Pure Lycopene from Tomato Preserves Extra Virgin Olive Oil from Natural Oxidative Events During Storage

Domenico Montesano, Lina Cossignani, Gilda D'Arco, Maria Stella Simonetti, and Pietro Damiani*

Dipartimento di Scienze Economico-Estimative e degli Alimenti, Sezione di Chimica Bromatologica, Università di Perugia, Italia

ABSTRACT: In this study, changes in an extra virgin olive oil treated with lycopene during storage were analyzed. Pure lycopene (0.5 and 1.0 mg) obtained from tomato was added to two separate bottles, each containing 100 mL of extra virgin olive oil, while another bottle containing the same oil was stored without any treatment. Samples enriched with pure lycopene showed PV remarkably lower than the sample without lycopene, and the total phenol contents were higher in treated oil samples than in the reference sample. In addition, a good correlation (r^2 = 0.969) between total phenol content and antioxidant power calculated as Trolox Equivalent Antioxidant Capacity (TEAC) was observed. The concentration of added lycopene decreased very slowly; after about 8 mon its residual value was over 60% with respect to the initial concentration.

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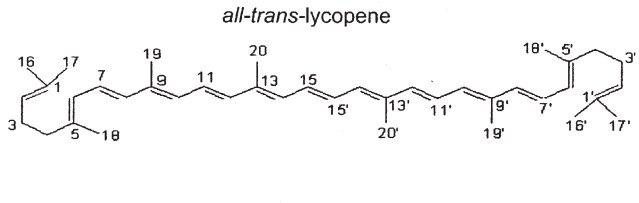
KEY WORDS: Antioxidant properties, β-carotene, carotenoids, HPLC-MS, lycopene, peroxide value, polyphenols, TEAC, α-tocopherol.



SCHEME 1

Carotenoids such as lycopene and β-carotene (Scheme 1) are principally responsible for the colors of vegetables and fruits. Like other carotenoids, lycopene, which provides the red color of tomatoes (*Lycopersicon esculentum*), is fat-soluble. In certain plants and microorganisms, lycopene serves as an accessory light-gathering pigment and as a protection against the toxic effects of oxygen and light (1). Lycopene may also protect humans against certain disorders, such as prostate cancer and perhaps some other cancers, and coronary heart disease (2–4).

As well as in tomatoes and tomato-based products, such as ketchup, pizza sauce, tomato juice and tomato paste, lycopene is found in watermelon, papaya, pink grapefruit, and pink guava. Furthermore, new lycopene-containing products have been placed on the market over the past years, including a reduced-fat margarine to which lycopene and lutein are added to produce a functional product. The lycopene in processed tomato products is more readily available to the body than that in fresh tomatoes. The average daily intake of lycopene is approximately 25 mg, with 50% in the form of processed tomato products (5,6). Various authors studied the correlations between oxidative stability and minor constituents of virgin olive oil (7) as well as the deterioration of natural antioxidant species of edible vegetable oils under deep-frying conditions (8). Moreover, Psomiadou and Tsimidou (9) studied the role of α-tocopherol, carotenoids, and chlorophylls in the oxidative stability of virgin olive oil. Research has also considered the relationship of total phenol content or of individual phenols with the oxidative stability of the oil, which is related to the flavor and shelf life of the oil (10–15). Marinova and Yashlieva (16) reported on the antioxidative activity of phenolic acids on TAG and FAME from olive oil. Papadopoulos and Boskou (17) tested the total polar fraction and individual phenols present in virgin olive oil for their antioxidant effect in refined olive oil and found that hydroxytyrosol and caffeic acid showed protection factors greater than BHT. Protocatechuic and syringic acids also have antioxidant activity. The aim of this work was to study the oxidative stability of extra virgin olive oil enriched with pure lycopene. For this reason several parameters for monitoring the changes of chemical characteristics of oil during storage were considered. In particular, the total phenolic fraction was determined by using the Folin-Ciocalteau method, antioxidant capacity by measuring the content of the *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD) radical cation, lycopene content by diode array detector (DAD)-HPLC coupled with atmospheric pressure chemical ionization (APCI)-MS, and the general oxidation status by the analysis of peroxide index of the oil. Pure crystalline lycopene was used to ensure that the variations in the oil are due exclusively to this substance and not to a mixture (generally an oleoresin) containing a percentage of lycopene. The pure lycopene added to

*To whom correspondence should be addressed at Dipartimento di Scienze Economico-Estimative e degli Alimenti, Sezione di Chimica Bromatologica, Università di Perugia, via Romana, 06126 - Perugia, Italy.
E-mail: chimbrom@unipg.it

the oil samples was obtained from tomato using a reliable extraction procedure (Montesano D., F. Fallarino, A. Bosi, L. Cossignani, M.S. Simonetti, P. Puccetti, and P. Damiani, unpublished data).

MATERIALS AND METHODS

Materials. We used extra virgin olive oil produced in the central region of Italy (Umbria). The oil was taken directly from the production line on the basis of a protocol established by the European Commission for Food Quality Policy on Protected Designation of Origin (EEC No. 2081/92), was obtained in the first 10 d of November, and was classified as coming from the first harvesting period. Its color was greenish, its aroma was fruity, and it had a bitter almond-like taste.

The extraction was performed at 24.5°C on November 11, 2004. Solvents and reagents used in the prechromatographic steps were reagent grade and the solvents for chromatography were HPLC grade. All organic solvents [analytical, uvasol (spectrophotometric) and HPLC grade], crystalline standard all-*trans*-lycopene, β-carotene, *N,N*-dimethyl-*p*-phenylenediamine dihydrochloride (DMPD), gallic acid, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), α-tocopherol, and BHT were purchased from Sigma-Aldrich (St. Louis, MO).

Methods. Extra virgin olive oil samples were prepared as follows: Sample 1 was stored without any treatment (control), Sample 2 was amended with 0.5 mg of pure lycopene/100 g of oil, and Sample 3 received 1.0 mg of pure lycopene/100 g of oil. Samples were analyzed just after their arrival in the laboratory (fresh oil), and were kept in amber glass bottles in darkness at ambient temperature during the experiment. The oil samples were monitored for 37 wk.

(a) Total phenolic content. The polyphenolic concentrations in the experimental oils were determined as follows: In a test tube approximately 2 g of oil (weighed to the nearest 0.1 mg) was diluted with 1 mL hexane, and then 2 mL of an extraction eluent mixture (methanol/water 60:40 vol/vol) was added. The tube was then shaken on a vortex mixer for 2 min and after centrifugation at 1500 × *g*, the methanol/water phase was transferred to a clean tube. The extraction step was repeated, then 1 mL hexane was added to the combined methanol/water extracts. The tube was shaken for 1 min and then centrifuged at 1500 × *g* to separate the two layers. The methanol/water phase was then transferred to a 100 mL round-bottomed flask and evaporated to dryness on a rotary evaporator (vacuum, 65°C). The residue was dissolved in 1 mL methanol, and 250 µL of each sample was introduced into a calibrated 25 mL flask as follows: 12.5 mL of deionized water, 1250 µL of Folin-Ciocalteu reagent (Sigma), 5 mL of a 20% solution of sodium carbonate (wt/vol) and distilled water to make up the total volume of 25 mL. The solution was agitated to homogenize it and left to stand for 30 min for the reaction to take place and stabilize. The absorbance at 765 nm was then determined in a 1 cm cuvette. The calibration curve was prepared with gallic acid solutions ranging from 0 to 1000 mg/L, and the results are given as gallic acid equivalents (GAE), or mg gallic acid/kg oil, in accordance with the literature (18).

$$DMPD_{(uncolored)} + \text{oxidant } (Fe^{3+}) + H^+ \rightarrow DMPD_{(purple)}^{\bullet+}$$

$$DMPD_{(purple)}^{\bullet+} + AOH \rightarrow DMPD_{(uncolored)}^{+} + AO$$

SCHEME 2

(b) Measurement of antioxidative ability by the DMPD method (Scheme 2). DMPD (100 mM) was prepared by dissolving 209 mg of DMPD in 10 mL of deionized water; 1 mL of this solution was added to 100 mL of 0.1 M acetate buffer, pH 5.25, and the colored radical cation ($DMPD^{\bullet+}$) was obtained by adding 0.2 mL of a solution of 0.05 M ferric chloride (final concentration 0.1 mM). One milliliter of this solution was placed directly in a 1-cm plastic cuvette, and its absorbance at 505 nm was measured. A standard solution of the antioxidant compound was prepared as follows: 1 mg/mL of Trolox was prepared by dissolving 0.1 g of Trolox in 100 mL of methanol. Fifty microliters of standard antioxidant or of polyphenol solution obtained from oil samples (as reported in the preceding paragraph) was added to the spectrometric cuvette containing 1 mL of the solution just described, and after 10 min of continuous stirring at 25°C the absorbance at 505 nm was measured. Buffered solution was placed in the reference cuvette. A dose-response curve was derived for Trolox by plotting the absorbance at 505 nm as a percentage of the absorbance of the uninhibited radical cation solution (blank) according to Equation 1:

$$\text{inhibition of } A_{505}\,(\%) = (1 - A_f/A_0) \times 100 \qquad [1]$$

where A_0 is the absorbance of uninhibited radical cation and A_f is the absorbance measured 10 min after the addition of antioxidant samples (19). Each oil sample was analyzed six times, and the values were expressed as mean ± SD ($n = 6$). By reference to the Trolox dose-response curve, the mean Trolox Equivalent Antioxidant Capacity (TEAC) value was derived for each sample according to Miller *et al*. (20).

(c) α-Tocopherol determination. α-Tocopherol was evaluated by HPLC-(DAD)-APCI-MS coupling, with the direct injection of an oil-in-hexane solution. HPLC analyses were carried out using an HPLC apparatus consisting of a Varian 9012 pump with on-line degasser (SCM 1000 T.S.P., Walton-on-Thames, United Kingdom) with a DAD (UV6000LP Spectra system; Thermo Separation Products, Stone, United Kingdom), detection at 295 nm. The column was a µ-Porasil, 5 µm, 30 cm × 3.9 mm i.d. (Waters, Milford, MA). The mobile phase consisted of hexane (A) and isopropanol (B); the samples were eluted according to the following gradient: 100% A for first 10 min then 95% A/5% B in 5 min; this percentage was maintained for 5 min and finally the hexane percentage (A) was increased to 100% again in 10 min to restore the initial conditions. The flow rate was 1 mL/min and run time 30 min. The sample injection volume was 20 µL. Mass spectra were monitored in the mass range *m/z* 200–700 on a Finnigan LC AQA

mass spectrometer equipped with an APCI interface (Finnigan, Manchester, United Kingdom). The APCI vaporizer temperature was held at 250°C. The corona discharge voltage was optimized to 3.5 kV. Detection was performed in the positive mode. Identification of α-tocopherol was achieved by comparing the retention time value with that of authentic standard. Initial and final α-tocopherol contents were then calculated from integrated peak areas of the samples using the calibration curve of an α-tocopherol standard. A stock solution of α-tocopherol was prepared by dissolving 10 mg of the analyte in 10 mL of methanol containing 0.05% of BHT. BHT is added to prevent tocopherol oxidation This solution was stored in aluminum foil-covered containers and kept at –20°C. Standard solutions of α-tocopherol prepared in the concentration range of 0.1 to 1.5 μg/mL showed a good linearity (0.994). The calibration curves were obtained by plotting the peak area of the analyte vs. its concentration. Each datum is the average of a minimum of seven determinations.

(d) Lycopene HPLC-DAD-APCI-MS determination. For the separation and identification of the two carotenoids β-carotene and lycopene (added), analysis by HPLC-DAD-APCI-MS coupling was performed. For chromatographic separation the same devices and conditions just described for tocopherol analysis were used. Detection was at 472 nm. The injection volume was 20 μL, and all the UV-vis spectra were recorded in the range from 380 to 700 nm and acquired by an Excalibur software system (ThermoQuest Corporation, Manchester, United Kingdom). Necessary precautions to avoid carotenoid degradation during analysis were taken, such as working under subdued light, using amber glassware or protecting with aluminum foil, and using N_2.

Mass spectra were monitored in the mass range *m/z* 200–700 on a Finnigan LC AQA mass spectrometer equipped with an APCI interface. The APCI vaporizer temperature was held at 450°C. The corona discharge voltage was optimized to 4.5 kV. The spectrometer was tuned to optimize the signal of *m/z* 537 ($[M + H]^+$ of β-carotene). Detection was performed in the positive mode. Identification of lycopene was achieved by comparing the retention time value with that of an authentic standard. Initial and final concentration of lycopene was calculated using the calibration curve plotted with different amounts of lycopene dissolved in hexane.

(e) Peroxide index determination. The oxidation process was evaluated by determining the peroxide index. This method quantifies the concentration of the immediate products of oxidation (peroxides) and therefore measures the degree to which oxidation is taking place at the present moment. About 2 g of crude oil was transferred to a 300 mL flask and then 40 mL of an acetic acid/chloroform (3:2 vol/vol) mixture and 2 mL of 10% KI were added. This mixture was left in darkness for 5 min and then 5 drops of starch indicator were added. Finally the solution was titrated with 0.01 N $Na_2S_2O_3$ (21). The peroxide index of the sample was expressed as mequiv O_2/kg. The values were the mean of at least six determinations.

RESULTS AND DISCUSSION

The total concentration of phenolic substances extracted from oil was determined using Folin-Ciocalteau reagent, and its variation was monitored for 37 wk (Fig. 1). The values are expressed as the mean ± SD (n = 6) using the equation $y = 0.0123x - 0.0345$ ($r^2 = 0.999$). The results show that lycopene is responsible for the preservation of phenolics. At time = 0 the phenolic content of the oil samples was about 700 mg/kg; at about 2 mon slight variations were recorded, while after about 6 mon polyphenols decreased to 400 mg/kg in Sample 1 (without lycopene). This value was constant for the successive 2 mon. For Samples 2 and 3 different trends were obtained: For the first 2 mon the polyphenol concentrations were similar to those in Sample 1, but after 6 mon there was about 500 mg polyphenols/kg oil in Samples 2 and 3. At the end of the 37-wk experiment, polyphenols were considerably more abundant in Samples 2 (450 mg/kg oil) and 3 (500 mg/kg) than Sample 1 (410 mg/kg).

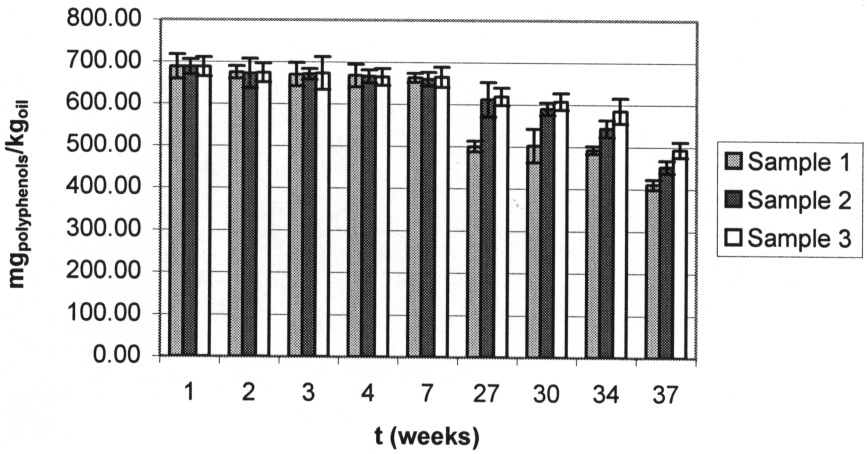


FIG. 1. Total phenolic index calculated for three sample oils as GAE (gallic acid equivalent). The values are the means of six determinations (n = 6). The error bars represent the SD.

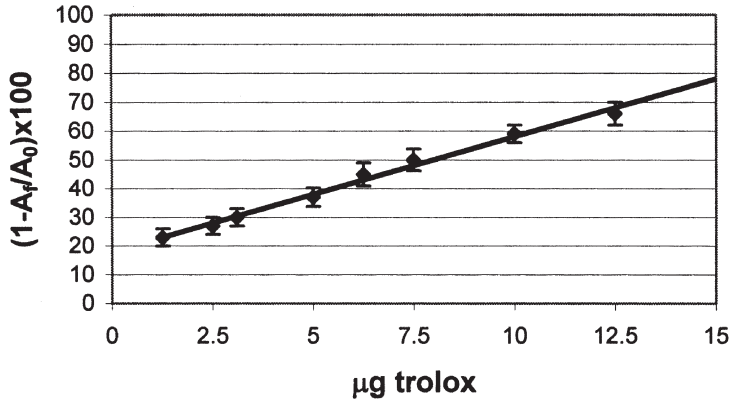


FIG. 2. Degree of inhibition of the absorbance at 505 nm as a function of the Trolox concentration. Values are means ± SD (n = 8).

The results of the dose-response curves obtained from eight different sets of experiments using Trolox are shown in Figure 2**.** The SD is very low and the dose-response curve is highly reproducible. Inhibition of the absorbance at 505 nm is linear between 0.2 and 12 µg of TROLOX. The relationship calculated within this range for the standard compound is presented in Equation 2:

$$A_{505}\ (\text{inhibition}) = 4.0\ (\mu\text{g of Trolox}) + 18.0 \qquad [2]$$
$$r^2 = 0.9911$$

The antioxidant ability of the oil samples was measured by using the DMPD method (see Materials and Methods section). The principle of the assay is that at an acidic pH and in the presence of a suitable oxidant solution DMPD can form a stable, colored radical cation (DMPD$^{\bullet+}$) (Scheme 2, step 1). The absorbance maximum for this species is at 505 nm. Antioxidant compounds (AOH) that are able to transfer a hydrogen atom to DMPD$^{\bullet+}$ produce a decoloration of the solution that is proportional to their amount (Scheme 2, step 2). This reaction is rapid (less than 10 min), and the end point, which is stable, is taken as a measure of the antioxidative efficiency. Therefore, this assay reflects the ability of a radical hydrogen donor to scavenge the single electron from DMPD$^{\bullet+}$. In Figure 3 the TEAC values of the three oil samples are shown. Sample dilution was selected to reduce the measurement within the appropriate part of the Trolox standard curve.

Note that Samples 2 and 3 (treated with lycopene) showed higher concentrations of polyphenols and higher values of TEAC than Sample 1. Preserving the phenolics content of oils by adding lycopene makes it possible to maintain the natural antioxidant power of oils during storage. At the end of 37 wk, the TEAC values of Samples 1–3 were 0.41, 0.52, and 0.51, respectively. There was a good correlation between the content of phenols and the TEAC of each oil sample: In Figure 4 the TEAC value of each oil vs. the respective amount of phenols is plotted. The correlation between these two parameters is more than 0.96. Therefore, by using the line equation given in the

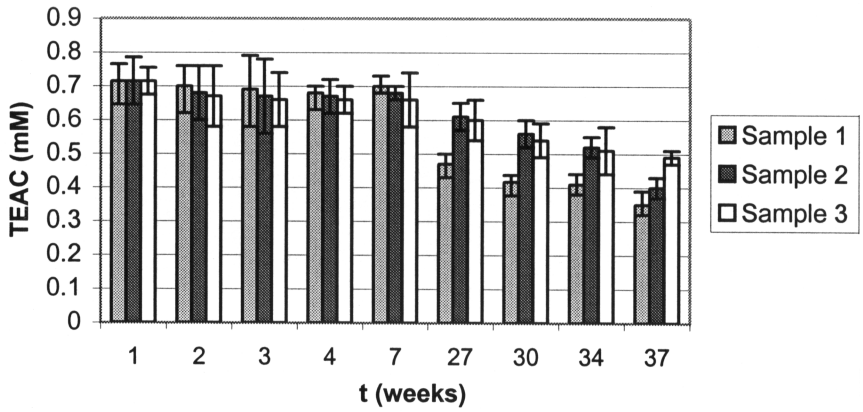


FIG. 3. Trolox Equivalent Antioxidant Capacity (TEAC) values of three sample oils. The results are expressed as mean ± SD (n = 6).

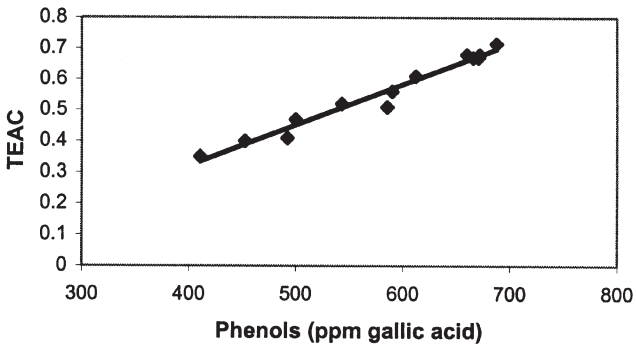


FIG. 4. Correlation between TEAC and amount of phenols for the three oil samples tested. The equation of the line is calculated to be $y = 0.0013x - 0.2033$ ($r^2 = 0.969$). For abbreviation see Figure 3.

caption for Figure 4, it is possible to calculate the TEAC of an oil from its phenol content.

To evaluate the effect of lycopene on the oxidative stability of olive oil, we determined the initial and final concentrations of α-tocopherol in our oil samples by HPLC-DAD-APCI-MS. In Figure 5 the HPLC-DAD-MS analysis of pure α-tocopherol is shown, and in Table 1 the results of analysis for α-tocopherol performed six times on oil samples are reported. The quantitative analysis was carried out using the following equation, obtained through regression analysis of data for the standard solutions: $y = 1.56 \times 10^6 - 9.50 \times 10^4$, $r^2 = 0.994$ (n = 7).

Standard α-tocopherol showed an absorption maximum at 292 nm, and its mass spectrum had a peak at *m/z* 431 $[M + H]^+$ without other characteristic fragmentation. Our results show that lycopene, in suitable concentrations, preserves the major part of vitamin E initially present in the oil. Note that at the end of the experiment α-tocopherol in Samples 1 and 2 had totally disappeared, and in Sample 3, with a higher initial lycopene concentration, about 60% of the initial concentration of α-tocopherol remained (Table 1). This result is notable because

TABLE 1
Initial and Final Concentrations of α-Tocopherol in Sample Oils[a]

	Initial concentration ± SD (mg/kg$_{oil}$)	Final concentration ± SD (mg/kg$_{oil}$)
Sample 1	115.89 ± 0.09	Absent
Sample 2	118.68 ± 0.11	Absent
Sample 3	119.15 ± 0.07	71.49 ± 0.04

[a]Calculated using the HPLC method described in the Materials and Methods section. Values are the means ± SD ($n = 6$).

vegetable fats and oils and products derived from them are the main sources of vitamin E-active compounds in the human diet. This topic was underlined in the latest North American guidelines that considered α-tocopherol to be the only biologically active form of vitamin E and discounted other vitamers (22). Not only is vitamin E an important natural antioxidant in foods, especially those rich in PUFA, but also, owing to its role as a scavenger of free radicals, it is believed to protect our bodies against degenerative malfunctions, mainly cancer and cardiovascular diseases (23–27). α-Tocopherol is traditionally considered to be the major antioxidant found in olive oil (25), where it constitutes about 90% of the total tocopherols. As such, its natural concentration varies from a few ppm up to 300 ppm (25,26); for these reasons it is worthwhile to preserve this molecule against degradation during storage.

With regard to HPLC analysis of lycopene and β-carotene, for a fast and unambiguous structural assignment, the coupling of HPLC to MS is an elegant solution, but since carotenoids belong to a rather nonpolar substance class, their ionization for MS detection is not simple. Since they lack a site for protonation, detection by ESI is difficult. Therefore, carotenoids have been investigated by APCI-MS. In Figure 6 is shown the HPLC-APCI-MS analysis of pure lycopene dissolved in hexane. In Figure 6C the spectrum registered by DAD and the mass spectrum by APCI source with related fragmentation (Fig. 6D) are reported. In Figure 7 the HPLC-APCI-MS analysis of pure β-carotene dissolved

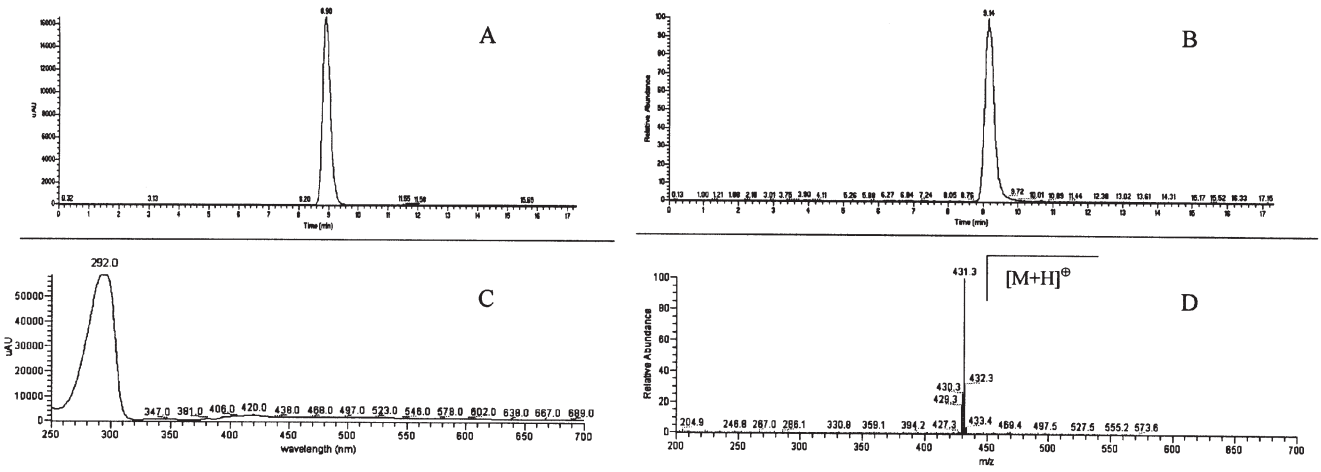


FIG. 5. HPLC-diode array detector-atmospheric pressure chemical ionization-mass spectrometry (HPLC-DAD-APCI-MS) coupling of an α-tocopherol standard. (A). Chromatogram acquired by DAD; (B) detection of α-tocopherol in positive-ion APCI-MS; (C) UV spectrum; (D) positive ion APCI mass spectrum.

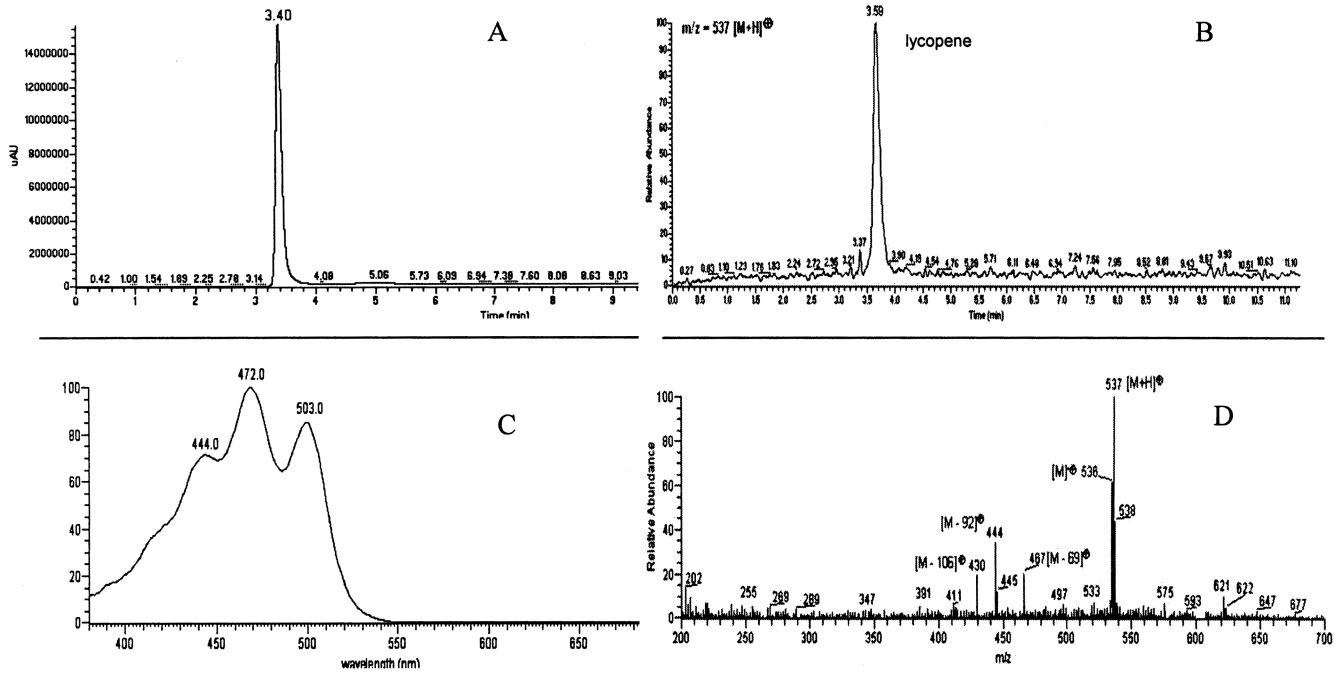


FIG. 6. HPLC-DAD-APCI-MS coupling of pure lycopene. (A) Chromatogram acquired by DAD. (B) detection of lycopene in positive-ion APCI-MS by extraction of the mass *m/z* 537 of the protonated molecule $[M + H]^+$; (C) UV spectrum; (D) positive ion APCI mass spectrum. For abbreviations see Figure 5.

in hexane is shown. In parts C and D of the figure are shown the spectrum recorded by DAD and the mass spectrum by APCI source with related fragmentation, respectively. The structural elucidation of these two isomers with mass spectrometry is very difficult because of the similar fragmentation patterns (28–30), but the two molecules are different with respect to the peak at *m/z* 467, which is present only in pure lycopene and which corresponds to fragment $[M - 69]^+$, identified as the end group of lycopene. This fragment is completely lacking in the spectrum of β-carotene because of its different end group: In β-carotene a ring is present as the terminal group whereas in lycopene the end groups are in open form. The other important radical ion, recorded at *m/z* 444 and identified as $[M - 92]^+$, is typical for both β-carotene and lycopene and is formed by free-radical fragmentation from the radical cation $[M]^{\bullet+}$. To aid structure determination, the UV spectra were recorded using a DAD. In evaluating the spectrum from 380 to 700 nm, β-carotene and lycopene can be identified because of their different absorption maxima.

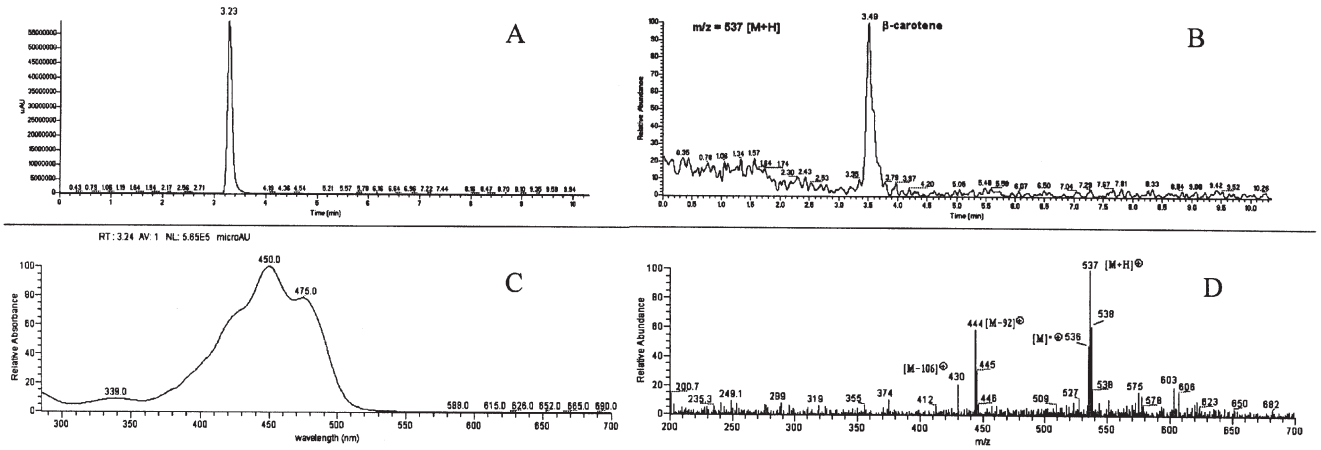


FIG. 7. HPLC-DAD-APCI-MS coupling of pure β-carotene. (A) Chromatogram acquired by DAD; (B) detection of lycopene in positive-ion APCI-MS by extraction of the mass *m/z* 537 of the protonated molecule $[M + H]^+$ (C) UV spectrum; (D) positive ion APCI mass spectrum is shown. For abbreviations see Figure 5.

TABLE 2
Initial and Final Concentrations of Lycopene in Sample Oils 2 and 3[a] (treated with lycopene, 0.5 and 1.0 mg/100 g, respectively)

	Initial concentration ± SD (mg/100 g_{oil})	Final concentration ± SD (mg/100 g_{oil})
Sample 2	0.58 ± 0.03	0.37 ± 0.01
Sample 3	1.05 ± 0.04	0.65 ± 0.02

[a]Calculated using the HPLC method described in the Materials and Methods section. Values are the means ± SD (n = 6).

The former had maxima at (423), 450, and 475 nm [The 423 nm value is relative to a shoulder that is not automatically integrated by DAD; it is calculated manually], while the latter had maxima at 444, 472, and 503 nm when measured under the same analytical conditions. In Figure 8 the separation between lycopene and β-carotene in the oil by means of HPLC is shown. This separation was important in order to monitor the variation of lycopene concentration in the three sample oils. In Figure 8A the separation between lycopene and β-carotene is shown whereas in Figures 8B and 8C the UV spectra of lycopene and β-carotene, respectively are shown; the spectra present the same absorption maxima as those of the relatively pure standard compounds. In Table 2 the results of lycopene concentration (mg/100 g) in added oil samples are reported.

The initial value for lycopene of Sample 2 was 0.58 mg/100 g whereas its final concentration was 0.37 mg/100 g. For Sample 3 initial and final concentrations were 1.05 and 0.65 mg/100 g, respectively (Table 2). From these data we can assert that lycopene added to olive oil survives very well. After 37 wk more than 60% remains with respect to the initial amount in both samples.

The results of the dose-response curves obtained by using pure lycopene from eight different sets of measurements are shown in Figure 9. The equation of the curve was: $y = 2 \times 10^9 x + 77.39 \times 10^4$ ($r^2 = 0.9999$).

We also determined the Peroxide Index during sample storage. The peroxide content present in a dietary fat or oil attests to its state of primary oxidation and thus its tendency to go rancid. Unsaturated FA react with oxygen forming peroxides, which determine a series of chain reactions whose end result is the formation of volatile substances having the characteristic rancid odor. These reactions are accelerated by high temperatures and by exposure to light and oxygen. Lower peroxide values are closely related to better-quality dietary oil and its state

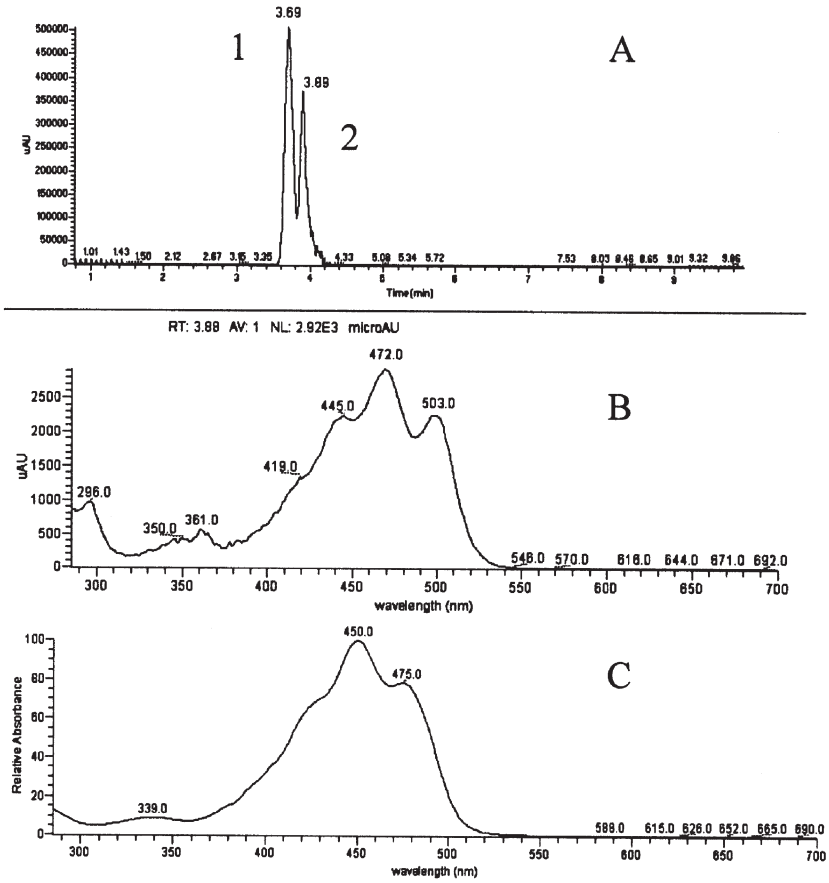


FIG. 8. HPLC separation of lycopene and β-carotene from Sample 2 (A); peak 1 is identified as β-carotene, peak 2 as lycopene. In (B) and (C) the UV spectra of lycopene and β-carotene, respectively, are shown.

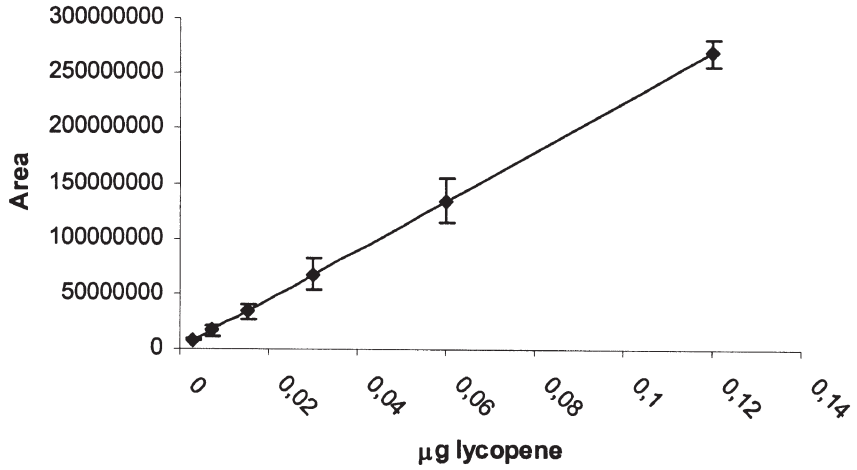


FIG. 9. Calibration curve plotted with different amounts of pure lycopene dissolved in hexane and measured with HPLC (volume of injection 20 µL), $y = 2 \times 10^9 \times + 77.39 \times 10^4$, $r^2 = 0.9999$ ($n = 8$).

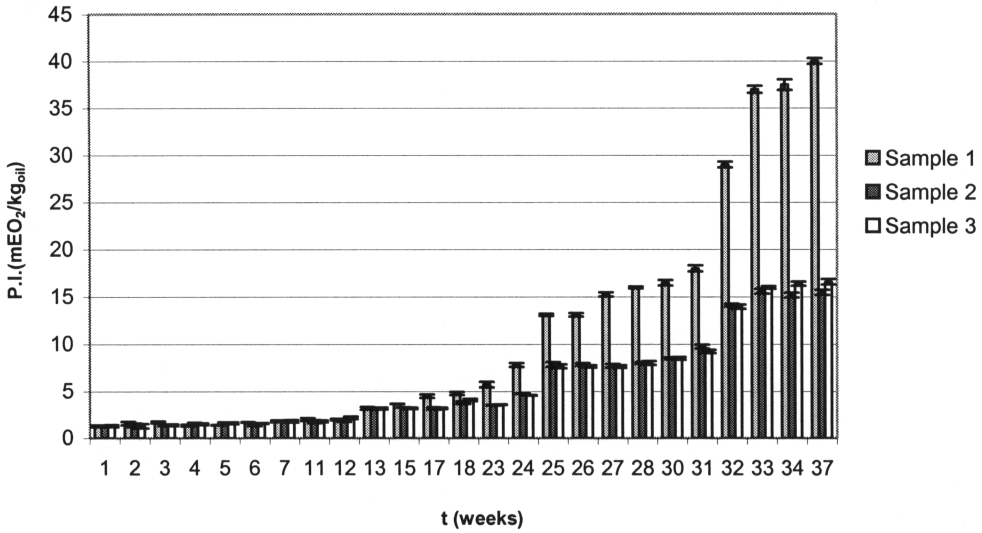


FIG. 10. Value of peroxide index (P.I.) during storage. The values shown are the mean of at least six determinations and are expressed as mean ± SD ($n = 6$).

of preservation. In Figure 10 the peroxide index for a period of 37 wk is shown. During the first 13 wk no remarkable variations were observed between the oil samples with added lycopene (Samples 2 and 3) and the reference sample (Sample 1). After this time different trends were seen: the peroxide index values of Sample 1 increased more over time than Samples 2 and 3. In particular, after 32 wk the peroxide index of Sample 1 was 29, which is over the maximum allowed by law (21), whereas the values for Samples 2 and 3 were both 14. At the end of 37 wk, Sample 1–3 had values of 40, 15, and 16, respectively.

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