilic fractions are separated and can be tested in aqueous and lipid phase oxidations; some synergistic interactions are maintained within fractions; fractions of particularly high activity may be identified and studied further e.g. extracting individual components. In this study, olive fractions as well as several phenolic standards have been subjected to antioxidant tests: in the aqueous phase using the phycoerythrin assay (DeLange & Glazer, 1989); and in the lipid phase using oxidation of linoleic acid and measurement of TBARS (Fernández, Pérez-Álvarez, & Fernández-López, 1997).

In order to further elucidate the structures of phenolics the fractions were subjected to LC-MS analysis. This technique has proven useful in identifying various known phenolics in olives (Ryan, Robards, Prenzler, & Antolovich, 1999) as well as novel compounds (Ryan, Robards, Prenzler, Jardine, Herlt, & Antolovich, 1999). While using standards to optimise conditions for detection of phenolics a number of important observations were made and these are included in this paper.

2. Materials and methods

2.1. Instrumentation

2.1.1. High-performance liquid chromatography (HPLC)

HPLC analysis was conducted using a Varian Star LC 9012 (Melbourne, Australia) solvent delivery system equipped with a 50 μl sample loop and a Star 9050 variable wavelength UV/VIS detector.

Semipreparative scale HPLC was performed using a Waters 510 HPLC pump (Sydney, Australia) with injection via a Rheodyne 7125 valve through a 2 ml sample loop. Column eluent was monitored using a spectrophotometer with semipreparative flow cell.

2.1.2. Liquid chromatography/mass spectrometry (LC/MS)

LC/MS was performed using a Finnigan aQa Thermo Quest LC/MS system and a Spectra system P4000 pump (Sydney, Australia) equipped with a 10 μl sample loop and photodiode array detection.

2.1.3. Fluorescence spectrophotometry

A Perkin Elmer HTS 7000 Bio Assay Reader (Melbourne, Australia) was used in the phycoerythrin-based antioxidant tests to measure fluorescence. Reaction occurred in a white plastic, 96 well plate at 37°C. Each well could hold 250 μl. The wavelengths for fluorescence excitation and emission were 492 and 595 nm, respectively.

2.2. Preparation of olive samples

Olives (*Manzanillo*) were picked randomly from the laterals in the crown joining the right hand co-dominant branch of one tree. The fruit picked had predominantly purple spotted skin. The collected olives, were immediately placed in liquid nitrogen and then freeze-dried (−47°C) for approximately 1 week, according to the moisture content of the fruit. Freeze-dried samples were placed in a freezer until ready for use. The freeze-dried olives were then pitted and blended in a general-purpose electric blender (Sunbeam Model PB-B, Sydney, Australia) to a fine powder. The powder was stored under nitrogen in plastic screw top jars, in a freezer at −18°C.

2.3. Preparation of olive extract

Freeze-dried olive (10 g) was measured into a beaker. Methanol:water (50:50, v/v; 50 ml) was added, and left for 30 min. The extract was filtered and washed with hexane (3×50ml) to remove oil. The extract was then filtered (0.45 μm) and diluted (1:10, v/v with 50:50, v/v methanol:water). Semipreparative scale HPLC was performed on undiluted extract.

2.4. HPLC analysis

Analytical-scale HPLC analyses of the olive extract and fractions were conducted on a C18 column (150×4.6 mm i.d.; 5 μm; Alltech, Sydney, Australia). The mobile phase involved a gradient of water:acetic acid (100:1, v/v) as solvent A and methanol:acetonitrile:acetic acid (95:5:1, v/v/v) as solvent B at a constant

flow rate of 1.5 ml min^{-1}. The gradient (Gradient I) comprised an initial isocratic period of 2 min at 5% B followed by a linear increase to 25% B over 10 min, with further stepwise linear increases to 40% B at 20 min, 50% B at 30 min and 100% B at 40 min, hold for 5 min and return to initial conditions over 10 min. UV detection was routinely performed at 280 nm.

Semipreparative scale HPLC was conducted on a YMC pack ODSAQ column (250×10 mm, 5 μm) using water:-methanol:acetic acid (95:5:1, v/v/v; Solvent C) and methanol:acetonitrile:acetic acid (95:5:1, v/v/v; Solvent D) with a flow rate of 4 ml min^{-1}. Gradient elution (Gradient II) involved a linear increase from 0% D to 21.1% D over 10 min followed by a linear increase to 36.8% D at 20 min, with further stepwise linear increases to 47.4% D at 30 min and 100% D at 40 min, held for 5 min and returned to initial conditions over 10 min. Six fractions of the olive extract were collected at the times shown in Fig. 1.

2.5. LC-MS

Phenolic standards used for instrument optimisation included oleuropein (Extrasynthese, Genay, France); tyrosol (Aldrich Chemical Co., Milwaukee, WI); gallic acid, chlorogenic acid, *p*-coumaric acid and vanillic acid (Sigma Chemical Co., St Louis, MO). Each standard was made up with methanol/water (50:50, v/v) to a concentration of 100 ppm and injected into a 50 μl loop using flow injection mode. HPLC analysis was conducted using a 10 μl sample loop and a C18 column (150×4.6 mm i.d.; 5 μm; Alltech, Sydney, Australia). The flow rate, cone voltage and probe temperature were systematically varied to determine optimum conditions as described in Section 3. The mobile phase solutions A and B with Gradient I were used for all separations.

2.6. Total phenols

The method used for the determination of total phenols using Folin Ciocalteu reagent was adapted from Shahidi and Naczk (1995). A diluted extract (0.5 ml of 1:10, v/v) or phenolic standard was mixed with Folin Ciocalteu reagent (5 ml, 1:10 diluted with nanopure water) and aqueous Na_2CO_3 (4 ml, 1 M). Solutions were heated in a 45°C water bath for 15 min and the total phenols were determined colorimetrically at 765 nm. The standard curve was prepared using 0, 50, 100, 150, 200, 250 mg l^{-1} solutions of gallic acid in methanol:-water (50:50, v/v). Total phenol values are expressed as gallic acid equivalents (mg g^{-1} dry mass) which is a common reference compound.

2.7. TBARS — linoleic acid assay

TBARS were determined using the linoleic acid system adapted from Kishida et al. (1993) in which linoleic acid (100 μl), copper chloride (0.05 mM, 300 μl) and test compounds (50 μl) were added to a test tube. The test tubes were then vortexed and placed in a shaking water bath at 37°C for 20 h. BHT (20 μl of 10 mM, dissolved in ethanol) was added to each tube to terminate the reaction (Esterbauer & Cheeseman, 1990).

Thiobarbituric acid was added to each test tube (freshly prepared; 0.67% w/v in 0.1M HCl, 3ml). To dissolve the thiobarbituric acid in HCl, sonication and momentary heating were required. The tubes were then vortexed, heated in a boiling water bath for 10 min, and allowed to cool. During the heating stage, the aqueous layer turned pink. The pink aqueous layer (2 ml) was removed, being careful to avoid any lipid phase, and added to a separate test tube containing butanol (2.5 ml) and vortexed. The absorbance of the butanol layer was measured at 532 nm. Butanol was used as the reagent blank.

The TBARS reaction mixture was examined by HPLC on a C18 column using isocratic elution with 95% Solvent A and 5% Solvent B. The reaction product was detected at 532 nm as a single compound eluting at 14.9 min with a shoulder at 14.4 min (results will be reported separately); additional peaks were observed in the ultraviolet trace at 250 nm.

2.8. Phycoerythrin fluorescence-based assay

The phycoerythrin fluorescence-based assay was conducted according to the method of Glazer (1990). Extracts, fractions and total fractions, were analysed at a range of concentrations varying from 20 g l^{-1} to 0.2 mg l^{-1}. Standard phenolic compounds were also examined over a range of concentrations. A stock solution of the Trolox standard (10 mM) was prepared in 50% (v/v) methanol-water and diluted as necessary.

2.8.1. Peroxyl free radical mediated system

Phycoerythrin (2 μl; 1.7×10^{-8} M), sodium phosphate buffer (178μl; pH 7.0 prepared by mixing 1 M sodium monohydrogen phosphate, 57.7 ml with 1 M sodium dihydrogen phosphate, 42.3 ml and diluting to 1 l) and antioxidant (1μl of phenolic compound, olive extract or fraction at various concentrations) were added to each well of the fluorescence spectrophotometer. 2,2′-Azo-bis(2-methylpropionamidine)dihydrochloride (AAPH) (20 μl, 40 mM freshly prepared in sodium phosphate buffer, pH 7.0) was added to start the reaction. Fluorescence readings were taken automatically every 2 min at 595 nm (excitation 492 nm) until the fluorescence readings had decreased by at least half.

2.8.2. Copper-ascorbate mediated system

Phycoerythrin (2 μl), sodium phosphate buffer (176 μl) and antioxidant (1 μl of phenolic compound, olive extract or fraction) were added, in order, to each well of

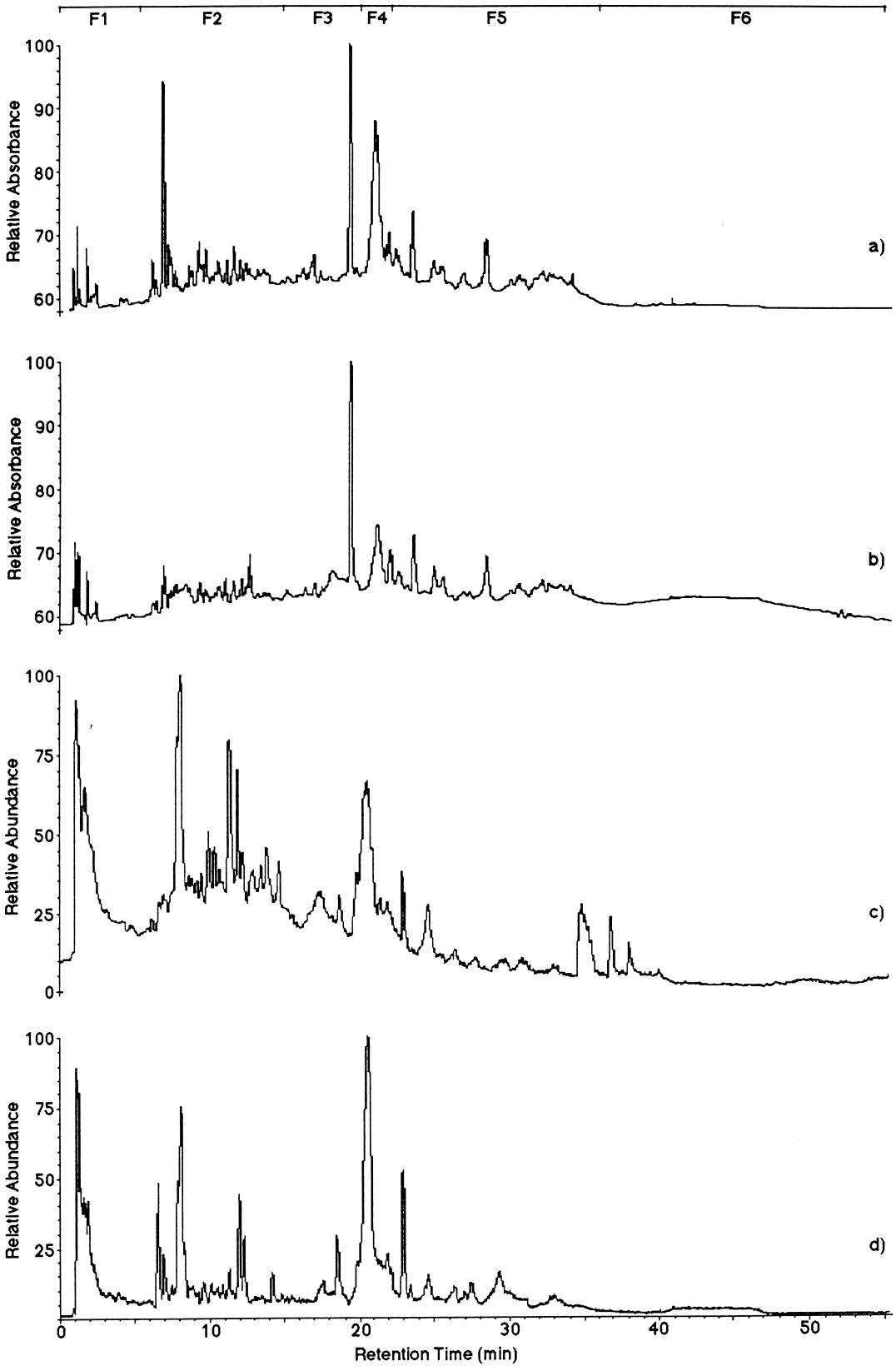

Fig. 1. Chromatogram of the extract obtained by analytical-scale separation using the C18 column and Gradient I with various methods of detection as (a) ultraviolet at 280 nm, (b) integrated response 190–400 nm, (c) total ion chromatogram, positive ion mode, and (d) total ion chromatogram, negative ion mode. The points of collection of the various fractions (F1, F2 ... F6) are indicated.

the fluorescence spectrophotometer. Ascorbic acid (2 μl, 0.036 M); followed by copper sulphate (20 μl, 4.5×10^{-6} M) were added to initiate the reaction. Fluorescence readings were recorded as for the peroxyl free radical-mediated system.

3. Results and discussion

The material obtained from freeze-dried olive by extraction with methanol-water (Tsimidou, 1998) represented 32% by mass of the original freeze-dried drupes.

3.1. Chromatographic analysis

Analysis of the methanolic extract by HPLC with photodiode array and either ESI or APCI detection (Fig. 1) revealed a complex mixture of phenolic compounds that was stable for up to 4 months when stored at 0°C. Several phenolic compounds have been identified in olive drupes (Ryan & Robards, 1998) and the compounds in Table 1 are representative of the diverse structural types. Retention data (Table 1) are typical of a reversed phase system.

Optimisation of mass spectral conditions of positive/negative ion mode, cone voltage and carrier flow rate were critical in LC-MS. Of less significance was the probe temperature. In general, ESI in negative ion mode, with a cone voltage of 30 V and a flow rate between 0.5 and 1 ml min^{-1}, provided the most successful mode for analysing the phenolic compounds. The negative ion mode of both APCI and ESI gave simpler spectra dominated by abundant pseudomolecular ion peaks whereas positive ion mode produced greater fragmentation, and hence, more structural information about the molecules. Fragmentation was enhanced by increasing cone voltage in both negative and positive modes and was significant for all compounds at values exceeding 50–75 V. Gallic acid, vanillic acid, tyrosol and *p*-coumaric acid were best identified using negative mode, while either positive or negative ion modes of APCI and ESI were suitable for oleuropein and chlorogenic acid. Tyrosol was particularly difficult to analyse, with identification of the pseudomolecular ion best achieved in APCI negative mode, 30 V cone voltage, flow rate of 1 ml min^{-1} and a probe temperature of 250°C. With all compounds, various solvent adducts and dimer ions were observed, depending on the compound and operating conditions.

Phenolic compounds identified conclusively in the extract included tyrosol, oleuropein, verbascoside and the dialdehydic form of the oleuropein aglycone (Bianco, Muzzalupo, Piperno, Romeo, & Uccella, 1999). Although hydroxytyrosol, and the substituted cinnamic acids were not confirmed, previous studies suggest that the presence of these compounds, albeit at significantly lower levels than oleuropein type compounds, is to be expected. Table 1 indicates the fraction in which these compounds would appear (if present). The presence of a number of phenolic glycosides (eg. luteolin-7-glucoside, quercetin-3-rhamnoside) which were not available as standards is also expected in Fraction 6. Compounds detected at 34, 36 and 38 min, respectively, in the total ion chromatogram (positive mode) of the olive extract were also detected in a blank run. These compounds were not detected by photodiode array detection.

Table 1
Retention times of phenolic compounds

Phenolic compound	Retention time (min)	Fraction in which compound would appear
Gallic acid	4.9	1
Tyrosol	11.5	2
4-Hydroxybenzoic acid	12.7	2
Chlorogenic acid	14.3	2
Vanillic acid, homovanillic acid	14.7	2
2,4-Dihydroxybenzoic acid	14.9	2
Caffeic acid	15.3	2
Syringic acid	16.0	2
Verbascoside	18.4	3
p-Coumaric acid	19.6	3
Ferulic acid	21.0	3
Dialdehydic form of oleuropein aglycone[a]	22.5	4
o-Coumaric acid	23.7	4
Oleuropein	25.7	5
Cinnamic acid	29.5	5

[a] Bianco et al., 1999.

3.2. Fractionation and measurement of total phenols

The phenolic extract (obtained by extraction with aqueous methanol) was fractionated by semipreparative scale HPLC into six fractions (Fig. 1) using 280 nm as a compromise wavelength (Ryan & Robards, 1998) for monitoring eluent. Chromatography of the individual fractions on an analytical column demonstrated a clean separation between fractions (data not shown). The material recovered from the preparative scale system represented 89% by mass of the sample injected onto the column (Table 2) and 29% by mass of the original freeze-dried olive powder. The masses represented by the different fractions decreased in the following order (Table 2): Fraction 1 > Fraction 2 > Fraction 5 > Fraction 4 > Fraction 3 > Fraction 6. From the integrated detector response (absorption at 190–400 nm), Fraction 3 contained the major peak in the extract but represented the smallest mass of the fractions with the exception of Fraction 6.

Total phenols, as determined by the Folin-Ciocalteu method, are reported (Table 2) as gallic acid equivalents (GAE) by reference to a standard curve

($y = 0.0051x - 0.0335$, $r^2 = 0.9974$). The levels of total phenols determined in this way are not absolute measurements of the amounts of phenolic materials but are in fact based on their chemical reducing capacity relative to an equivalent reducing capacity of gallic acid. The different responses of the fractions in this assay may arise from the variety of phenolics found in the olive. For instance, it has been reported (Frankel, Waterhouse, & Teissedre, 1995) that the reducing capacity is enhanced by ortho or para orientation of the phenolic hydroxy groups.

The freeze-dried extract had a total phenol content, as measured by Folin Ciocalteu reagent, of 150 mg GAE per g extract. Data are also reported for the individual fractions relative to the mass of the particular fraction and to the mass of freeze-dried extract. Based on measurement of total phenols, Fraction 1, which represented the bulk of the material, comprised predominantly non-phenolic compounds. This is not unexpected as the fraction contained material eluted in the void volume and is thus likely to include polar substances such as sugars. An alternative, but less likely explanation, is that phenolic material in Fraction 1 was unable to reduce Folin Ciocalteu reagent. The bulk of the phenolic species was contained in Fractions 2–5, and particularly, Fraction 3. The latter contained the predominant peak (as measured at 280 nm) in the chromatogram of the extract and contributed most to the total phenolic pool of the olive. Based on previous studies (Ryan, Robards, Prenzler, Jardine, Herlt, & Antolovich, 1999; Ryan, Robards, & Lavee, 1999a, 1999b), compounds that are likely to be present in the various fractions are identified in Table 1.

3.3. Antioxidant activity

The relative ability of the fractions and extract to act as antioxidants was tested in a lipid system and an aqueous system using linoleic acid and phycoerythrin, respectively, as the oxidisable substrates. The antioxidant effects of 11 phenolic compounds were also investigated at different concentrations to relate activity to structural effects.

3.4. Lipid system

The linoleic acid system was a two-phase system involving copper chloride in aqueous solution, and the linoleic acid as a separate layer. In this procedure (Kishida et al., 1993), malondialdehyde (MDA) and other thiobarbituric acid reactive substances (TBARS) are formed as a result of peroxidation of the linoleic acid. It is postulated that the formation of MDA from fatty acids with less than three double bonds, such as linoleic acid, occurs via the secondary oxidation of primary carbonyl compounds (e.g. 2-nonenal) (Fernández et al., 1997). Examination of the reaction mixture by thin layer chromatography and LC-MS showed a single product that was quantified at 532 nm following extraction of the aqueous phase with n-butanol. The intensity of colour formed during this assay is apparently related to the amount of MDA produced. However, the lipid phase interfered with the measurement of the TBARS reaction product due to absorption at 447 nm. This was easily eliminated by selecting the aqueous phase prior to extraction with n-butanol although centrifugation of the extract was equally successful.

The antioxidant activity of various phenolic compounds on the oxidation of linoleic acid is expressed as percent inhibition of TBARS formation (Table 3). All compounds exhibited antioxidant activity in the range 10–1000 mg l^{-1} with the exception of 4-hydroxybenzoic acid which showed a pro-oxidant behaviour at the lowest concentration of 10 mg l^{-1}. Caffeic acid, cinnamic acid and 2,4-dihydroxybenzoic acid showed a positive, linear dose-response inhibition of oxidation over the range 10–1000 mg l^{-1}, i.e. the higher the addition level, the higher the antioxidant activity. For the remaining compounds, the relationship between concentration and percent inhibition was more complex, showing in many

Table 2
Physical appearance, recoveries and total phenol contents of the fractions isolated by preparative scale high-performance liquid chromatography

Olive fraction	Appearance	Mass of fraction (mg)	Recovery relative to total mass injected (%)	Recovery relative to mass of original dried olive (%)	Total phenols[a] (mg GAE[b] per g dried fraction)	Total phenols[a] (mg GAE per g original dried olive powder)
Fraction 1	Yellow syrup-like	658	63.3	21	3.2	0.2
Fraction 2	Brown	108	10.3	3.4	100	3.4
Fraction 3	Purple/brown	36	3.5	1.1	390	4.4
Fraction 4	Purple	49	4.7	1.5	240	3.7
Fraction 5	Brown	68	6.5	2.1	280	5.9
Fraction 6	Light yellow	5.5	0.53	0.2	0.6	Trace
Summation of Fractions 1–6		925	89	29		

[a] Relative error, 5%.
[b] GAE, gallic acid equivalents.

cases a maximum response at some intermediate concentration. Caffeic acid was the most effective antioxidant in the lipophilic system and it was the only antioxidant that showed a significant reduction in oxidation at all concentrations. Meyer et al., (1998b) also reported that hydroxycinnamic acids and their esterified derivatives exerted considerable antioxidant potency towards the copper-catalysed human LDL oxidation in vitro, with caffeic acid and its esterified derivatives being the most active compounds.

It is difficult to assign an order of antioxidant activity to the compounds because of the differential responses at the three concentrations. Nevertheless, at an antioxidant concentration of 100 mg l^{-1}, the phenolic compounds decreased in antioxidant activity in the order caffeic acid > tyrosol ≅ naringin ≅ chlorogenic acid > vanillic acid > 4-hydroxybenzoic acid ≅ *p*-coumaric acid ≅ gallic acid > naringenin > 2,4-dihydroxybenzoic acid ≅ oleuropein. These compounds may be classified as simple phenols (tyrosol), benzoic acids (vanillic, 4-hydroxybenzoic, 2,4-dihydroxybenzoic and gallic acids), hydroxycinnamic acids (*p*-coumaric, caffeic and chlorogenic acids), flavonoids (naringenin and naringin) and iridoids (oleuropein). Further distinction may be made by the number of hydroxyl and methoxy groups, relative acidity (benzoic acids versus hydroxycinnamic acids) and the solubility/partitioning behaviour of the phenol between the aqueous and lipid phases.

A number of structural features seems to be important in determining this trend. The foremost consideration is the extent and nature of the hydroxylation pattern of the aromatic ring. The presence of a single hydroxyl group, in either the benzoic (4-hydroxybenzoic acid) or hydroxycinnamic acids (*p*-coumaric acid), confers limited activity. On the other hand, the presence of an *o*-diphenol, as in caffeic acid and chlorogenic acid, enhances the ability of the phenolic to act as an antioxidant. *o*-Diphenols, such as these, may act as potent metal chelators (Meyer et al., 1998b). Glycosidic linkage of the caffeic acid, as in chlorogenic acid, reduced activity. Decreased antioxidant activity of chlorogenic acid is in agreement with data observed in other assays (Chen & Ho, 1997; Meyer et al., 1998b). The decreased antioxidant activity of chlorogenic acid may be explained by its enhanced water solubility. It is therefore more favourably partitioned in the aqueous phase. The effect of dihydroxylation is site-specific as it did not enhance activity in the case of 2,4-dihydroxybenzoic acid. Addition of an *o*-methoxy group, in the case of vanillic acid (versus 4-hydroxybenzoic acid), increased antioxidant activity due to improved stabilisation of the phenoxyl radical.

It is generally assumed that the hydrogen donor ability and inhibition of oxidation are enhanced by increasing the number of hydroxyl groups in the phenol. However, gallic acid, having three hydroxyl groups, did not exhibit exceptional antioxidant activity. This may be related to the hydrophilic character of gallic acid (Jacobsen, Schwarz, Stöckmann, Meyer, & Adler-Nissen, 1999), which reduced partitioning in the lipid phase.

Both (±)-naringenin and naringin exhibited antioxidant activity but at a lower level than that of caffeic

Table 3
Percent inhibition of the copper(II)-induced oxidation of linoleic acid by various phenolic substances[a]

Compound	Percent inhibition[a]		
	Antioxidant concentration 1	Antioxidant concentration 2	Antioxidant concentration 3
Caffeic acid	72	56	29
Chlorogenic acid	14	25	21
p-Coumaric acid	46	17	18
Gallic acid	12	13	12
2,4-Dihydroxybenzoic acid	7	3	0
4-Hydroxybenzoic acid	15	16	−4
Vanillic acid	12	20	12
Oleuropein	19	0	12
Tyrosol	20	33	20
(±) Naringenin	30	8	21
Naringin	19	29	16
Extract	94	91	82
Fraction 1	0.3	33	15
Fraction 2	89	−11	−40
Fraction 3	90	46	−30
Fraction 4	91	88	48
Fraction 5	90	83	20
Fraction 6	Insufficient sample	−77	−49

[a] Data are reported as the mean of triplicate determinations with a 5% method error. A negative sign represents a pro-oxidant effect. Antioxidant concentrations 1–3 correspond to 1000, 100 and 10 mg l^{-1}, respectively of standard or 200,000, 20,000 and 2000 mg l^{-1}, respectively of fraction.

acid, with naringin being more effective than naringenin. Meyer, Heinonen, and Frankel (1998a) found that the flavonoid, quercetin, had a lower antioxidant activity in LDL oxidation than caffeic acid. The reduced activity of naringenin relative to naringin was unexpected and was reversed at a higher antioxidant concentration of 1000 mg l^{-1}. Flavonoid aglycones, such as (±)-naringenin, have been reported (Cook & Samman, 1996) to be more effective at inhibiting malondialdehyde production than their corresponding glucosides where the sugar moiety reduces the antioxidant efficiency of adjacent hydroxyl groups due to steric hindrance.

The olive extract exhibited significant antioxidant activity (Table 3) in the linoleic acid system with Fraction 4 displaying the highest activity, followed by Fractions 5 and 3. These fractions also had the highest concentration of total phenols expressed as GAE. It seems unlikely that oleuropein in Fraction 5 can account for the activity of this fraction and the presence of another active compound must be suspected. Some fractions, particularly at higher dilution, exhibited pro-oxidant activity. Nevertheless, the original extract exhibited exceptional antioxidant activity at all measured concentrations in the linoleic acid system. Thus, synergistic effects may be important in the ability of a fraction to contribute to the antioxidant activity. Hydroxytyrosol and oleuropein derivatives have been shown (Visioli, Bellomo, Montedoro, & Galli, 1995) to inhibit the copper(II)-mediated oxidation of low density lipoprotein in vitro.

3.5. Aqueous system

Antioxidant activity can be measured as the ability to scavenge free radicals generated in an aqueous phase. The highly fluorescent protein phycoerythrin (PE) has been used as the target of free radical damage (DeLange & Glazer, 1989; Glazer 1990; Wang et al., 1996). Peroxyl radicals, generated by the thermal decomposition of AAPH, quench the fluorescence of the phycoerythrin while addition of an antioxidant that reacts rapidly with peroxyl radicals inhibits the loss of fluorescence intensity and this inhibition is proportional to the antioxidant activity. Alternatively, PE is used to assess the effectiveness of antioxidants against hydroxyl radicals. Hydroxyl radicals are generated from an ascorbate $-Cu^{2+}$ system at copper-binding sites on PE causing site-specific damage to the macromolecule.

The water soluble antioxidant trolox was used as an antioxidant standard in this study (Wang et al., 1996).

3.5.1. AAPH mediated oxidation

The fluorescence decay, due to peroxyl radical attack on PE, was zero order. Trolox inhibited the free radical attack, as seen by a delay in the loss of fluorescence, and therefore a decrease in the slope of the curve. The reaction kinetics differed greatly between the phenolic compounds (Fig. 2) with a limited number (chlorogenic, *p*-coumaric and vanillic acids) exhibiting a concentration-dependent inhibition of the fluorescence decay over the entire concentration range from 0.06 to 2 mM. This differential behaviour of the antioxidants suggests that different reaction mechanisms may be operative, depending on the antioxidant concentration. It is also likely that the reaction products derived from the phenolic compounds differ and this aspect is being investigated.

A common practice is to report data as either a Trolox equivalents (Wang et al., 1996) or as some measure of the lag phase induced by the antioxidant (Guo, Cao, Sofic, & Prior, 1997). However, a lag phase was not observed experimentally, which has also been reported previously (Cao, Verdon, Wu, Wang, & Prior, 1995). Results are reported as T_{50} values, i.e. the time required for the fluorescence to decay to 50% of its original value where a larger T_{50} indicates better antioxidant function.

The T_{50} is concentration-dependent as shown in Fig. 3. For comparison, the T_{50} value of trolox, at a concentration 5×10^{-4} M, was 108 min. The antioxidant behaviour divides the compounds into three groups as 2,4-dihydroxybenzoic acid, gallic acid, vanillic acid and oleuropein; 4-hydroxybenzoic acid, caffeic acid, cinnamic acid, tyrosol, naringin and naringenin; *p*-coumaric acid and chlorogenic acid. In the first group, the T_{50} of the phenolic compound is greater than or equal to that of Trolox, at all concentrations, while, in the second group, it is less than the corresponding value for trolox at all concentrations. Thus, the first group of compounds show greater antioxidant activity than Trolox while that of the second group is lower than Trolox. In the case of *p*-coumaric acid and chlorogenic acids, their relative effectiveness is determined by concentration.

There is a significant difference from the behaviour seen in the linoleic system. Three of the most effective antioxidants in the lipid system, namely, caffeic acid, tyrosol and naringin, are now in Group 2 and exhibit minimal activity. On the other hand, 2,4-dihydroxybenzoic, gallic and vanillic acids now exhibit significant activities compared with the relatively low activity seen in the lipid system. Salah, Miller, Paganga, Tijburg, Bolwell, and Rice-Evans (1995) also showed that gallic acid has significant activity against radicals generated in the aqueous phase. However, their observation that the compounds with the most hydroxyl groups exerted the greatest activity was not substantiated in the present study. In the case of flavonoids, increasing the number of hydroxyl groups does not necessarily enhance activity (Rice-Evans, Miller, & Paganga, 1996). The monohydroxy derivative, 4-hydroxybenzoic acid, showed no activity against peroxyl radicals generated in the aqueous phase, consistent with the electron withdrawing capacity of the para-carboxyl functional group. In contrast, dihydroxylation in the *ortho* and *para* position

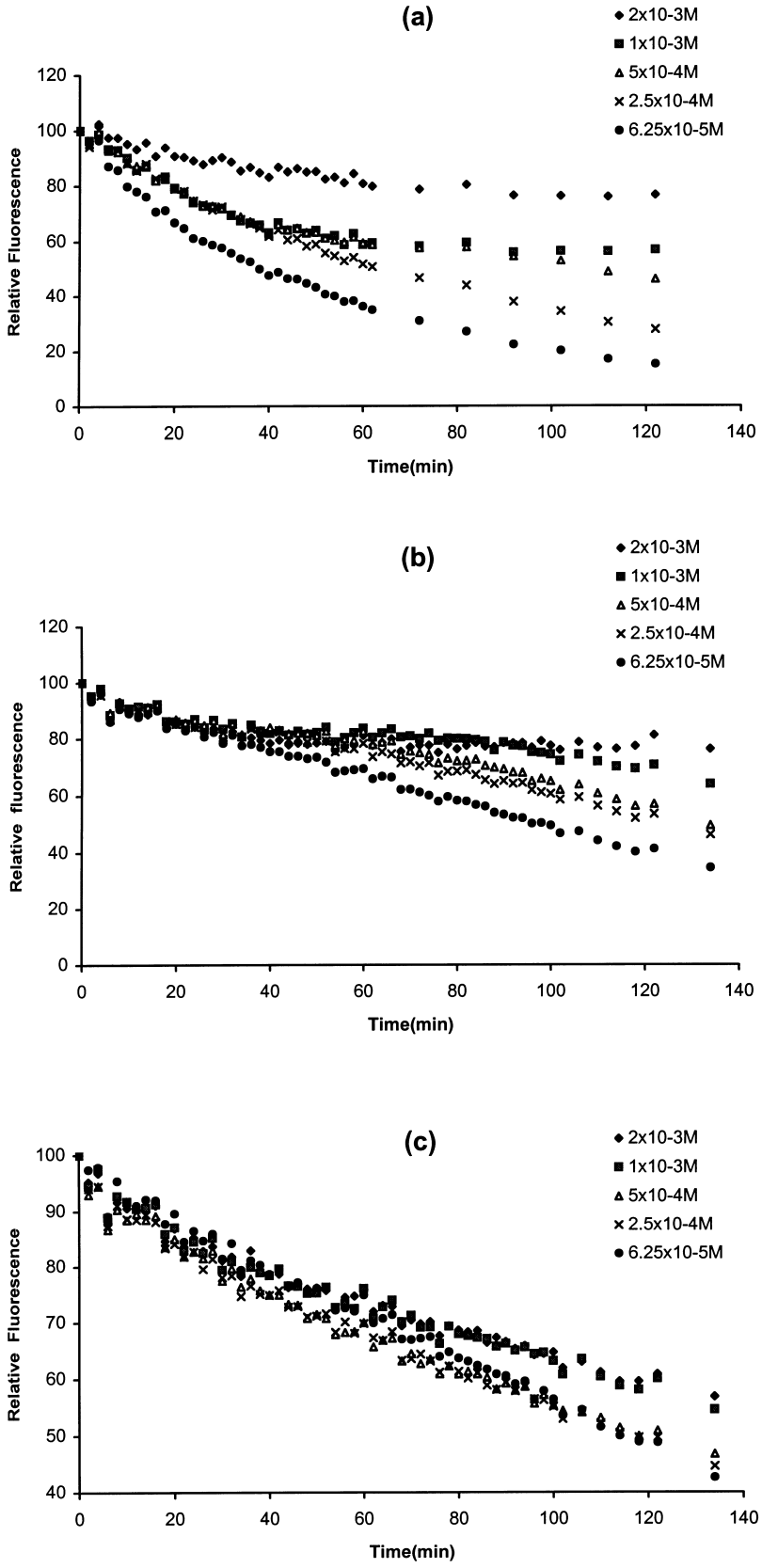

Fig. 2. Kinetics of the AAPH-initiated decomposition of phycoerythrin for representative compounds (a) chlorogenic acid, (b) gallic acid and (c) 2,4-dihydroxybenzoic acid.

(2,4-dihydroxybenzoic acid) enhanced activity relative to the monohydroxy derivative. Insertion of an ethylenic group in *p*-coumaric acid enhanced activity relative to 4-hydroxybenzoic acid, as did the presence of the phenolic group (*p*-coumaric acid versus cinnamic acid).

The olive extract exhibited antioxidant activity in this system (Fig. 3) and this was seen in the individual fractions, primarily in Fractions 5, 2 and 3, consistent with the phenolic content of these fractions and the relatively high concentration of oleuropein in Fraction 5. The latter showed the same concentration-dependent behaviour as seen with the oleuropein standard. There is some distinction in the behaviour in the aqueous system. The later-eluting and more lipophilic Fraction 5 exhibited greater relative activity in the aqueous system where, at lower concentrations (Fig. 3), it has the highest activity of the fractions and extract.

3.5.2. Copper(II)-ascorbate mediated oxidation

The copper(II) ascorbate initiated decay of fluorescence of PE showed a T_{50} of 11 min, whereas Trolox inhibited formation of hydroxyl free radicals by scavenging of the radicals giving a T_{50} value of 23 min at a Trolox concentration of 5×10^{-4} M. Of the phenolic compounds, vanillic acid, gallic acid, 2,4-dihydroxybenzoic acid and oleuropein most effectively scavenged hydroxyl radicals (Table 4), as shown by T_{50} values approximating that of Trolox at similar concentrations. This is consistent with their activity in the peroxyl radical system. These compounds were amongst the lowest in activity in the lipid system. The olive extract inhibited oxidation of PE via scavenging of hydroxyl radicals only at the highest concentration of 20,000 mg l^{-1}. The individual fractions inhibited oxidation at all concentrations examined but it was only in Fractions 2 and 3 at 20,000 mg l^{-1} that significant activity was observed. Fraction 4 exhibited a pro-oxidant effect at concentrations exceeding 20,000 mg l^{-1} (data not reported) and this was the only fraction where this was observed.

In comparing the effectiveness of antioxidants in the two aqueous systems, it is apparent that similar trends are observed. As noted above, 2,4-dihydroxybenzoic acid, gallic acid, vanillic acid, and oleuropein were effective free radical scavengers in both hydroxyl and peroxyl systems. Compounds that showed little activity in the peroxyl system also showed little activity in the hydroxyl system (4-hydroxybenzoic acid, caffeic acid, cinnamic acid, tyrosol, naringin and naringenin). Chlorogenic acid showed concentration dependent antioxidant activity in the peroxyl system, and approached the activity of Trolox at higher concentrations in the hydroxyl system. On the other hand, *p*-coumaric acid was significantly less active against hydroxyl radicals than peroxyl radicals.

Table 4
Inhibition of the copper(II)-ascorbate mediated oxidation of phycoerythrin. Results are expressed as T_{50} values for different concentrations of phenolic compound or olive extract

Compound	T_{50} (min)[a]				
	Concentration 1	Concentration 2	Concentration 3	Concentration 4	Concentration 5
Caffeic acid	11	12	12	12	12
Chlorogenic acid	19	17	16	16	14
Cinnamic acid	16	16	16	20	20
p-Coumaric acid	13	14	14	16	15
Gallic acid	23	23	23	19	19
2,4-Dihydroxybenzoic acid	19	20	19	22	19
4-Hydroxybenzoic acid	21	16	15	16	15
Oleuropein	20	20	21	19	20
Tyrosol	11	12	11	13	12
(±)-Naringenin	16	16	16	16	15
Naringin	16	16	16	16	15
Vanillic acid	23	20	23	20	21
Extract	22	13	13	12	13
Fraction 1	27	17	17	16	16
Fraction 2	140	22	18	18	18
Fraction 3	100	21	19	19	19
Fraction 4	25	20	20	20	20
Fraction 5	33	22	20	20	18
Fraction 6	22	22	18	20	20

[a] Data are reported as the mean of triplicate determinations (± 2 min). Concentrations 1–5 correspond to an antioxidant concentration of 1×10^{-3} M, 5×10^{-4} M, 2.5×10^{-4} M, 1.25×10^{-4} M, 6.25×10^{-5} M phenolic compound, respectively, or 20,000 mg l^{-1}, 2000 mg l^{-1}, 200 mg l^{-1}, 20 mg l^{-1}, 2 mg l^{-1} of olive extract/fraction, respectively.

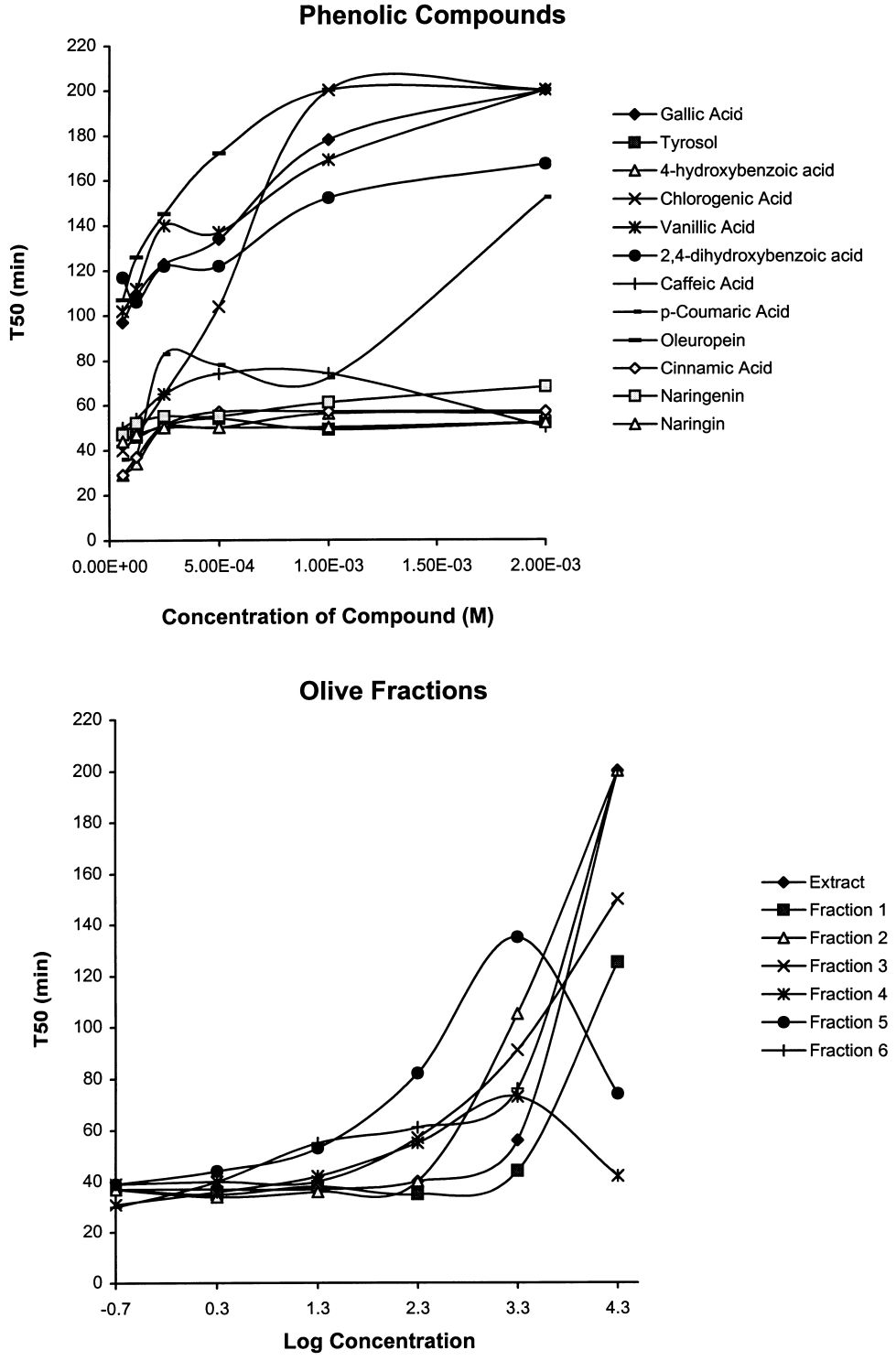

Fig. 3. Effect of antioxidant concentration on T_{50} as determined in a peroxyl radical system generated by AAPH from phycoerythrin.

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