Characterization of the Total Free Radical Scavenger Capacity of Vegetable Oils and Oil Fractions Using 2,2-Diphenyl-1-picrylhydrazyl Radical

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The total free radical scavenger capacity (RSC) of 57 edible oils from different sources was studied: olive (24 brands of oils), sunflower (6), safflower (2), rapeseed (3), soybean (3), linseed (2), corn (3), hazelnut (2), walnut (2), sesame (2), almond (2), mixture of oils for salad (2), "dietetic" oil (2), and peanut (2). Olive oils were also studied according to their geographical origins (France, Greece, Italy, Morocco, Spain, and Turkey). RSC was determined spectrophotometrically by measuring the disappearance of the radical 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^•$) at 515 nm. The disappearance of the radical followed a double-exponential equation in the presence of oils and oil fractions, which suggested the presence of two (fast and slow) groups of antioxidants. RSC was studied for the methanol-soluble phase ("methanolic fraction", MF) of the oil, the fraction nonsoluble in methanol ("lipidic fraction", LF), and the nonfractionated oil ("total oil"; TF = MF + LF). Only olive, linseed, rapeseed, safflower, sesame, and walnut oils showed significant RSC in the MF due to the presence of phenolic compounds. No significant differences were found in the RSC of olive oils from different geographical origins. Upon heating at 180 °C the apparent constant for the disappearance of RSC (k_T) and the half-life ($t_{1/2}$) of RSC for MF, LF, and TF were calculated. The second-order rate constants (k_2) for the antiradical activity of some phenolic compounds present in oils are also reported.

INTRODUCTION

The consumption of fruits, vegetables, red wines, juices, etc. (rich in antioxidant compounds) provides protection against diseases, including cancer and cardio- and cerebrovascular diseases (Ames, 1983; Steinberg et al., 1991; Wang et al., 1996). This protection can be explained by the capacity of these antioxidants to scavenge free radicals, which are responsible for the oxidative damage of lipids, proteins, and nucleic acids (Aruoma, 1998).

There is a consensus of the health-beneficial aspects of the Mediterranean-style diet in which olive oil is the principal source of fat (Assmann et al., 1997). Consumption of olive oil has been reported to be also effective in the prevention of coronary heart disease (CHD) (Baudet et al., 1986; Grundy, 1986; Visioli and Galli, 1995) and against some cancers (Miller et al., 1983; World Health Organization, 1992; Hill et al., 1995).

Antioxidants in oils are important in the stabilization of free fatty acids (Six, 1994; Baldioli et al., 1996). The antioxidant activity of phenols and other compounds present in oils has been well and widely studied by several authors (Gordon and Kourimská, 1995; Baldioli et al., 1996; Litridou et al., 1997; Visioli and Galli, 1998; Visioli et al., 1998; Yoshida and Takagi, 1999).

Despite the above studies regarding the antiradical activity of compounds present in oils, to our knowledge, a kinetic approach to study the free radical scavenger capacity of oils and oil fractions has not been carried out. This kinetic study is based on two assay conditions: initial radical concentration (2,2-diphenyl-1-piucylhydrazyl radical), $[DPPH^•]_i$ > initial antioxidant concentration ($[AH]_i$) (for oils and oil fractions) and $[DPPH^•]_i$ < $[AH]_i$ for standard phenolic compounds that can be present in oils. This study involves the comparison of the total free radical scavenger capacity (RSC) of oils from different sources and origins. The study is carried out by measuring spectrophotometrically the disappearance of the free radical DPPH$^•$ to quantify the RSC of the methanol-soluble fraction (MF), the methanol-nonsoluble fraction (LF), and "total" oil (TF; oil without fractionation, TF = MF + LF). The effect of temperature (180 °C) on RSC of the different oils is also studied. Thin-layer chromatography (TLC) experiments were carried out to corroborate spectrophotometric results and as rapid visualization of the main phenols responsible for the antiradical activity. Moreover, the second-order rate constants (k_2) for the free radical scavenger activity of some phenolic compounds present in oils (protocatechuic acid, oleuropein, hydroxytyrosol, syringic acid, α-tocopherol, sinapic acid, sesamol, and caffeic acid) are reported.

MATERIALS AND METHODS

Reagents. Free stable DPPH radical (DPPH$^•$), sesamol, sinapic acid, syringic acid, protocatechuic acid, caffeic acid,

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tyrosol, phospholipids (phosphatidylcholine, phosphatidyl-ethanolamine, and phosphatidylserine), and α-tocopherol were purchased from Sigma (St. Louis, MO). Oleuropein was acquired from Roth (Karlsruhe, Germany). Hydroxytyrosol was obtained according to the procedure of Soler-Rivas et al. (unpublished results). All other reagents were of analytical grade and also supplied by Sigma. Experiments were always performed on freshly made up solutions. DPPH• was dissolved in either methanol or ethyl acetate. The free radical showed the same molar absorptivity (12500 M^{-1} cm^{-1} at 515 nm; Brand-Williams et al., 1995) and stability in both organic solvents. The radical, in the absence of antioxidant compounds, was stable for >1 h of normal kinetic assay.

Sample Preparation. Oils (57 edible oils) from different sources (14 sources) and origins (France, Greece, Italy, Morocco, Spain, and Turkey, only for olive oils) were purchased in local supermarkets in The Netherlands as well as in their original countries. The different oils were treated with methanol to assay the nonlipidic fraction. Five hundred microliters of the different oils was mixed with 500 μL of methanol. The mixture was vigorously stirred for 1 h and further centrifuged at 3000*g* for 5 min to better separate both methanolic (MF) and lipidic (LF) phases. The total RSC of the MF of the different oils was assayed spectrophotometrically at 515 nm, as described under Spectrophotometric Assays, with DPPH• previously dissolved in methanol.

Different solvents were used to assay the RSC of LF and TF: hexane, chloroform, acetone, ether, and ethyl acetate. The best result was achieved with ethyl acetate, and therefore the RSC of the LF and TF was assayed, as described under Spectrophotometric Assays, with DPPH• previously dissolved in ethyl acetate.

To determine the RSC of TF, the oil was taken directly from the bottle without further treatment. The use of ethyl acetate was critical to dissolve completely the hydrophobic compounds (nonsoluble in methanol) of LF and TF. The initial concentration of antioxidants in the medium was always lower than that of DPPH• ([DPPH•]$_i$ > [AH]$_i$). This condition was fulfilled when some DPPH• concentration remained in the medium at the end of the reaction ([DPPH•]$_r$).

The total RSC was calculated as the DPPH• concentration (micromolar) that was removed by the different volumes of the oils and oil fractions (microliters). Final RSC values corresponded to the mean of at least two separate experiments for at least two different bottles of the same oil (four experiments per oil and oil fraction).

The RSC values of phenolic compounds, which can be present in oils, were also analyzed. Phenolic standards were dissolved in methanol and the phospholipids in ethyl acetate.

Graphs, Mathematical Fits, and Statistical Treatment. Graphs and fitting of the experimental data were carried out by using a Gauss–Newton algorithm (Marquardt, 1963) implemented in the Sigma Plot 2.01 program for Windows (Jandel Scientific, 1994). One thousand data points were taken per spectrophotometric recording of the disappearance of DPPH• in the presence of the MF, LF, TF, and phenolic compounds.

Oils and Oil Fractions. The experimental data for the disappearance of DPPH• in the presence of oil and oil fractions were fitted by nonlinear regression (NLR) to the double-exponential equation

$$\text{DPPH}^\bullet = \text{DPPH}_1^\bullet \, e^{-\lambda_1 t} + \text{DPPH}_2^\bullet \, e^{-\lambda_2 t} + \text{DPPH}_r^\bullet \quad (1)$$

where DPPH• is the radical concentration at any time, the parameters DPPH$_1^\bullet$ and DPPH$_2^\bullet$ are the radical concentrations at time zero for the simple uniexponential equations that form the double exponential, and λ_1 and λ_2 are the first-order rate constants for the simple uniexponential equations that form the double-exponential equation. Finally, DPPH$_r^\bullet$ is the remaining DPPH• concentration in the medium because of the antioxidant depletion (Figure 1).

Second-order rate constants (k_2) were not calculated for the oils and oil fractions because the condition to calculate this
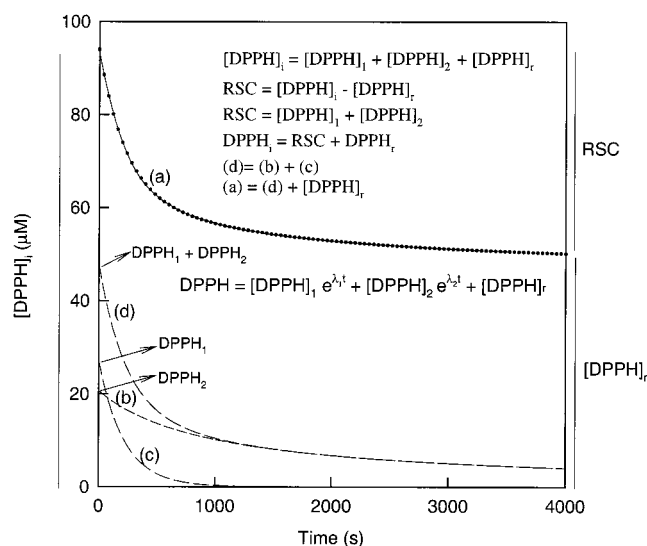

Figure 1. Spectrophotometric recording for the disappearance of DPPH• in the presence of MF of olive oil: curve a, (—) experimental data; (···) NLR fitting of experimental data to eq 1. Curves b and c are the uniexponential curves that form curve a. Curve d = curve b + curve c. Conditions were 93 μM [DPPH•]$_i$ and 5 μL of MF of a Spanish olive oil. λ = 515 nm.

constant ([DPPH•]$_i$ < [AH]$_i$) could not be reached in many of the sample oils.

Initial estimates for the four-parameter curve (eq 1) were carried out according to the method of Ratkowsky (1989). However, the equation proposed to fit the experimental values also accepted "expected-value" parameters as initial estimates (i.e., graphic estimation of the parameters (DPPH$_1^\bullet$, DPPH$_2^\bullet$, λ_1, and λ_2) by using the experimental plot of DPPH• versus time (*t*) (Ratkowsky, 1989).

Commonly accepted fitting criteria for NLR, such as the value of the norm (the lower the norm, the better the fit), was applied in all of the fits to the different equations. Moreover, the best kinetic model function that fits the experimental data will lead to the most even distribution of residuals, that is, the differences between observed and predicted responses (residual values scattered close to 0 value) (Forsyth et al., 1999). Figure 2 shows the NLR fitting of experimental data to different equations (uniexponential, double exponential, and second order) as well as the residual plots depending on the equation chosen.

Phenolic Compounds. Second-order rate constants (k_2) were determined for some phenolic compounds present in oils (protocatechuic acid, syringic acid, oleuropein, hydroxytyrosol, caffeic acid, sesamol, sinapic acid). In this case, under pseudo-first-order conditions, ([DPPH•]$_i$ < [AH]$_i$), DPPH• was depleted in the medium following the equation

$$\text{DPPH}^\bullet = \text{DPPH}_0^\bullet \, e^{-k_{obsd} t} \quad (2)$$

where DPPH• is the radical concentration at any time, DPPH$_0^\bullet$ is the radical concentration at time 0, and k_{obsd} is the pseudo-first-order rate constant. This constant (k_{obsd}) was linearly dependent on the concentration of phenolic compound (antioxidant), and from the slope of this plot, the second-order rate (k_2) was determined (Mukai et al., 1993; Shi and Niki, 1998). Experiments were carried out in triplicate. The initial estimations of DPPH• and k_{obsd}, which are necessary to fit the experimental data by NLR to eq 2, were obtained from the slope and ordinate on the origin of the straight line resulting from the fit by linear regression of the experimental data of DPPH• and *t* to

$$\ln \text{DPPH}^\bullet = \ln \text{DPPH}_0^\bullet - k_{obsd} t \quad (3)$$

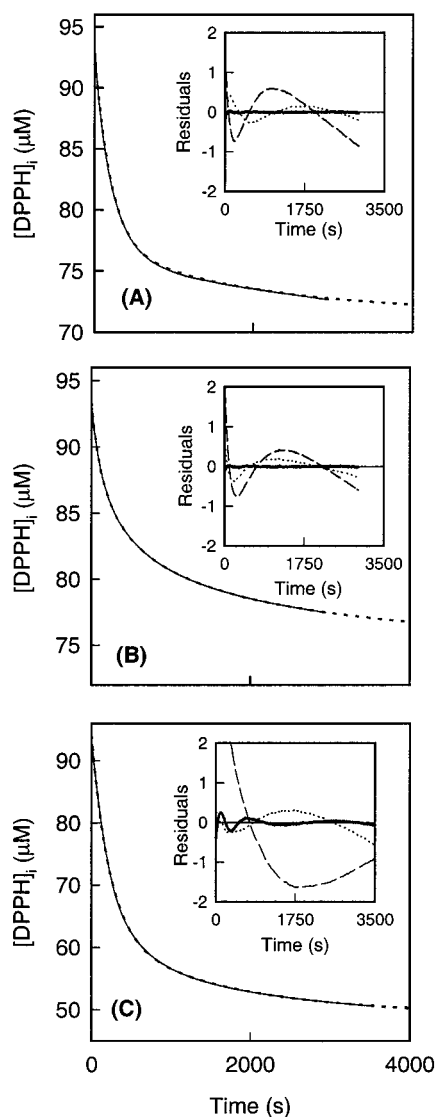**650** *J. Agric. Food Chem.,* Vol. 48, No. 3, 2000

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Figure 2. NLR fitting of experimental data to eq 1 in the scavenging of DPPH• by olive oil: (—) experimental data; (•••) NLR fitting; (A) MF, 93 μM [DPPH•]$_i$, 5 μL of MF of a Spanish olive oil; (B) LF, the same conditions as in MF but 5 μL of LF and [DPPH•]$_i$ dissolved in ethyl acetate; (C) 5 μL of TF, same conditions as in LF. (Insets) In (A–C), insets show the curve fitting according to the residuals values criterion. NLR fittings to a double-exponential equation (—) (eq 1), to a second-order equation (- - -), and to an uniexponential equation (•••). The fitted model is best when the residual values are close to 0.

Spectrophotometric Assays. The determination of the antioxidant capacity of different compounds can be carried out by means of a number of procedures that measure the induction period of lipid oxidation by the Rancimat method to test oxidative stability in oils (Baldioli et al., 1996; Chen and Ho, 1997); the inhibition of hexanal formation during copper-catalyzed human low-density lipoprotein (LDL) oxidation (Meyer et al., 1998); the suppression of light emission in chemiluminescent-based procedures (Whitehead et al., 1992; Mantle et al., 1998); the disappearance of free radicals (ABTS•, DPPH•, galvinoxyl radical, etc.) by the effect of radical scavengers (Brand-Williams et al., 1995; Miller and Rice-Evans, 1996; Sánchez-Moreno et al., 1998; Shi and Niki, 1998), etc. In the present work, kinetic assays were carried out by measuring the disappearance of DPPH• at 515 nm. DPPH• was dissolved in methanol to assay both MF and phenolic compounds and in ethyl acetate to assay LF, TF, or phospholipids. The standard assay mixture for MF, LF, and TF contained 995 μL of methanol or ethyl acetate with DPPH• (93 μM) and 5 μL of the corresponding sample (MF, LF, TF, and phospho-

lipids). When a larger volume of sample was used, the possible dilution in the cuvette was taken into account (the largest volume of sample assayed was 40 μL). DPPH• concentration was 20 μM (970–995 μL) in the assay of the phenolic compounds, the concentration of which ranged from 60 to 300 μM (5–30 μL of different stock concentrations). The final volume in all of the assays was 1 mL. The spectrophotometric assays were recorded in an ultraviolet–visible Perkin-Elmer Lambda-2 spectrophotometer (Überlingen, Germany), on-line interfaced to a Pentium-100 microcomputer (Ede, The Netherlands). Temperature was controlled at 25 °C with a circulating bath with heater/cooler and checked using a precision of ±0.1 °C. The reference cuvette contained all of the components except the radical.

Effect of Temperature. The oils were incubated at 180 °C, which is a common temperature in frying processes (Gordon and Kourimská, 1995). Temperature was controlled by an ETS-D2 digital thermometer (IKA-TRON, The Netherlands). Aliquots were removed after different incubation times and treated with methanol to separate both MF and LF fractions. The effect of the temperature on the RSC was studied on MF, LF, and TF. The experimental data were fitted by NLR to a decreasing uniexponential equation (by using the same software described above)

$$RSC = RSC_0 \, e^{-k_T t} \qquad (4)$$

where RSC is the instantaneous total antiradical capacity, RSC_0 is RSC at time 0, k_T is the apparent first-order rate constant, which describes the velocity of the disappearance of RSC because of the effect of temperature, and t is the incubation time at 180 °C.

The parameter $t_{1/2}$ was also calculated. This parameter ($t_{1/2}$) gives the elapsed time to reduce RSC to 50%. Therefore, this parameter gives the half-life for the RSC of the oil and can be obtained by using the expression $t_{1/2} = \ln 2/k_T$. The initial estimations of RSC_0 and k_T, which are necessary to fit the experimental data by NLR, were obtained from the slope and ordinate on the origin of the straight line resulting form the fit by linear regression of the experimental data of RSC eq 1 and t to

$$\ln RSC = \ln RSC_0 - k_T t \qquad (5)$$

Thin-Layer Chromatography (TLC) Experiments. MF fractions (10 μL) were applied on silica gel 60 F254 TLC aluminum sheets (20 × 20 cm) (Merck, Darmstadt, Germany) at one of the extremes to run the chromatography (TLC). A mixture of toluene/ethyl acetate/formic acid (5:4:1) (v/v/v) was used as the mobile phase. When the front arrived at the end, the sheet was dried out and stained with 0.4 mM DPPH• in methanol.

RESULTS AND DISCUSSION

Scavenging Effect on DPPH•. The accepted way to inhibit lipid oxidation by antioxidants is through their antiradical activity. The model of scavenging stable DPPH• is a widely used method to evaluate antioxidative activities in a relatively short time compared to other methods (Brand-Williams et al., 1995; Chen and Ho, 1997; Sánchez-Moreno et al., 1998):

$$DPPH^{\bullet} + (AH)_n \rightarrow DPPH-H + (A^{\bullet})_n \qquad (6)$$

The new radical formed (A•) can mainly follow radical–radical interaction to render stable molecules via radical disproportionation (collision of radicals with abstraction of an atom by one radical to another, DPPH• + A• → DPPH–A; A• + A• → A–A), although these secondary reactions are greatly hindered (Chimi et al., 1991; Aruoma, 1998).

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Therefore, the disappearance of DPPH• is an index to estimate RSC in the medium. Absorbance decreases as a result of a color change from purple to yellow as the radical is scavenged by antioxidants through donation of hydrogen to form the stable DPPH−H (eq 6). In the presence of an antioxidant, a decrease in the absorbance at 515 nm was measured until the antioxidant is depleted under our assay conditions for oils ($[DPPH•]_i > [AH]_i$).

The subindex "n" in eq 6 is related to the presence of two antioxidants or two groups of antioxidants that differ in their velocity (fast and slow) to scavenge the radical. This might explain why the experimental recordings can be fitted better to a double-exponential equation. Therefore, eq 6 could be represented as

$$DPPH• + (AH)_1 \xrightarrow{\lambda_1} DPPH−H + (A•)_1 \quad (7)$$

$$DPPH• + (AH)_2 \xrightarrow{\lambda_2} DPPH−H + (A•)_2 \quad (8)$$

and then the overall equation that describes the disappearance of DPPH• in the presence of two groups of antioxidants $(AH)_1$ and $(AH)_2$ according to the fitting criteria and the mathematical criteria explained under Materials and Methods (Figure 2) becomes eq 1.

The term $DPPH•_r$ is added to express the amount of radical that remains in the medium after antioxidant depletion (Figure 1).

The rate constants (λ_1 and λ_2) are to be considered as a measure of the speed of DPPH• disappearance. Taking into account that $[DPPH•]_i > [AH]_i$, this means that all of the antioxidant reacts with the radical and, therefore, λ_1 and λ_2 are an index of the speed of antioxidant disappearance (the higher the value of λ, the quicker the disappearance of the antioxidant).

It is important to understand the meaning of the parameters $DPPH•_1$ and $DPPH•_2$. Figure 1 (curve a) shows a spectrophotometric recording of the disappearance of DPPH• in the presence of total olive oil (also applicable to oils with RSC in both MF and LF). This curve was fitted by NLR to eq 1. Curves b and c are the simple uniexponential equations (eqs 7 and 8), which form the double-exponential equation (curve d). Therefore, curve a is the sum of curves b and c and the residual DPPH• ($[DPPH•]_r$) (Figure 1). The disappearance of DPPH• is proportional to the amount of the antioxidants. Therefore, we can assume that the amount of DPPH• that disappears is equal to the RSC of the antioxidants present in the medium. The parameters $DPPH•_1$ and $DPPH•_2$ represent the RSC, which operates through the uniexponential equations which form the double-exponential equation. At the start of the assay ($t = 0$), $DPPH•_1 + DPPH•_2 + DPPH•_r = DPPH•_i$. At final time, the antioxidant is depleted after reaction with DPPH• and then $DPPH• = DPPH•_r$. This was due to the assay condition $[DPPH•]_i > [AH]_i$, which allowed all of the antioxidants to take part in the reaction with DPPH•. The amount of DPPH• removed from the medium is equivalent to the RSC of the oil. Thus, RSC is equal to $DPPH•_i − DPPH•_r$. Taking into account previous consideration we can assume that RSC = $DPPH•_1 + DPPH•_2$ (Figure 1).

Under the above-cited assay condition ($[DPPH•]_i > [AH]_i$), the free radical DPPH• disappears in the assay

Table 1. Second-Order Rate Constants (k_2, μM^{-1} s^{-1} × 10^{-4}) for the Free Radical Scavenger Activity of Some Standard Phenolic Compounds That Can Be Present in Oils[a]

compound	k_2	compound	k_2
sinapic acid	12.0 ± 1.1	caffeic acid	2.6 ± 0.2
α-tocopherol	8.4 ± 0.7	protocatechuic acid	1.6 ± 0.1
oleuropein	6.2 ± 0.5	syringic acid	1.0 ± 0.1
hydroxytyrosol	5.7 ± 0.4	tyrosol	NS[b]
sesamol	4.0 ± 0.3	vanillic acid	NS

[a] Conditions were 20 μM DPPH•; the concentration of phenolic compounds ranged from 60 to 300 μM. [b] NS, antioxidant activity was not significant for these compounds.

medium depending on [AH] (Brand-Williams et al., 1995; Shi and Niki, 1998).

For standard phenolic compounds, the pseudo-first-order conditions ($[DPPH•]_i < [AH]_i$) could be applied. In this case, taking into account eq 6

$$-\frac{d[DPPH•]}{dt} = k_{obsd}[DPPH•] = k_2[AH][DPPH•] \quad (9)$$

The pseudo-first-order rate constant, k_{obsd}, was linearly dependent on antioxidant (AH) concentration (results not shown). From this plot, the second-order rate constant k_2 was determined for some phenolic compounds present in oils (Table 1). The order of antiradical effectiveness, according to k_2 values, was sinapic acid > α-tocopherol > oleuropein > hydroxytyrosol > sesamol > caffeic acid > protocatechuic acid > syringic acid in our assay conditions. Other compounds such as tyrosol and vanillic acid presented negligible antiradical capacity.

There are many previous studies about the antiradical effectiveness of phenolic compounds. The sequence obtained for this effectiveness greatly varies depending on the assay method and the way in which the results are analyzed (Chimi et al., 1988, 1991; Le Tutour and Guedon, 1992; von Gadow et al., 1997; Sánchez-Moreno et al., 1998; Visioli et al., 1998). In these works, a different order for the sequence of antioxidant−antiradical effectiveness can be found, with either the same or different assay methods. For instance, our results regarding the effectiveness of α-tocopherol, oleuropein, and hydroxytyrosol do not agree with those of Visioli et al. (1998), who expressed the antiradical effectiveness in terms of EC_{50} (the amount of antioxidant needed to decrease by 50% the initial radical concentration). However, our results do agree with those of Le Tutour and Guedon (1992), who also compared the antiradical effectiveness in a kinetic study according to second-order rate values.

RSC in Oils and Oil Fractions. *"Methanolic" Fraction (MF).* To study separately the contribution of the phenolic compounds to the RSC in the different oils, an extraction in methanol was carried out. Experimental recordings for the disappearance of DPPH• in the presence of MF of the different oils were fitted to eq 1 (Figures 2A and 3A). RSC was linearly dependent on the volume of the oil fraction (Figure 3B). Antiradical activity was observed in MF of safflower ≈ sesame > rapeseed > walnut ≈ olive > linseed oils. In the rest of the oils, negligible antiradical capacity was observed in the MF (Table 2; Figure 4). The mean of the different oils assayed is shown. Safflower and sesame oils showed the highest antiradical capacity in MF (Shahidi et al.,
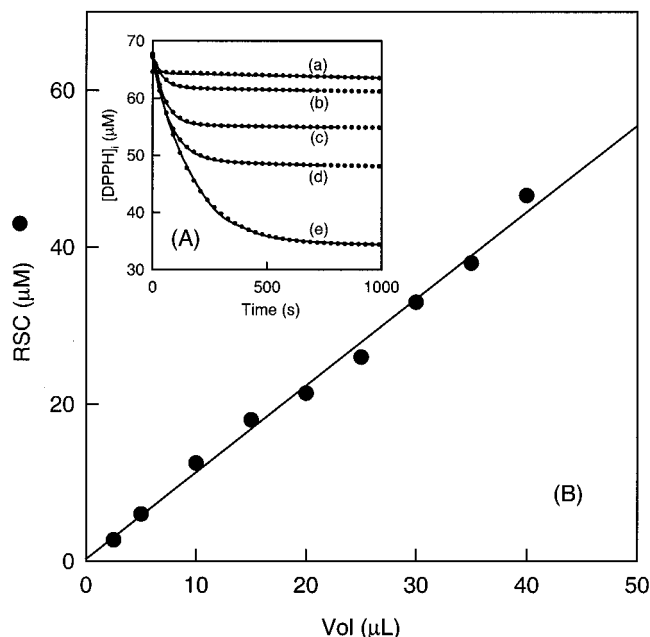
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Figure 3. Dependence of RSC on olive oil volume: (A) spectrophotometric recordings for the disappearance of DPPH$^\bullet$ in the presence of different volumes of MF of olive oil. Conditions were 68 μM [DPPH$^\bullet$]$_i$ and, curve a, 2.5 μL; curve b, 5 μL; curve c, 10 μL; curve d, 15 μL; and curve e, 30 μL; (B) linear dependence of RSC on MF olive oil ($\bullet$) experimental data; ($-$) linear regression fitting. Conditions were the same as in (A).

Table 2. Total TSC of Oil (TF) and Oil Fractions [Methanolic (MF) and Lipidic (LF)]^a

oil source^a	RSC$_{TF}$	RSC$_{MF}$	RSC$_{LF}$
olive	27.2 ± 9.3	8.3 ± 5.0	11.3 ± 4.1
rapeseed	94.6 ± 7.0	13.8 ± 8.9	59.7 ± 9.6
walnut	70.1 ± 9.0	8.5 ± 0.7	56.2 ± 9.8
safflower	71.7 ± 5.3	25.5 ± 2.2	37.5 ± 5.0
linseed	81.1 ± 13.2	7.1 ± 1.5	46.9 ± 8.5
sesame	78.4 ± 11.8	24.5 ± 3.5	40.1 ± 5.6
sunflower	75.3 ± 9.1	0.2 ± 0.02	70.2 ± 6.1
soybean	112.5 ± 27.1	0.1 ± 0.05	110 ± 26.5
hazelnut	60.4 ± 5.5	0.1 ± 0.05	59.8 ± 8.1
almond	62.5 ± 8.2	0.4 ± 0.1	62.2 ± 8.4
corn	87.3 ± 16.5	0.3 ± 0.1	86.7 ± 15.5
peanut	38.9 ± 18.5	0.1 ± 0.05	37.9 ± 18.2
dietetic oil	55.1 ± 9.5	0.1 ± 0.05	54.3 ± 9.6
oil for salad	55.3 ± 11.2	0.1 ± 0.05	54.1 ± 10.8

^{a} The mean values of the different oils for each source are shown. Conditions were 93 μM DPPH$^\bullet$ and 5 μL of the corresponding oil or oil fraction. (The assay medium contained methanol for MF and ethyl acetate for TF and LF.)

1992). Only one brand of Spanish olive oil showed a similar antiradical capacity.

The compounds mainly responsible for the antiradical capacity were visualized by TLC. After staining of the sheet with DPPH$^\bullet$, compounds with antiradical capacity appeared as yellow bands (Figure 7). R_f values of the bands were calculated and compared to those of standards. MF of rapeseed oil showed a band with an R_f = 0.61. The main phenolic compound in this oil is sinapic acid (Xu and Diosady, 1997); when this compound was developed on TLC its R_f was the same. Therefore, this compound could be mainly responsible for the RSC in the rapeseed MF. Sesamol showed a band with R_f = 0.82, the same as the more intense band in the sesame oil. Two other minor components were also visualized in this oil (with R_f = 0.62 and 0.59). These phenolic compounds might be sesamolin and sesamolinol, as

reported elsewhere (Shaidi and Wanasundra, 1992). The MF of olive oil showed five intense bands with R_f = 0.65, 0.6, 0.53, 0.43, and 0.18. Syringic and protocatechuic acids showed bands with R_f = 0.65 and 0.6, respectively. The band with R_f = 0.53 could be mostly caffeic acid, but the intensity of this band increased further if the sheet was observed 1 h later because of the influence of tyrosol. The latter appeared at the same R_f as caffeic acid, but its very low antiradical activity needed a longer time to reduce the radical and to show its yellow band. The band with R_f = 0.43 was identified as hydroxytyrosol. The lowest band (with R_f = 0.17) was oleuropein. This compound could be detected only in few of the MF of olive oils and as a faint band possibly because it was present in low concentration. Vanillic acid presented an R_f = 0.67, and it was difficult to visualize on TLC because of its low RSC. All of these compounds have been previously reported as constituents of olive oil (Montedoro et al., 1993). Safflower oil showed a very intense band with an R_f = 0.62, indicating the presence of a compound with high RSC. So far, such a compound has not been previously reported. At the present, the identity of this compound and the others present in low quantities in walnut and linseed oils is being studied. The rest of the oils (sunflower, corn, soy, almond, and hazelnut) showed no yellow bands and negligible RSC in their MF fractions.

The phenolic fraction of the assayed olive oils showed similar bands, but their intensity when applied at the same concentration was different (results not shown), indicating differences in the phenolic content between olive oils. This could also be observed spectrophotometrically because a significant standard deviation regarding their RSC in MF was observed. Less variation in RSC of MF was observed for Turkish and Greek olive oils. In general, mean values of RSC of MF for olive oils were rather similar and, therefore, it was not possible to correlate a higher or lower antiradical capacity of MF to the origin of the olive oil except for analyzed Turkish oils, which showed lower RSC (Figure 4). The great diversity of methods utilized at the present for the oil extraction (virgin oil), from the traditional style (ground by mills) to the modern technologies (centrifugation or percolation), might explain the large standard deviation in the phenolic content (Ranalli et al., 1999).

"Lipidic" Fraction (LF). After extraction with methanol, the most hydrophobic phase was assayed. In this case, the assay medium contained ethyl acetate to dissolve the hydrophobic compounds. Experimental recordings for the disappearance of DPPH$^\bullet$ in the presence of LF of the different oils were fitted to eq 1 (Figure 2B). In this case, the double-exponential response could be due to the presence of tocopherols and phospholipids (Koga and Terao, 1995), which had different degrees of efficiency in the scavenging of the radical (results not shown). Considerable antiradical capacity was observed for all of the oils tested and with less standard deviation than in MF (Table 2).

The use of ethyl acetate to dissolve DPPH$^\bullet$ has not been previously reported. The stability and molar absorptivity of DPPH$^\bullet$ were the same as in methanol solution. This allowed us to characterize the antiradical effectiveness of the methanol-nonsoluble compounds.

The antiradical capacity of phospholipids and α-tocopherol of this LF was assayed. RSC of phospholipids was much lower than that of α-tocopherol (results not shown). RSC of LF of the different oils followed the

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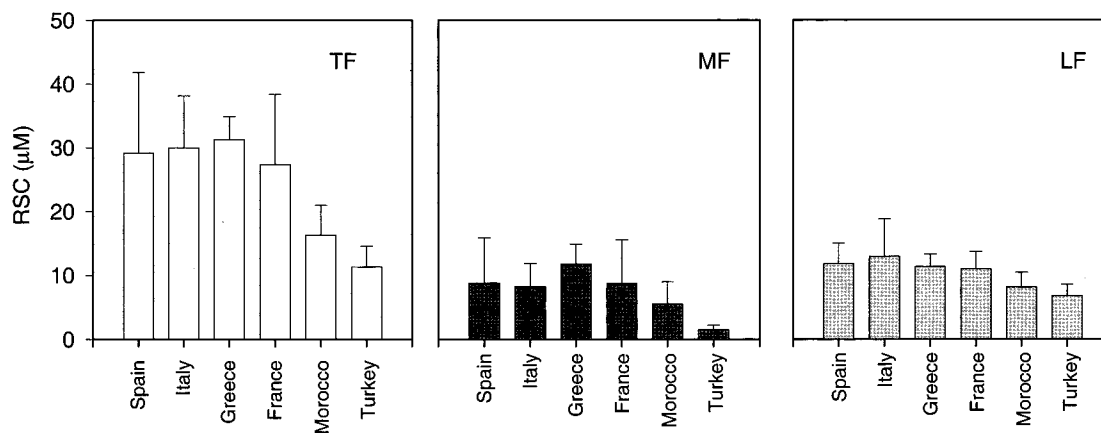*J. Agric. Food Chem.,* Vol. 48, No. 3, 2000 **653**

Figure 4. RSC of olive oils and oil fractions from different countries. Conditions were the same as in Figure 1.
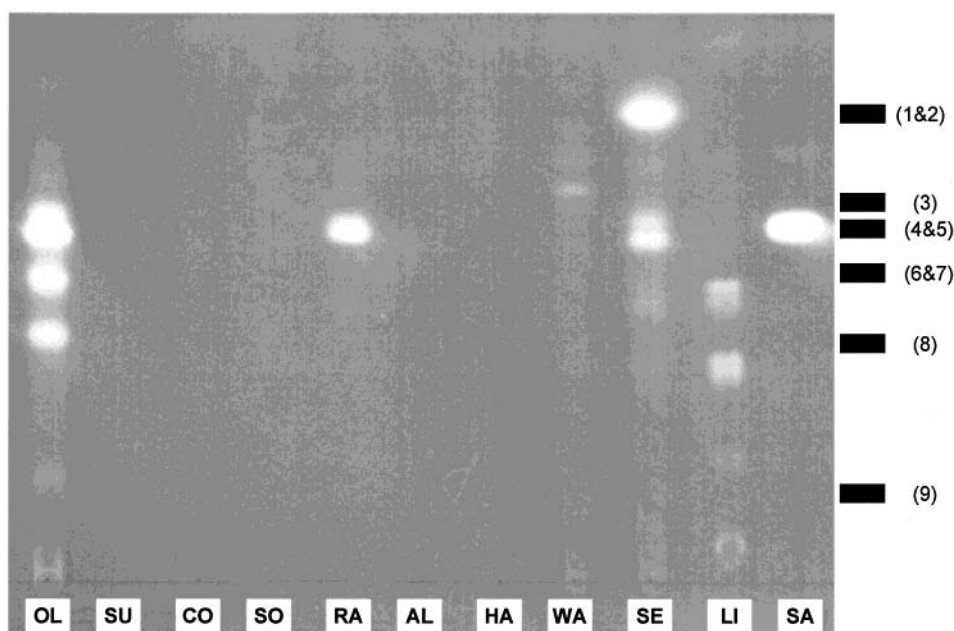

Figure 5. TLC stained with a DPPH• solution in methanol. MFs (10 μL) of oils from different sources were applied [see Materials and Methods for details; MF of olive (OL), sunflower (SU), corn (CO), soy (SO), rapeseed (RA), hazelnut (HA), walnut (WA), sesame (SE), linseed (LI), and safflower (SA) oils]. Standards were sesamol (**1**, $R_f = 0.82$), vanillic acid (**2**, $R_f = 0.82$), syringic acid (**3**, $R_f = 0.65$), sinapic acid (**4**, $R_f = 0.61$), protocatechuic acid (**5**, $R_f = 0.6$), caffeic acid (**6**, $R_f = 0.53$), tyrosol (**7**, $R_f = 0.53$), hydroxytyrosol (**8**, $R_f = 0.43$), and oleuropein (**9**, $R_f = 0.17$).

sequence according to their different tocopherol contents. Tocopherols have different efficiencies as antioxidants: $\delta > \gamma \approx \beta > \alpha$ (Six, 1994). The same sequence was obtained for the RSC of LF of the different oils. Therefore, it can be suggested that the RSC of LF of the different oils is mainly due to their different concentrations and types of tocopherols. The highest RSC was observed for soybean oil (rich in δ-tocopherol; Netherlands Standard Institute, 1989; Salunkhe et al., 1992) and the lowest for virgin olive oil (low concentration of tocopherol, 97% of α-tocopherol of total tocopherols, the least efficient as antioxidant; Netherlands Standard Institute, 1989).

"Total" Fraction (TF). RSC for each oil, without fractionation, was higher than the arithmetic sum of the experimental recordings corresponding to MF and LF. This could be due to the synergistic effect of the different antioxidants present in both MF and LF fractions (Figure 6; Koga and Terao, 1995). The RSC of oils was mainly due to the type and content of tocopherols because of the minor contribution of the phenolic compounds except for olive oils (Table 2).

Hazelnut oil has been proposed as a potential alternative to olive oil because of its balanced composition of fatty acids, similar to that of olive oil. Moreover, hazelnut oil is less susceptible to sunlight, and it has a higher tocopherol content than olive oil (Contini et al., 1997). However, hazelnut oil lacks RSC in the MF (Table 2), which could be involved in the health-beneficial aspects described for olive oil (Visioli and Galli, 1998).

Despite these health-benefits effects, olive oil represented only 2% of the global consumption of fats and oils in the world during the years 1996–1997, an increase of only 9% in its consumption since 1989. Soybean oil was the most consumed oil all over the world, with 22% of consumption and an increase of 27% since 1989. The highest increases in consumption were for rapeseed oil, with 44% (12% of global consumption), and corn oil, with 40% (2% of global consumption). Among the vegetable oils assayed here, linseed and sesame oils were the least consumed (0.8 and 0.7%, respectively), with changes in their consumption of +15% and −1%, respectively (O'Mara, 1998).
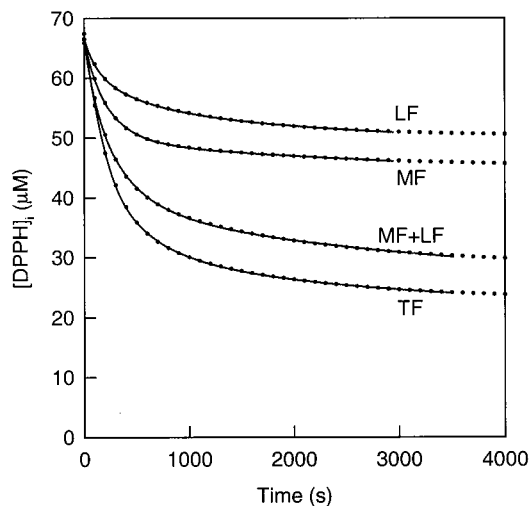
Figure 6. Synergistic effect of oil fractions: (—) experimental data; (···), NLR fitting of experimental data to eq 1. Conditions were the same as in Figure 2.
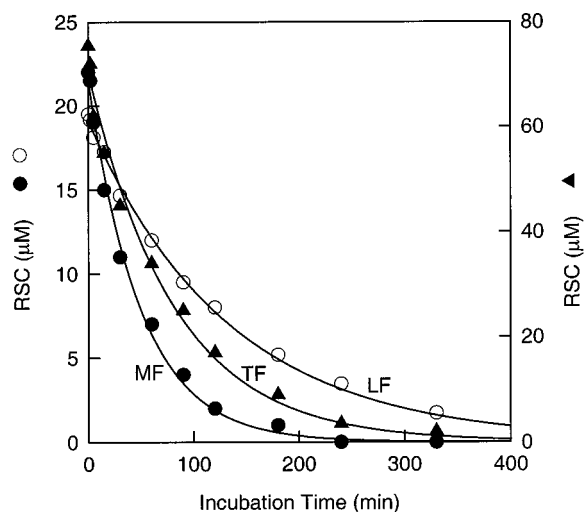

Figure 7. Influence of temperature on RSC of olive oil: (●) MF; (○) LF; (▲) TF; (—) NLR fittings of experimental data to eq 4. Conditions were the same as in Figure 1. Temperature = 180 °C.

The relatively low RSC found in olive oils with respect to other oils might seem to decrease the importance of olive oil as source of antioxidants, but, on the contrary, this low content in RSC is due to the phenolic compounds, which, apart from their antiradical activity, have also other healthy effects on humans (Beuchat and Golden, 1989).

Effect of Temperature on RSC. These experiments were focused on the study of the different effects of temperature on RSC oils and fraction oils. For this purpose both the apparent constant, which describes the
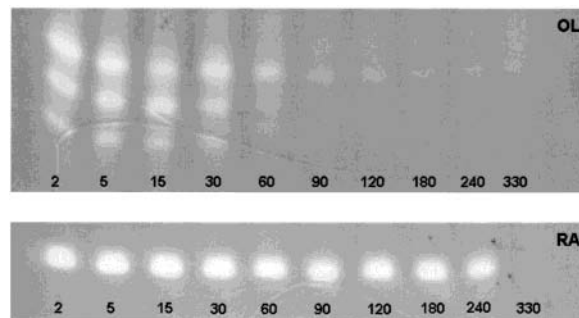

Figure 8. TLC stained with a DPPH• solution in methanol (see Materials and Methods for details): MF of (OL) olive oil (10 μL) and (RA) rapeseed oil (5 μL) during 5.5 h of incubation at 180 °C. The numbers indicate the incubation time (in minutes).

disappearance of RSC with incubation time (k_T), and $t_{1/2}$ (elapsed time to reduce RSC to 50%) at 180 °C were calculated. The most representative oils were selected for this study.

Both k_T and $t_{1/2}$ were highly dependent on the oil source and oil fraction (Table 3). The higher the k_T value, the lower the RSC resistance to temperature, and the lower the $t_{1/2}$ value, the lower the RSC resistance. Olive oil showed the least thermostable RSC among the oils tested (Figure 7; Table 3). The most representative fraction for the health of human consumers (TF) showed a $t_{1/2}$ value of 56 min, which means that after ~1 h of frying olive oil, its RSC decreases by 50%. The RSC for TF was an average of that for LF and MF. For MF the value was lower (35 min) and for LF higher (107 min). This can be explained because of the relatively high resistance of tocopherols to temperature (Figure 7; Table 3; Yoshida and Tagaki, 1999). In the MF (Figure 8, OL) after 60 min, the remaining RSC of this fraction was apparently due mostly to protocatechuic acid because during the first hour of incubation, the rest of the phenolic compounds, hydroxytyrosol, tyrosol, and syringic and caffeic acids, were degraded.

These results do not contradict the usefulness of olive oil for frying processes. It is known that fats and oils undergo various deleterious changes including hydrolytic, oxidative, isomerization, and polymerization reactions during heat treatment at elevated temperature. However, olive oil shows a high resistance to these nondesirable changes (Aggelousis and Lalas, 1997; Kiritsakis, 1998). Our study demonstrates that the antiradical capacity of olive oil is affected by high temperature (Figures 7 and 8), which does not mean that olive oil is unstable at this temperature.

RSC of MF in safflower and rapeseed oils was more resistant to temperature than the MF of olive oil, in particular, safflower MF, in which the $t_{1/2} = 433$ min (Table 3). In these cases, the RSC was mainly due to

Table 3. Effect of Temperature on the Total RSC of Oil (TF) and Oil Fractions [Methanolic (MF) and Lipidic (LF)][a]

	TF		MF		LF	
oil source	k_T (min^{-1}) × 10^{-3}	$t_{1/2}$ (min)	k_T (min^{-1}) × 10^{-3}	$t_{1/2}$ (min)	k_T (min^{-1}) × 10^{-3}	$t_{1/2}$ (min)
olive	12.4 ± 1.1	55.9 ± 4.9	20.0 ± 1.6	34.6 ± 2.8	6.5 ± 0.5	106.6 ± 8.0
sunflower	4.2 ± 0.2	165.0 ± 8.0			4.3 ± 0.2	160.2 ± 8.0
corn	2.3 ± 0.1	301.4 ± 13.0			2.2 ± 0.1	315.1 ± 14.1
rapeseed	4.6 ± 0.2	150.7 ± 6.0	8.5 ± 0.5	81.5 ± 5.1	3.5 ± 0.2	198.0 ± 11.0
soybean	1.6 ± 0.1	385.1 ± 23.0			1.7 ± 0.1	407.7 ± 23.1
safflower	2.8 ± 0.2	247.5 ± 18.0	1.6 ± 0.1	433.2 ± 30.0	4.1 ± 0.3	169.1 ± 16.0

[a] Conditions were 93 μM DPPH• and 5 μL of the corresponding oil or oil fraction; incubation time ranged from 0 to 330 min at 180 °C. (The assay medium contained methanol for MF and ethyl acetate for TF and LF.)

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the unidentified compound of safflower MF ($R_f = 0.62$) and sinapic acid (showing a high resistance to temperature) rather than to other possible degradation products with RSC. When the MFs of these oils were visualized on TLC, a unique band with R_f values corresponding to those compounds was found constant in intensity during the selected incubation time. Only in the MF of rapeseed was sinapic acid degradation observed after 5.5 h of incubation at 180 °C (Figure 8, RA). This resistance was even higher for sesame oil (results not shown) in which $t_{1/2}$ could not be calculated because no disappearance of sesamol was observed in the assay time (Yoshida and Tagaki, 1999).

RSC in TF and LF of sunflower, corn, and soybean oils (which lacked phenolic compounds) presented a high resistance to temperature (Table 3). It is known that tocopherols present different thermostabilities: $\alpha < \beta < \gamma \approx \delta$ (Yoshida and Tagaki, 1999). The sequence of the $t_{1/2}$ values was in accordance with that of the content and type of tocopherols in the different oils (Tables 2 and 3). This also agrees with the lowest $t_{1/2}$ for LF in olive oils because of the relatively low content of tocopherol (97% of α-tocopherol, the least resistant to temperature; Yoshida and Tagaki, 1999).

ABBREVIATIONS USED

AH, antioxidant; [AH]$_i$, initial antioxidant concentration; DPPH•, 2,2-diphenyl-1-picrylhydrazyl radical; [DPPH•]$_i$, initial DPPH• concentration; k_2, second-order rate constant; DPPH•$_1$ and DPPH•$_2$, radical concentrations at time zero for the simple uniexponential equations that form the double-exponential eq 1; [DPPH•]$_r$, remaining DPPH• concentration after antioxidant interaction; k_{obsd}, pseudo-first-order constant; k_T, first-order constant for the disappearance of RSC with temperature; λ_1 and λ_2, first-order rate constants for the simple uniexponential equations that form the double-exponential eq 1; LF, lipidic fraction, methanol nonsoluble fraction; MF, methanolic fraction, methanol-soluble fraction of oils; NLR, nonlinear regression; RSC, total free radical scavenger capacity; TF, "total" fraction (MF + LF), oil without fractionation; TLC, thin-layer chromatography; $t_{1/2}$, elapsed time to decrease RSC to 50% with temperature.

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