Extraction and Identification of Natural Antioxidant from *Sideritis euboea* (Mountain Tea)

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The dried aerial parts of the mountain tea *Sideritis euboea* were extracted using *n*-hexane, methanol, diethyl ether, ethyl acetate, and *n*-butanol. The residues were tested for their antioxidant activity on sunflower oil at 50 °C under UV light. The oxidation of the sunflower oil was measured using PV, absorbance $E_{1cm}^{1\%}$, and malondialdehyde by high-performance liquid chromatography (HPLC). The butanol extract showed the highest antioxidant activity and was further fractionated by silica and cellulose column chromatography and finally by HPLC. The activity of the final fraction on a range of vegetable oils was compared to that of common used antioxidants (BHT, α-tocopherol) using DPPH•, the Rancimat method, and the Schaal oven test. At a level of 400 ppm, the extracted kaempherol showed the highest antioxidant activity among all antioxidants tested. The final fraction was identified (using UV, ^{1}H NMR, ^{13}C NMR, mass spectroscopy, and melting point) as 3,5,7,4′-tetrahydroxy flavone (kaempherol).

KEYWORDS: *Sideritis euboea*; mountain tea; natural antioxidants; isolation; ^{1}H NMR; ^{13}C NMR; melting point; ultraviolet absorption spectroscopy; 3,5,7,4′-tetrahydroxy flavone; kaempherol

INTRODUCTION

The growing consumer preference for "natural" products forces the fats and oils industry increasingly to seek natural sources for antioxidants rather than investing in synthetic ones. Doubts about the safety and use of synthetic antioxidants first arose in the early 1960s. Toxicological evaluations of butylated hydroxytoluene (BHT) and other synthetic antioxidants have been considered in several WHO/FAO publications (*1*). Butylated hydroxyanisole (BHA) and BHT, the most widely used antioxidants, have unsurpassed efficiency in various food systems besides their high stability, low cost, and other practical advantages. However, their use in food has been declining due to suspected action as a promoter of carcinogenesis as well as due to a general rejection of synthetic food additives. Nevertheless, these antioxidants are approved for food use, but because of the above concerns, the level of use is strictly regulated (*1*).

Extracts of many plants, such as spices, herbs, cocoa shells, coffee beans, oats, tea, beans, sesame oil, tomatoes, rose hips, osage orange, amla fruit, onions, peppers, olive leaves, and soybeans, have shown to have various degrees of antioxidant activity in different fats and oils. The activity of these plants and their extracts can be attributed to the presence of flavonoid compounds, phospholipids, tocopherols, and ascorbic acid (*2*).

In the past few years the phytochemistry of the genus *Sideritis labiatae* (mountain tea) has been investigated and various di- and triterpenoids, sterols, coumarins, and flavonoids aglycone and glycosides have been identified. The flavonoids from species growing in the Iberian peninsula and Canary islands have been extensively studied, because extracts of the aerial parts of these species are used in folk medicine for their anti-inflammatory and antirheumatic properties (*3*).

Rios et al. (*4*) reported that flavonoids from *Sideritis javalabrensis* (growing in Spain) are reducing agents able to interact with free radical species (of relevance to autoxidation mechanism). The genus *Sideritis (Labiatae)* is of great botanical and pharmacological interest. More than 10 species grow in Greece, e.g., *Sideritis euboea*, which is found in the Euboea island region near Athens. The dried inflorescences of a number of species of *Sideritis* are used to prepare a popular beverage in the Balkan countries and especially in Greece, the so-called mountain tea (*5*).

The *Sideritis* species are self-growing, hardy, extremely tolerant of drought, and can grow in the rocky soil of Greek mountains. Greek people have used them as flavoring additives and preservatives in olive oil. Since various flavonoids have been identified in *Sideritis* species and it is known that these have high antioxidant activity, it is possible that the *Sideritis euboea* can be used as a source of natural food antioxidants, with economic benefit, especially for Greece.

This study sets out to isolate and identify natural components from the *Sideritis euboea*, which can be used as natural

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antioxidants, and to assess their effects on the storage life of a range of vegetable oils.

EXPERIMENTAL PROCEDURES

Extraction of Plant Material. The method used was adapted from Rios et al. (*4*). The air-dried aerial parts of *Sideritis euboea* (4.8 kg) were defatted with 4 L of *n*-hexane. After filtration, the *n*-hexane was removed with the aid of a Heildolph VV 2000 rotary evaporator and a WB 2000 water bath (Heildolph-Elektro, Kelheim, Germany), equipped with a Büchi B-160 Vacobox pump (Büchi Laboratorius-Technik AG, Flawil, CH 9230, Switzerland) (47 °C, 150 rpm, and 400 mbar vacuum), yielding a brown residue of 38.6 g (fraction S1).

The defatted plant material was then extracted using methanol. The methanol was evaporated using the rotary evaporator (55 °C, 150 rpm, and 300 mbar vacuum), yielding a black gum of 1056 g. This methanol extract was dried in an oven at 60 °C for 2 h, yielding a residue of 1001 g.

The methanol residue (1001 g) was dissolved in 2 L of distilled water, shaken vigorously for five minutes, and extracted with 6 L of diethyl ether for 2 min in a separating funnel. The contents of the funnel were allowed to separate. The top layer (diethyl ether) was removed, and the solvent was evaporated with the aid of the vacuum rotary evaporator (45 °C, 90 rpm, and 300 mbar vacuum). The remaining gum was left at room temperature (30 °C) for 24 h to yield a dark residue of 34.96 g (fraction S2).

The aqueous layer of the separating funnel was extracted with 1800 mL of ethyl acetate. The ethyl acetate layer was removed, and the solvent was evaporated with the aid of rotary evaporator (55 °C, 90 rpm, and 250 mbar vacuum). The remaining gum was transferred to a vacuum oven at 60 °C for 2 h. After drying, the remaining brown residue weighed 29.18 g (fraction S3).

The remaining aqueous layer of the separating funnel was extracted with 1800 mL of saturated *n*-butanol. The organic layer was removed, and the solvent was evaporated with the aid of vacuum rotary evaporator (80 °C, 90 rpm, and 150 μbar vacuum). The remaining gum was transferred to a vacuum oven at 80 °C for 2 h. After drying, a residue of 171 g was collected (fraction S4).

Column Chromatography Using Silica of Fraction S4. The method used was adapted from Rios et al. (*4*). The fraction S4 (171 g) was chromatographed on a glass column (3 cm inside diameter (i.d.) and 50 cm in length) filled with silica (Kieselgel 60, 70-230 Mesh, Merck Ltd, Darmstand, Germany). The elution solvent was chloroform: methanol (7:3). In total, 59 samples of 50 mL were collected in dark glass bottles.

Thin-Layer Chromatography (TLC) Using Silica Plates. The method was adapted from Rios et al. (*4*). The 59 fractions from silica column chromatography were monitored by TLC, using plates (20 × 20 cm²) precoated with Silica gel F254 (layer thickness 0.25 mm, Merck Ltd, Darmstand, Germany). The mobile phase was chloroform:acetic acid:methanol (90:5:5). After completion of the run, the plate was dried thoroughly in the oven at 105 °C for 3 min and, after cooling, was sprayed using a 1% methanolic solution of diphenylboric acid 2-amino ethyl ester (C₄H₁₆BNO—Naturstoffreagenz A or Neu's reagent, Sigma Chemicals Co., St. Louis, USA), which is adequate for identification of flavonoids by the color produced (*1*). The developed spots were observed under UV light (366 nm) to identify any flavonoids present.

Column Chromatography Using Cellulose of Fraction TLCS3. The fraction TLCS3 was chromatographed on the same glass column filled with microcrystalline cellulose (20—100 μm, Merck Ltd, Darm-stand, Germany). The elution solvent was acetic acid:water (15:85). In total 22 samples of 25 mL each were collected in dark glass bottles.

Thin-Layer Chromatography (TLC) Using Cellulose Plates. The 22 fractions from cellulose column chromatography were monitored by TLC, using plates (20 × 20 cm²) precoated with cellulose (layer thickness 0.1 mm, Merck Ltd). The mobile phase was acetic acid:water (15:85). After run, the plates were dried thoroughly in the oven at 110 °C for 4 min and, after cooling, were sprayed with 1% Neu's reagent. The spots were observed under UV light at 366 nm.

Determination of the Antioxidant Activity of the Isolated Fractions Using the UV-Accelerated Method. The fractions were tested for their antioxidant activity on sunflower oil (C16:0, 6.4%; C18:0, 3.6%; C18:1, 25.1%; C18:2, 63.5%), olive residue oil (C16:0, 10.3%; C16:1, 0.7%; C18:0, 2.9%; C18:1, 72.2%; C18:2, 12.1%; C18:3, 1.3%), corn oil (C16:0, 10.8%; C18:0, 1.8%; C18:1, 28.9%; C18:2, 56.8%; C18:3, 0.7%), and soybean oil (C16:0, 11.0%; C18:0, 3.7%; C18:1, 19.5%; C18:2, 57.2%; C18:3, 7.3%) (all purchased from the local market) using the UV-accelerated method as described by Lalas and Tsaknis (*1*). The extent of oxidation of the oils was assessed using peroxide value (PV), UV absorption of 232 nm (conjugated dienes), and determination of malondialdehyde (MDA) by high-performance liquid chromatography (HPLC). The method used for the determination of PV was adopted from Lea (*6*). The method used for the determination of specific extinction ($E_{1cm}^{1\%}$) at 232 nm (conjugated dienes) was adopted from IUPAC (*7*). The method used for the determination of MDA by HPLC was adopted from Tsaknis et al. (*8*) HPLC was performed using a Waters System consisting of a Waters 600E HPLC pump and a Waters 486 Tunable Absorbance Detector (Millipore Corporation, Waters Chromatography Division, Massachusetts, MA 01757, USA) (detection limit: 6.3×10^{-8} mol/kg of oil). The system was equipped with a Waters μ-bondapack C18 (3.9 mm × 30 cm) column. In all methods the antioxidant activity of the various fractions was compared to commercial antioxidants including α-tocopherol and BHT (both purchased from Sigma Chemicals Co., St. Louis, USA).

Preparative-Scale HPLC Fractionation of Fraction TLCC5. The method described below is a modification based on the method of Rios et al. (*4*). The fraction TLCC5 (1.63 g) was diluted to 5 mL with methanol in a glass screw-capped test tube. The fractionation was carried out with a mobile phase made up of solvent A (methanol and trifluoroacetic acid, 99.95:0.05 v/v) and solvent B (water and trifluoroacetic acid, 99.95:0.05 v/v), using an HPLC Waters 600E HPLC pump set to deliver the mobile phase at a flow rate of 1.9 mL/min through a Waters Zorbax RX-C18 semipreparative column (9.4 mm × 250 mm, Zorbax porous silica microsphere, particle size 5 μm). The elution profile was: 0 min, 70% B in A (linear gradient); 15 min, 60% B in A (linear gradient); 30 min, 50% B in A (linear gradient); 52 min, 40% B in A (linear gradient). Eluting species were detected with a Waters 486 Tunable Absorbance Detector set at 255 nm. Ten samples of 0.5 mL were injected onto the column. The column pressure was 2200 ± 20 psi. Data from the detector were automatically integrated using a computer integrator running Waters Baseline 815 software.

Six fractions were collected from each injection, with the aid of a Waters Fraction Collector, in 100-mL dark glass bottles, and those with the same retention time were combined and named according to their order of exit as HPLC 1—6. The solvents of the above fractions were removed with the aid of a vacuum rotary evaporator (120 mbar, 80 °C). The residues of the six fractions were tested for their antioxidant activity (using the above-described UV-accelerated method), and the purity of the compound with the highest antioxidant activity (HPLC3) was checked with a Waters Zorbax RX-C18 analytical column (4.6 mm × 250 mm, Zorbax porous silica microsphere, particle size 5 μm), using the same conditions as described above for the semipreparative column but with a flow rate of 1 mL/min (5 mg of this compound was dissolved with 5 mL of methanol). This solution was injected onto the column through a 20-μL loop of the Reodyne valve and only one peak was detected.

Determination of the Antioxidant Activity of Fraction HPLC4 using the Rancimat Method. The method used to test the antioxidant of isolated fractions was a modification of the Lalas and Dourtoglou (*2*) method. Sunflower oil (2.5 mg) and added fractions (the weight of the fractions was designed to give a final concentration in the sunflower oil of either 200 or 400 ppm) were accurately weighed into the reaction vessel of the Rancimat 679 (Metrhom LTD, Herisau, CH 9101, Switzerland) along with another vessel with a sample of sunflower oil without antioxidant (control). Absolute ethanol (5 mL) was added in each of the vessels and mixed well. The conditions were set at 110 °C and 20 L/h. The protection factor (PF) was calculated as PF = (induction period with antioxidant)/(induction period without antioxidant).

Determination of Hydrogen Donation Ability of Fraction HPLC4. The method used was adapted from Yen and Hsieh (*9*). Methanolic 2,2-diphenyl-1-picrylhydrazyl (1.0 mL, 0.01 mM, DPPH•) solution was

Natural Antioxidants from *Sideritis euboea*

J. Agric. Food Chem., Vol. 53, No. 16, 2005 **6377**

added to 4.0 mL of sample solution. The mixture was shaken vigorously and left to stand for 30 min at 25 °C (±0.8 °C). The absorbance (A) of this solution was measured at 517 nm against a control comprising of 4.0 mL of methanol and 1.0 mL of 0.1 mM methanolic DPPH$^\bullet$ solution. The absorbance (B) and (C) at 517 nm of the sample solution and the DPPH$^\bullet$ solution against 5 mL methanol was also measured.

The results were calculated using the following formula: % disappearance = $(A + B)/(C) \times 100$

where A is absorbance of DPPH$^\bullet$ against sample + DPPH$^\bullet$, B is absorbance of sample against methanol, and C is absorbance of DPPH$^\bullet$ against methanol.

Schaal Oven Test of Fraction HPLC4. The method described below is a modification based on the method of Kiritsakis et al. (*10*). Oil (10 g) and the added antioxidant were accurately weighed into a glass Petri dish (87 mm i.d. and 15 mm in height). Absolute ethanol (5 mL) were added and mixed with the aid of a glass rod. The Petri dishes were placed into the oven and held at a constant temperature of 65 °C for 6 days. The oils were then transferred to 50-mL dark glass bottles, to which nitrogen was added, and then stored at −20 °C until required for analysis.

Identification of Compound HPLC4. The identification of fraction HPLC4 was done using the following methods: ^{1}H NMR, ^{13}C NMR, mass spectroscopy (MS), melting point (mp), and UV absorption spectroscopy.

For the ^{1}H NMR and ^{13}C NMR determinations, the sample was prepared according to El-Ansari et al. (*11*), and 25 mg of sample were dissolved in 0.5 mL of hexadeuteriodimethylsulfoxide (DMSO). The solution was then analyzed using a Varian spectrometer model Gemini 2000 (Varian, Palo Alto, California, USA) at 300 MHz.

The positive electron impact (EI) method used for MS was adapted from Hedin and Phillips (*12*). Spectra were taken at 70 eV in the EI mode with a VG Mass-Lab 20-250 automated mass spectrometer (VG MassLab, Manchester, United Kingdom). The sample was introduced into the source of the instrument via a direct insertion probe. The source was maintained at a temperature of 200 °C, and the probe was ballistically heated from ambient temperature to 300 °C.

The method used for the determination of the mp was adapted from Markham (*13*) and carried out on a 535 Büchi apparatus.

The method used for UV spectroscopy was adapted from Markham (*13*) and carried out on a Hitachi U-3210 spectrophotometer (Hitachi Co., Tokyo, Japan).

RESULTS AND DISCUSSION

Extraction of Fractions from *Sideritis euboea*. Five solvents (*n*-hexane, methanol, diethyl ether, ethyl acetate, and *n*-butanol) were used in order to separate the flavonoids from the plant material (4800 g), according to their polarity, yielding four fractions: S1 (*n*-hexane residue, 38.60 g), S2 (diethyl ether residue, 34.96 g), S3 (ethyl acetate residue, 29.18 g), and S4 (*n*-butanol residue, 171.00 g).

Antioxidant Activity of Extracts. The four fractions (S1−S4) isolated from the plant material were tested for their antioxidant activity on sunflower oil, which was oxidized using the UV-accelerated method. The methods used to measure the extent of lipid oxidation of the sunflower oil were PV, absorbance $E_{1cm}^{1\%}$ at 232 nm, and determination of MDA by HPLC. None of the above methods can judge oil deterioration adequately in all situations on its own. Although, the combination of them can assess the extent of oxidation in both the beginning (primary products: PV and specific extinction at 232 nm) and the end (secondary products: MDA) (*1*). The antioxidant activity of the various fractions was compared to commercial antioxidants including α-tocopherol and BHT. The results (**Table 1**) indicated that the samples of sunflower oil containing 200 and 400 ppm of the *n*-butanol fraction (S4) showed the least oxidation. Therefore, this fraction seemed to be a better antioxidant than BHT and α-tocopherol at a level of 200 and 400 ppm.

Table 1. Antioxidant Activity of Fractions of *Sideritis euboea* Using Sunflower Oil[a]

sample	PV (mEq O_2 kg^{-1} oil)	$E_{1cm}^{1\%}$	MDA by HPLC (mol MDA kg^{-1} oil)
sunflower oil (SO)	324.1 (1.7)	36.23 (0.03)	5.67×10^{-6} (0.055)
SO + 200 ppm S1	276.3 (1.3)	30.67 (0.05)	4.71×10^{-6} (0.093)
SO + 400 ppm S1	254.1 (0.5)	27.01 (0.04)	4.30×10^{-6} (0.054)
SO + 200 ppm S2	264.4 (0.7)	29.54 (0.08)	4.47×10^{-6} (0.016)
SO + 400 ppm S2	243.3 (0.9)	26.61 (0.06)	4.06×10^{-6} (0.271)
SO + 200 ppm S3	211.5 (1.5)	23.06 (0.05)	3.34×10^{-6} (0.036)
SO + 400 ppm S3	184.2 (0.9)	19.60 (0.09)	2.83×10^{-6} (0.003)
SO + 200 ppm S4	141.7 (0.8)	13.98 (0.03)	2.05×10^{-6} (0.007)
SO + 400 ppm S4	123.5 (1.6)	11.82 (0.06)	1.60×10^{-6} (0.022)
SO + 200 ppm BHT	152.1 (1.0)	16.13 (0.03)	2.20×10^{-6} (0.016)
SO + 200 ppm α-tocopherol	163.4 (0.9)	17.30 (0.04)	2.47×10^{-6} (0.070)
sunflower oil (SO)	307.7 (2.5)	32.06 (0.03)	5.26×10^{-6} (0.091)
SO + 200 ppm TLCS1	139.1 (1.0)	13.98 (0.06)	1.94×10^{-6} (0.048)
SO + 200 ppm TLCS2	165.4 (0.7)	16.79 (0.07)	2.56×10^{-6} (0.154)
SO + 200 ppm TLCS3	94.2 (0.6)	8.17 (0.05)	1.06×10^{-6} (0.051)
SO + 200 ppm TLCS4	144.2 (0.6)	14.87 (0.01)	2.07×10^{-6} (0.048)
SO + 200 ppm TLCS5	158.7 (1.5)	16.09 (0.05)	2.29×10^{-6} (0.135)
SO + 200 ppm TLCS6	198.8 (0.5)	20.83 (0.04)	3.22×10^{-6} (0.073)
SO + 200 ppm TLCS7	152.8 (0.7)	15.29 (0.08)	2.16×10^{-6} (0.091)
SO + 200 ppm BHT	142.8 (1.9)	12.17 (0.01)	1.94×10^{-6} (0.036)
SO + 200 ppm α-tocopherol	155.3 (2. 9)	15.86 (0.01)	2.18×10^{-6} (0.048)
sunflower oil (SO)	334.4 (2.1)	37.16 (0.07)	5.74×10^{-6} (0.018)
SO + 200 ppm TLCC1	262.2 (2.1)	28.16 (0.07)	4.30×10^{-6} (0.112)
SO + 200 ppm TLCC2	218.3 (1.3)	23.64 (0.07)	3.78×10^{-6} (0.123)
SO + 200 ppm TLCC3	245.4 (1.4)	25.94 (0.04)	3.81×10^{-6} (0.176)
SO + 200 ppm TLCC4	188.3 (2.5)	19.38 (0.05)	2.85×10^{-6} (0.124)
SO + 200 ppm TLCC5	134.2 (1.1)	12.53 (0.03)	1.52×10^{-6} (0.101)
SO + 200 ppm TLCC6	174.5 (0.7)	17.28 (0.06)	2.45×10^{-6} (0.070)
SO + 200 ppm TLCC7	196.7 (1.2)	19.46 (0.04)	2.88×10^{-6} (0.060)
SO + 200 ppm TLCC8	204.3 (1.9)	22.04 (0.03)	3.24×10^{-6} (0.065)
SO + 200 ppm BHT	140.3 (1.4)	13.00 (0.05)	1.70×10^{-6} (0.131)
SO + 200 ppm α-tocopherol	166.1 (1.7)	17.00 (0.04)	2.42×10^{-6} (0.093)
sunflower oil (SO)	329.6 (1.2)	35.73 (0.03)	5.71×10^{-6} (0.237)
SO + 200 ppm HPLC1	214.8 (1.8)	22.84 (0.08)	3.01×10^{-6} (0.035)
SO + 200 ppm HPLC2	198.3 (1.8)	19.76 (0.05)	2.77×10^{-6} (0.060)
SO + 200 ppm HPLC3	227.6 (0.7)	23.89 (0.07)	3.37×10^{-6} (0.105)
SO + 200 ppm HPLC4	153.2 (2.6)	15.28 (0.06)	2.17×10^{-6} (0.098)
SO + 200 ppm HPLC5	219.3 (1.7)	22.90 (0.02)	3.81×10^{-6} (0.091)
SO + 200 ppm HPLC6	204.2 (3.0)	20.09 (0.05)	2.92×10^{-6} (0.154)
SO + 200 ppm BHT	141.2 (1.3)	14.55 (0.01)	2.00×10^{-6} (0.103)
SO + 200 ppm α-tocopherol	172.1 (2.2)	16.98 (0.01)	2.38×10^{-6} (0.094)

[a] Values are means of triplicate determinations. Standard deviations are given in parentheses.

Column and TLC Chromatography Using Silica of Fraction S4. The *n*-butanol fraction (S4) that showed the highest antioxidant activity was further separated by silica column chromatography, and 59 fractions of 50 mL were collected. These fractions were monitored on silica TLC plates, using Neu's reagent to locate the components at 366 nm (*14*). The components of the 59 fractions were classified according to their R_f values, yielding, after combinations, seven fractions which were named TLCS1−7 (**Table 2**). The appropriate combined fractions were concentrated using a vacuum rotary evaporator (150 mbar vacuum, 70 °C).

The seven fractions (TLCS1−7) isolated from the *n*-butanol fraction (S4) were tested for their antioxidant activity on sunflower oil, which was oxidized using the accelerated UV method. The changes of PV, absorption $E_{1cm}^{1\%}$ at 232 nm, and MDA by HPLC indicated that the samples of sunflower oil containing the TLCS3 fraction showed the lowest increase in oxidation (**Table 1**).

The fraction TLCS3, which showed the highest activity, consisted of three zones (**Table 2**). There is a relationship between the spot colors and flavonoids structure. The blue-green color has been observed in flavonols with a free 3-OH and with or without a free 5-OH. The deep-purple color has been observed in flavones with a 5-OH and a 4′-OH or for 3-OH substituted

Table 2. Classification of Fractions from Column Chromatography According to Their R_f Values

fraction	R_f values	combined fractions	amount (g)	observed colors at 366 nm[a]
		Silica Column Chromatography		
TLCS1	0.93–0.97	1–4	30.6	blue-green, yellow, light-blue
TLCS2	0.59–0.61	5–8	29.0	brown, red, green, yellow
TLCS3	0.41–0.45	9–10	9.6	blue-green, deep-purple, pale-yellow
TLCS4	0.28–0.34	11–12	2.4	orange, red
TLCS5	0.23–0.25	13–20	5.2	dark, blue, green
TLCS6	0.16–0.22	21–31	1.5	brown
TLCS7	0.12–0.16	32–59	2.7	orange, red
		Cellulose Column Chromatography		
TLCC1	0.94–0.98	1–5	2.89	green, weak-blue, yellow, weak-red, orange, green-yellow
TLCC2	0.91–0.93	6–7	1.89	red, green, weak-blue, orange, light-yellow, yellow
TLCC3	0.35–0.38	8–9	0.67	orange, red, green
TLCC4	0.30–0.33	10	1.05	green, light orange, blue
TLCC5	0.25–0.29	11–14	1.63	yellow-green, blue, weak-red
TLCC6	0.17–0.19	15–18	0.77	weak-blue, orange
TLCC7	0.12–0.15	19–20	0.36	yellow-green
TLCC8	0.9–0.11	21–22	0.03	yellow-green

[a] Using Neu's reagent.

flavonols and 4′-OH chalcones lacking B-ring hydroxyl groups, and the pale-yellow color was found to be due to dihydroflavonols lacking a free −OH (*15*).

Column and TLC Chromatography Using Cellulose of Fraction TLCS3. The fraction TLCS3 was further fractionated by cellulose column chromatography, and 22 fractions of 25 mL were collected. These fractions were monitored on cellulose TLC plates, using Neu's spray reagent under UV light at 366 nm. The 22 fractions were classified according to their R_f values, yielding, after combinations, eight fractions that were named TLCC1−8 (**Table 2**). The appropriate combined fractions were concentrated using a vacuum rotary evaporator (150 mbar vacuum, 70 °C).

The above residues were tested for their antioxidant activity on sunflower oil (using the UV-accelerated method). The changes of PV, absorption $E_{1cm}^{1\%}$ at 232 nm, and MDA by HPLC indicated that the samples of sunflower oil containing the fraction TLCC5 showed the smallest increase in extent of oxidation (**Table 1**). Furthermore, this fraction was a better antioxidant when compared with BHT and α-tocopherol.

Fraction TLCC5 when it was observed on the TLC plates, under UV light (366 nm), showed three colored zones, yellow-green, blue, and weak-red. The yellow-green color indicates the presence of 4′-OH flavonoids (*16*).

HPLC of Fraction TLCC5. Fraction TLCC5 was fractionated by HPLC. Six fractions were separated and named HPLC1−6. The solvent was removed using the rotary evaporator, and the amounts (g) and colors (TLC) of residues obtained were HPLC1 (0.061, red), HPLC2 (0.097, pink), HPLC3 (0.069, brown), HPLC4 (0.454, yellow), HPLC5 (0.126, green), and HPLC6 (0.216, light-brown).

The above residues were tested for their antioxidant activity on sunflower oil (using the UV-accelerated method). The changes of PV, specific absorption at 232 nm, and MDA by HPLC indicated that compound HPLC4 (retention time 44.87 min) showed the highest antioxidant activity among the six fractions and α-tocopherol. However, HPLC4 showed a lower antioxidant activity when compared with BHT. This was an interesting result as the original fraction TLCC5 showed a higher antioxidant activity than BHT. One explanation for this effect is that the compounds of TLCC5 act synergistically to give the antioxidant effect. Kikugawa et al. (*17*) reported that, when phenolic antioxidants are used in combination, they can exhibit synergistic effects. BHT, 2-BHA, and mixtures of the two

exhibited different hydrogen donating capabilities to the stable radical, DPPH•. 2-BHA donated 0.75−1.2 hydrogen atoms, and BHT donated much less hydrogen. The synergistic effect of hydrogen donation to DPPH• (2.78 hydrogen atoms) was observed with a combination of 2-BHA and BHT. A synergistic effect was observed with a mixture of tocopherols and BHT, as a result of catalytic activation of the hydrogen donating capability of the BHT by tocopherols. Similar synergistic effects have been observed when 2,6-di-*tert*-butylphenols are combined with 4-methoxyphenol derivatives. Shervin (*18*) reported that phenolic antioxidants act synergistically when used together. Haumann (*19*) reported synergism between protein hydrolysates and BHA, BHT, PG, α-tocopherol, caffeic acid, and hydroquinone.

The purity of the compound HPLC4 was checked using a Waters Zorbax RX C-18 analytical column, and only one peak was detected by UV detector at 255 nm.

Hydrogen Donation Ability of Fraction HPLC4. The antiradical activity of fraction HPLC4 was compared with that of the commonly used antioxidants BHT, BHA, and propyl gallate using the stable radical (DPPH•). The rate percent of disappearance of DPPH• after 30 min was 93.8, 91.0, 90.1, and 59.0 for BHT, HPLC4, BHA, and propyl gallate, respectively. When copper ions (Cu^{2+}) were added, small changes were observed for the BHT, HPLC4, and BHA (95.5, 92.4, and 91.7, respectively), while in contrast the propyl gallate showed a significant increase (79.6) in the hydrogen donation ability. The chelating activity of fraction HPLC4 did not appear to be significant in the hydrogen donation to DPPH• when copper was present.

The above results confirmed that the fraction HPLC4 acted as an H-atom donor to the DPPH• radical. Pratt and Hudson (*20*) reported that the main evidence that the flavonoids work mainly as primary antioxidants is their ability to work equally well in metal catalyzed and unanalyzed systems. Gordon (*21*) suggested also that the primary antioxidants are usually compounds donating a hydrogen atom to lipid free radicals in order to produce a relative stable radical.

The hydrogen-donating capabilities of DPPH• differed among the antioxidants. BHT, BHA, and HPLC4 showed equal effects on the DPPH• radical, while in contrast propyl gallate showed a much higher effect (approximately 30% higher).

Rancimat Method of Fraction HPLC4. The order of effectiveness of HPLC4, BHT, and α-tocopherol on the protec-

Natural Antioxidants from *Sideritis euboea*

J. Agric. Food Chem., Vol. 53, No. 16, 2005 **6379**

Table 3. Antioxidant Activity of Fraction HPLC4 on Sunflower Oil Using the Schaal Oven Test and on a Range of Vegetable Oils Using the UV-Accelerated Method[a]

sample	PV (mEq O_2 kg^{-1} oil)	$E_{1cm}^{1\%}$	MDA by HPLC (mol MDA kg^{-1} oil)
Schaal Oven Test			
sunflower oil (SO)	165.0 (1.5)	14.01 (0.08)	2.13×10^{-6} (0.146)
SO + 200 ppm BHT	76.1 (0.9)	3.65 (0.05)	0.38×10^{-6} (0.073)
SO + 200 ppm α-tocopherol	92.2 (1.7)	5.47 (0.03)	0.38×10^{-6} (0.100)
SO + 200 ppm fraction HPLC4	90.0 (1.9)	5.31 (0.04)	0.62×10^{-6} (0.111)
SO + 400 ppm fraction HPLC4	59.2 (1.6)	1.36 (0.02)	0.12×10^{-6} (0.047)
UV-Accelerated Method on a Range of Vegetable Oils			
sunflower oil (SO)	336.2 (2.7)	36.18 (0.04)	5.92×10^{-6} (0.098)
SO + 200 ppm BHT	148.4 (1.8)	14.28 (0.05)	1.96×10^{-6} (0.048)
SO + 200 ppm α-tocopherol	168.1 (2.3)	16.56 (0.03)	2.45×10^{-6} (0.002)
SO + 200 ppm fraction HPLC4	164.4 (1.3)	15.98 (0.01)	2.31×10^{-6} (0.145)
SO + 400 ppm fraction HPLC4	137.6 (1.4)	12.82 (0.06)	1.93×10^{-6} (0.068)
olive-residue oil (ORO)	225.4 (2.2)	20.08 (0.05)	2.87×10^{-6} (0.125)
ORO + 200 ppm BHT	175.3 (2.0)	14.95 (0.03)	1.72×10^{-6} (0.004)
ORO + 200 ppm α-tocopherol	197.7 (1.7)	16.32 (0.04)	2.26×10^{-6} (0.093)
ORO + 200 ppm fraction HPLC4	186.2 (1.9)	15.51 (0.06)	2.18×10^{-6} (0.006)
ORO + 400 ppm fraction HPLC4	162.1 (2.3)	12.79 (0.03)	1.54×10^{-6} (0.167)
corn oil (CO)	290.1 (1.2)	31.73 (0.07)	5.56×10^{-6} (0.005)
CO + 200 ppm BHT	227.2 (1.8)	25.07 (0.06)	4.18×10^{-6} (0.006)
CO + 200 ppm α-tocopherol	252.8 (1.4)	26.91 (0.04)	4.86×10^{-6} (0.004)
CO + 200 ppm fraction HPLC4	241.7 (2.4)	25.41 (0.03)	4.50×10^{-6} (0.041)
CO + 400 ppm fraction HPLC4	209.5 (1.0)	23.44 (0.07)	4.12×10^{-6} (0.017)
soybean oil (SBO)	358.1 (2.8)	40.15 (0.04)	7.49×10^{-6} (0.009)
SBO + 200 ppm BHT	244.2 (2.4)	26.34 (0.09)	5.30×10^{-6} (0.014)
SBO + 200 ppm α-tocopherol	279.6 (1.4)	30.67 (0.05)	6.12×10^{-6} (0.023)
SBO + 200 ppm fraction HPLC4	263.8 (2.0)	29.39 (0.01)	5.31×10^{-6} (0.007)
SBO + 400 ppm fraction HPLC4	226.8 (1.5)	25.02 (0.08)	4.57×10^{-6} (0.098)

[a] Values are means of triplicate determinations. Standard deviations are given in parentheses.

tion of sunflower oil as determined by the Rancimat method was found to be as follows: 400 ppm HPLC4 > 200 ppm BHT > 200 ppm HPLC4 > 200 ppm α-tocopherol > 400 ppm α-tocopherol > 600 ppm HPLC4. The PF values were 1.79, 1.56, 1.29, 1.23, 1.11, and 1.07, respectively. Fraction HPLC4 showed a much lower PF value at a level of 600 ppm (PF = 1.07).This was also observed with the α-tocopherol at a level of 400 ppm (PF = 1.11).

It has been observed that the activity of some phenolic antioxidants does not increase linearly with increasing concentration. At sufficiently high levels of addition it may even become a pro-oxidant. Among the natural antioxidants, this fact has to be considered mainly with α- and β-tocopherol. At room temperature, α-tocopherol does not show pro-oxidant activity below approximately 600−700 mg/kg fat. When the temperature is increased, the formation of antioxidant radicals accelerates more rapidly than the autoxidation of the substrate. Consequently, the antioxidant becomes a pro-oxidant through the increased decomposition of hydroperoxides. When the antioxidant concentration is increased the number of antioxidant radicals formed exceeds the number of available free and peroxide radicals (*22*).

Schaal Oven Test of Fraction HPLC4. The effect of the antioxidants BHT and α-tocopherol each at a level of 200 ppm and the HPLC4 at a level of 200 and 400 ppm on the rate of deterioration of sunflower oil was studied using the Schaal oven test at 65 °C for 6 days (144 h).

The changes of PV, absorption $E_{1cm}^{1\%}$ at 232 nm, and MDA concentration by HPLC indicated (**Table 3**) that the samples of sunflower oil containing HPLC4 at a level of 400 ppm showed the highest antioxidant activity among all the antioxidants tested, while the fraction HPLC4 at a level of 200 ppm had a lower antioxidant activity than that of 200 ppm BHT but a higher antioxidant activity than 200 ppm α-tocopherol.

UV-Accelerated Method on a Range of Vegetable Oils. The comparative effects of the antioxidants BHT, α-tocopherol, and HPLC4 with respect to retardation of autoxidation of sunflower oil, olive residue oil, corn oil, and soybean oil, at a temperature of 50 °C, under UV light for 12 h were studied. The results (**Table 3**) confirmed that the order of effectiveness of the added antioxidants increase as follows: 400 ppm fraction HPLC4 > 200 ppm BHT > 200 ppm fraction HPLC4 > 200 ppm α-tocopherol. The results shown that the fraction HPLC4 at a range of 400 ppm gave the highest protection and α-tocopherol the lowest. The results are in line with those of the Rancimat method.

The rate of oxidation of the above oils, as shown by the change in PV, absorption $E_{1cm}^{1\%}$ at 232 nm, and MDA by HPLC was lower in olive-residue oil than in corn oil, sunflower oil, and soybean oil. The explanation is that the rate of oxidation is relating to the degree of unsaturation of the fatty acids present in the vegetable oil; thus linolenic acid with three double bonds is more susceptible than oleic with one double bond (*23*). The monounsaturated fatty acid content of the olive residue oil is about 80% in contrast with soybean oil, which contains only 18−26% monounsaturated fatty acid content, and moreover it contains 5.5−10% linolenic acid, while the range of this acid in olive residue oil is <1.5% (*24*).

The 200-ppm HPLC4 gave the highest protection against autoxidation to the sunflower oil (PF 2.26) following by soybean oil (PF 1.37), olive-residue oil (PF 1.29), and corn oil (PF 1.25) (the PF was calculated using the absorbance $E_{1cm}^{1\%}$ values).

Various tests were then performed to establish the identity of HPLC4.

UV Spectrum. The UV spectrum of the compound HPLC4 dissolved in methanol exhibited two major absorption peaks in the region 240−400 nm (although the UV spectra are reproduced for the range of 220−500 nm, only the maxima for those peaks/shoulders occurring at wavelengths longer than 240 nm are indicated, Marby et al. (*15*). The λ_{max} of the band I (300−380 nm) was 365.8 nm, and the λ_{max} of the band II (240−280 nm) was 266.4 nm. These are in agreement with those reported by Markham and Chari (*25*) for the 3,5,7,4′-tetrahydroxy flavone (kaempherol), which shows λ_{max} of 367 and 266 nm for the bands I and II, respectively.

The methanol spectrum, particularly the position of band I, provides information about the type of flavonoid as well as its oxidation pattern. Band I of flavones occurs in the range 304−350 nm, whereas band I of 3-hydroxyflavones (flavonols) appears at a longer wavelength (352−385 nm) (*14*).

^{1}H NMR and ^{13}C NMR Spectroscopy. The ^{1}H NMR spectrum of fraction HPLC4 showed two doublet protons at the region of 6.20 (H-6, coupling constant, J:2 Hz) and 6.40 (H-8, J:2 Hz) ppm. The protons at C-6 and C-8 of flavonols which contain the common 5,7-dihydroxy substitution pattern give rise to two doublets in the range 6.0−6.5 ppm. The H-6 doublet occurs consistently at higher field than the signal for the H-8 (*15*).

In the same region of the spectrum (6.90 ppm) another two proton signals were also observed which show "coupling" and are bonded to C-5′ and C-3′ atoms. These two doublet protons (3′,5′−H) have a coupling constant of 8 Hz. Fang et al. (*26*) suggested also the existence of H-3′ and H-5′ doublet protons in the downfield near the C-6 atom of flavonoids. Marby et al. (*15*) reported that the doublet for the C-3′ and C-5′ protons appears upfield from the C-2′ and C-6′ protons and generally falls in the range 6.65−7.1 ppm for all types of flavonoids. The

position of the C-2′ and C-6′ doublets appears at lower field (7.1−8.1 ppm) than the C-3′ and C-5′ doublet.

Two doublet protons were also recorded in the NMR spectrum at 8.05 ppm (2′,6′-H) with a coupling constant of 8 Hz. Fang et al. (*26*), also reported these protons in the same region (8.08 ppm) using CCl$_4$ as solvent in the NMR spectrum of 5,7,8,4′-tetrahydroxy-3,6-dimethoxyflavone.

Additionally four single protons were recorded at 9.45, 10.15, 10.82, and 12.47 ppm, which represent four hydroxyl groups bonded with four different carbon atoms. The signal at 12.47 ppm is a typical one for a C-5 hydrogen-bonded hydroxyl group (*15*).

The extracted sample HPLC4 was compared with authentic 3,5,7,4′-tetrahydroxy flavone (Kaempherol) (Fluka AG, Chemicals Fabric, Bucks, Switzerland). Both their spectrums showed an unexpected signal at 5.75 ppm. The explanation is that the solvent used (DMSO) rapidly absorbs atmospheric moisture, and the signal obtained from the absorbent H$_2$O often obscures NMR signals resulting from some of the flavonoid protons (*25*).

When D$_2$O was added to the DMSO solvent of the sample, the proton signals at 9.45, 10.15, 10.82, and 12.47 ppm were not present, indicating that these protons are hydroxyl protons. Markham (*14*) reported that more helpful in the localization of a hydroxyl is the direct correlation of the one proton singlets, which disappear upon addition of D$_2$O, with the hydroxyl protons they represent.

The following signals were observed in the ^{13}C NMR spectrum of HPLC4 δ (ppm): 93.0 (8-C), 97.5 (6-C), 102.05 (10-C), 115.0 (3′-5′-C), 121.0 (1′-C), 129 (2′,6′-C), 135.0 (3-C), 146.5 (2-C), 155.5 (9-C), 160.02 (4′-C), 160.08 (5-C), 163.0 (7-C), 175.0 (4-C).

These results are similar to those reported by Markham and Chari (*25*) for the 3,5,7,4′-tetrahydroxy flavone [(93.5 (8-C), 98.2 (6-C), 103.1 (10-C), 115.4 (3′-5′-C), 121.7 (1′-C), 129.5 (2′,6′-C), 135.6 (3-C), 146.8 (2-C), 156.2 (9-C), 159.2 (4′-C), 160.7 (5-C), 163.9 (7-C), 175.9 (4-C)].

^{13}C−^{1}H coupling data have been used to good effect in the distinction of C-6 from C-8 signals and C-5 from C-9 signals. The degree of coupling identifies each carbon and demonstrates that C-5 resonates downfield from C-9 and that C-6 resonates downfield from C-8 (*25*). The reverse of the order for H-6 and H-8 resonances take place in the ^{1}H NMR (*15*).

The carbonyl carbon, C-4, resonates at around 175−178 ppm, when the carbonyl is not hydrogen bonded, but in the presence of hydrogen-bonding to a 5-hydroxyl group it moves downfield to about 182 ppm. When a 3-hydroxyl is present as well as a 5-hydroxyl, the resonance returns to about 176 ppm, but with the 3-hydroxyl alone, the resonance appears at about 171−173 ppm (*25*).

The HPLC4 spectrum as well as the authentic 3,5,7,4′-tetrahydroxy flavone spectrum showed two unexpected signals at 54.5 and 158.5 ppm due to the DMSO absorbing water. In contrast to proton resonance signals, the intensity of a C-13 resonance signal does not necessarily reflect the number of carbon it represents and so integration of ^{13}C NMR spectra is rarely of value (*25*).

The ^{1}H NMR and ^{13}C NMR spectrums confirmed that the isolated HPLC4 compound was 3,5,7,4′-tetrahydroxy flavone (kaempherol).

MS. The mass spectrum of the extracted HPLC4 fraction showed a value of 286 for the base peak (M$^+$) [molecular ion (3,5,7,4′-tetrahydroxy flavone)] and 258, 229, 213, 184, 153, 136, 121, 105, 93, and 69 for 10 fragments. The same base peak was shown also in the authentic 3,5,7,4′-tetrahydroxy

flavone sample, which showed the same fragments but with higher intensities. One likely reason for this is that the spectrum may be affected by acquired impurities in the sample during the isolation procedure.

Wollenweber (*27*) reported that in the case of flavones and flavonols the silica dissolved from the adsorbent can cause very annoying artifact peaks during mass spectral analysis.

These results are in agreement with those reported by Hedin and Phillips (*12*) who suggested that the fragments with values of 258 ([M − 28]$^+$), 153 ([A$_1$ + 1]$^+$), 121 (B$_2$$^+$), and 229 (corresponding to loss of 57 [C$_2$HO$_2$]$^+$) represent very useful ions which confirm the 3,5,7,4′-tetrahydroxy flavone structure.

mp. The mp of the isolated HPLC4 was shown to be within a range of 273−276 °C, while the mp of the authentic 3,5,7,4′-tetrahydroxy flavone compound obtained from Fluka was in the range of 275−277 °C. When the two samples were mixed, the melting point was 274−276 °C.

If the flavonoids are crystalline, one of the best methods of comparison is by melting point and mixed melting points determination. Pure flavonoids possessing the same or closely similar melting points may or may not be the same compounds, but the melting point of the mixture will be markedly depressed, often 20−30 °C below the melting point of either compound, if the compound are different (*25*).

The rate of bath heating should be very low as the melting point is approached (about 1°/min), otherwise, the temperature of the mercury in the thermometer bulb and the temperature of the crystals in the capillary will each be an unknown amount below the temperature of the bath and probably not equal to each other. This is because of the slowness with which heat energy is transferred by conduction (*28*).

All the above results confirmed the authenticity of HPLC4 as 3,5,7,4′-tetrahydroxy flavone (kaempherol).

In conclusion, the use of kaempherol (consisting of fraction HPLC4 and present in a quantity of 218.75 ppm in the original dried plant material) as a potential natural antioxidant and a possible substitute of artificial ones should be considered. The *Sideritis euboea* is a self-growing plant, hardy, extremely tolerant to drought, and can grow in the rocky soil of Greek mountains. The production and exploitation of a potential natural antioxidant from that species could be of economic benefit.

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