HPLC Analysis of Phenolic Antioxidants, Tocopherols and Triglycerides

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Triglycerides, together with nine synthetic phenolic antioxidants most commonly used to prevent oxidation of edible oils and fats, as well as the natural antioxidants tocopherols and α-tocopherol acetate, were separated by high performance liquid chromatography by means of a reversed phase C_{18}-column and gradient elution with water/acetonitrile/methanol/isopropanol. Besides dilution of the oil with isopropanol/hexane, no further example preparation was required. UV detection was applied. The synthetic antioxidants propyldodecylgallate, octyldodecylgallate, dodecylgallate, 3-*tert*-butyl-4-hydroxyanisol, *tert*-butylhydroquinone, 3,5-*di-tert*-butyl-4-hydroxytoluene, 2,6-*di-tert*-butyl-4-hydroxymethylphenol, 2,4,5-trihydroxybutyrophenone and nordihydroquaiaretic acid, as well as α- and δ-tocopherol and α-tocopherol acetate were base-line separated; β- and γ-tocopherol, however, eluted together. The triglycerides, detected at $\lambda = 215$ nm, were separated according to their partition number. The absorption at $\lambda = 215$ nm revealed saturated and unsaturated triglycerides. The absorption at $\lambda = 280$ nm indicated triglycerides with conjugated unsaturation, relating information about refining and heat treatment of the oil. Oxidized unsaturated triglycerides showed absorption at $\lambda = 230$ nm. Triglycerides of ricinoleic acid, a hydroxymonounsaturated acid, gave identical UV spectra. The simultaneous detection of antioxidants and triglycerides may be used to study inhibition effects by antioxidants in oils.

KEY WORDS: Autoxidation of triglycerides, HPLC analysis of sunflower oil, phenolic antioxidants, separation by HPLC, tocopherols, triglycerides, vitamin E.

Synthetic antioxidants are phenolic substances widely used as additives in foods, especially in oils and fats, in order to delay or prevent oxidative deterioration. The phenolic protection is usually accomplished by interrupting the free radical pathway of lipid oxidation (1). In various countries, restrictions exist concerning the use of antioxidants (2).

Vitamin E, mainly consisting of α-, β-, γ and δ-tocopherol, is the naturally occurring antioxidant in edible oils and fats. It may be present up to 2,000 ppm, but usually its concentration lies between 100–300 ppm (3). The tocopherols act in the same manner as the synthetic antioxidants.

Several high performance liquid chromatography (HPLC) methods (2–9) have been reported to separate phenolic food antioxidants. A reversed phase chromatography system and UV absorbance detection has been adopted by AOAC (10). According to the AOAC official methods of analysis (1,10), antioxidants in oils and fats are analyzed by dissolving the sample in hexane and the antioxidants are partitioned into acetonitrile. The solution is concentrated and diluted with isopropanol and injected into the liquid chromatograph equipped with UV detection at 280 nm. The separation of the antioxidants propyldodecylgallate (PG), *tert*-butylhydroquinone (TBHQ), nordihydroquaiaretic acid (NDGA), 3-*tert*-butyl-4-hydroxyanisol (BHA), octyldodecylgallate (OG), dodecylgallate (DG), 3,5-*di-tert*-butyl-4-hydroxytoluene (BHT) was achieved by gradient elution with distilled water and 30–100% acetonitrile in the presence of 5% acetic acid. It has been stated that the extraction procedure causes some recovery problem for TBHQ (2). Another problem with reversed phase elution systems was the coelution of certain antioxidants, *e.g.*, 2,6-*di-tert*-butyl-4-hydroxymethylphenol (Ionox-100) and PG or DG and BHT (4,10). When acetonitrile is replaced by methanol, overlapping of 2,4,5-trihydroxybutyrophenone (THBP) and TBHQ, as well as BHT and DG, occurred (4).

Tocopherols and tocopherol esters have been analyzed by normal or reversed phase HPLC systems applying UV absorbance or fluorometric detection (11). Tocopherols are usually determined in oils without previous extraction of the antioxidants. The determination of tocopherols and tocotrienols in vegetable oils and fats by direct HPLC has been described by Pocklington and Dieffenbacher (12).

Oil triglycerides (TGs) have been separated effectively by reversed phase HPLC, allowing clear differentiation of different oils. Excellent separations of the TGs were obtained with propionitrile as eluent (13). Reviews on the subject have been published (14,15). To our knowledge there is only one report dealing with the simultaneous HPLC analysis of antioxidants and tocopherols (9), and there is no report on TG detection in the same HPLC run as antioxidants and tocopherols.

In the present study, synthetic phenolic antioxidants, tocopherols and TGs were separated in the same run. In order to obtain a better separation of the antioxidants, the AOAC method (10) was modified by changing the content of methanol in acetonitrile. To prevent tailing of the antioxidants, the water eluent was adjusted with phosphoric acid. For the elution of the less polar fractions isopropanol was added. The oil sample was dissolved in isopropanol/hexane and injected.

EXPERIMENTAL PROCEDURES

Apparatus. The liquid chromatographic system consisted of a Hewlett-Packard HP1090 system (Hewlett-Packard, Palo Alto, CA) with a HP1040A diode array UV detector, a Perkin Elmer ISS-100 autosampler (Perkin Elmer, Norwalk, CT), a HP9836C work-station with a HP7958 hard disc drive and a HP7470A plotter. A reversed phase Nucleosil 120-5 C_{18}-column (125 × 4 mm, 5 μ particle size, cat. no. 720,051) and a precolumn (cat. no. 720,999, Macherey-Nagel, Düren, Germany) were used.

Reagents and chemicals. All solvents were HPLC grade. The phenolic antioxidants were purchased from Fluka AG (Buchs, Switzerland). The tocopherols were obtained from

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Merck (Darmstadt, Germany) and the TGs triolein, trilinolein and trilinolenin were from Sigma (St. Louis, MO). The oil used was a commercial sunflower oil.

Preparation of standards and samples. Isopropanol/hexane (4:1) was used to prepare all standard and oil solutions. This solvent mixture was proposed by Wolf for lipid extraction instead of methanol/chloroform (16). Stock solutions were prepared of the individual antioxidants and tocopherols containing an accurately known concentration of about 50 mg/mL of each substance. A solution with the synthetic phenolic antioxidants and a second solution with the tocopherols, containing accurately about 5 mg/mL of each substance, were made up. Working solutions containing accurately about 300 mg/mL TGs, 50 μg/mL of each synthetic antioxidant and 300 μg/mL of each tocopherol were used and 20 μL were injected with the autosampler.

Eluents. The following eluting solvents were used: A, water + phosphoric acid (pH 3); B, acetonitrile/methanol (7:5); and C, isopropanol.

TABLE 1

Solvent Gradient

Time (min)	Solvents: A	B	C
0	70	30	0
25	0	100	0
Hold			
30	0	100	0
45	0	40	60
Hold			
63	0	40	60
64	0	100	0
67	70	30	0
Hold (equilibration)			
77	70	30	0

The solvent gradient used is shown in Table 1. The flow rate was 1 mL/min. The absorbance was measured at wavelengths of 215 and 280 nm. The spectral data of each peak were recorded in the 210–330 nm range.

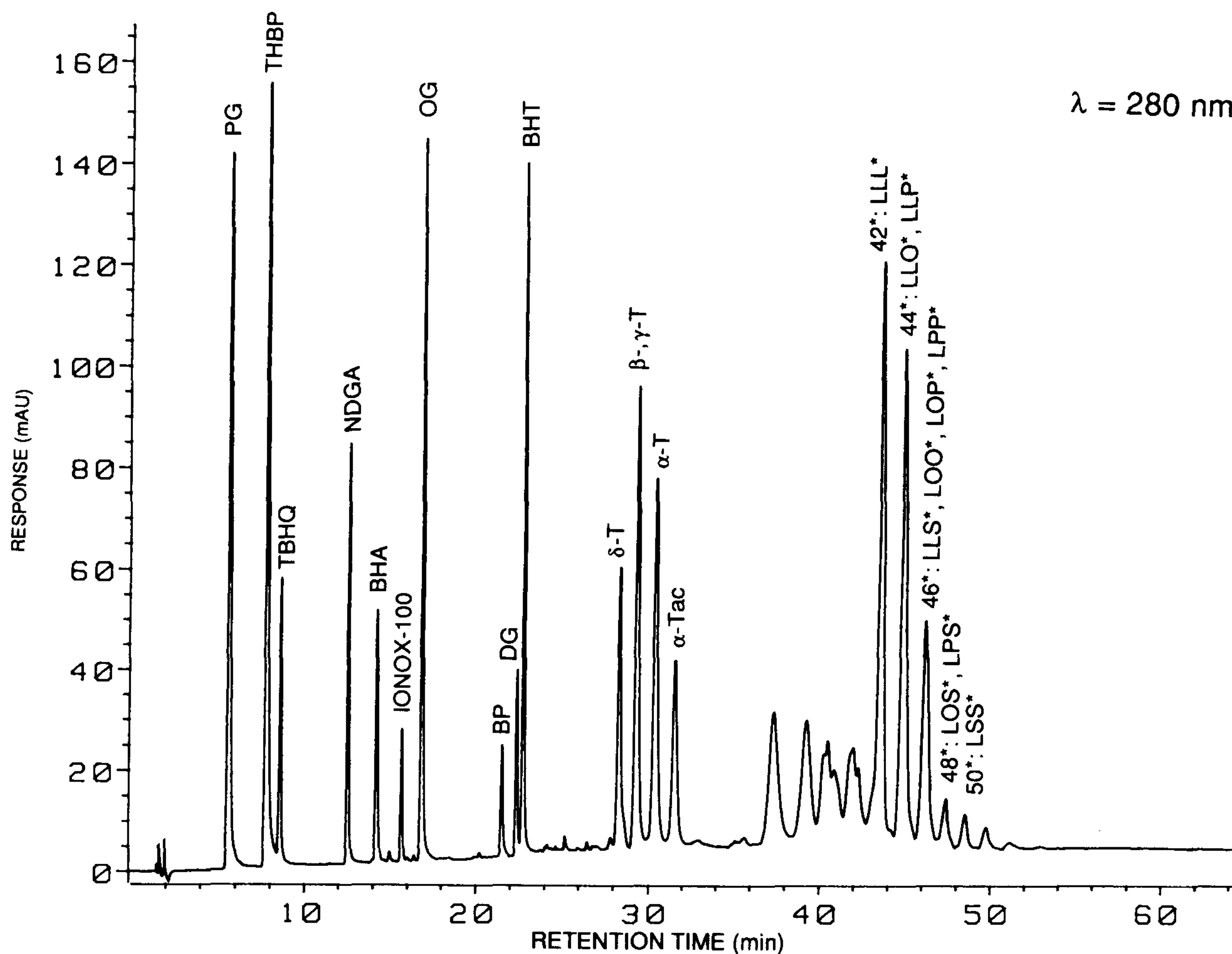

FIG. 1. HPLC of synthetic antioxidants, tocopherols and triglycerides with conjugated double bonds. Partition number (PN): 42*, LLL*; 44*, LLO*, LLP*; 46*, LLS*, LOO*, LOP*, LPP*; 48*, LOS*, LPS*; 50*, LSS*. Detection: UV at λ = 280 nm. See Text for HPLC conditions. P, 16:0; S, 18:0; O, 18:1 and L, 18:2.

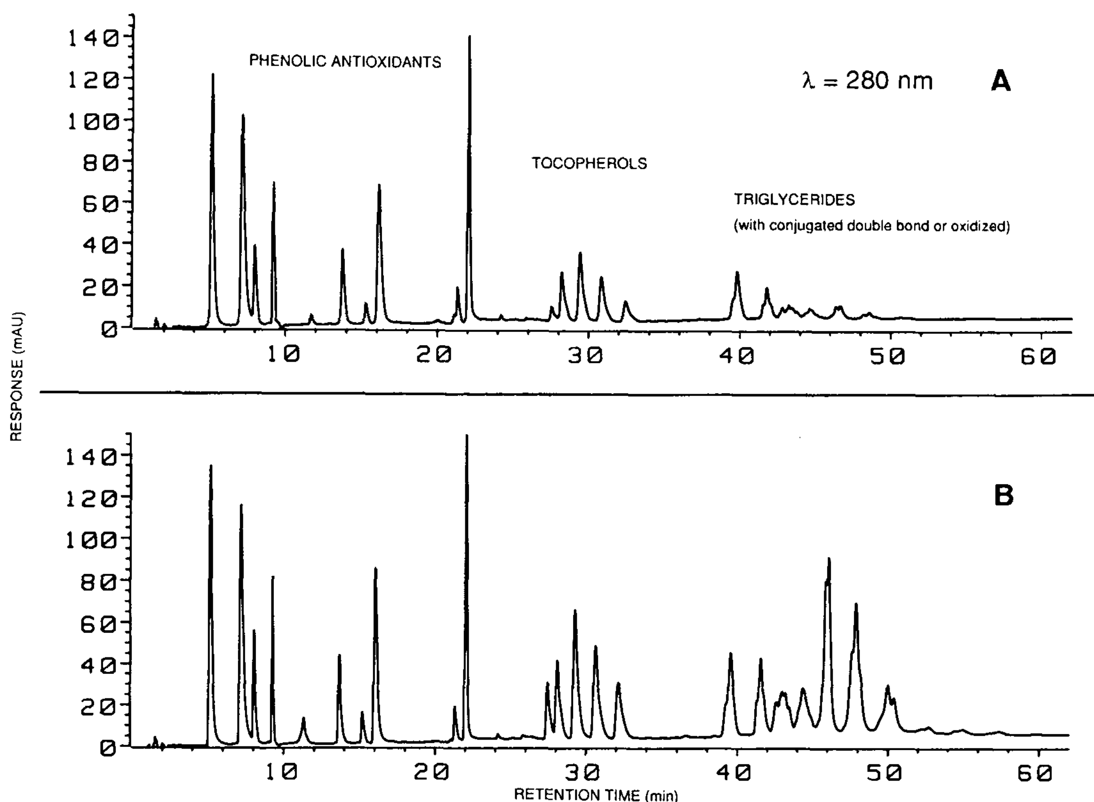

FIG. 2. HPLC of synthetic phenolic antioxidants, tocopherols and triglycerides (λ = 280 nm). A, extraction with acetonitrile from hexane, according to AOAC method 20.009–20.013 (1984). B, dilution of the oil with hexane/isopropanol. See Figure 1 for peak identification, text for HPLC conditions.

RESULTS AND DISCUSSION

In the present study the separation of phenolic antioxidants, tocopherols and TGs was carried out on a reversed phase C_{18}- column by gradient elution with water (pH 3)/acetonitrile/methanol/isopropanol. The best separation of the antioxidants was obtained by using a ratio of methanol in acetonitrile of 5:7 (solvent B, Table 1). Methanol was effectively used as polar modifier to promote the separation of THBP and TBHQ as well as BHT and DG. The water eluent was adjusted to pH 3 with phosphoric acid to prevent tailing of the antioxidants (solvent A, Table 1). The less polar fractions, including the triglycerides, were eluted by addition of isopropanol (solvent C, Table 1).

Synthetic antioxidants. Figure 1 shows a chromatogram measured at 280 nm. The wavelengths of maximum absorption of individual antioxidants as obtained from the spectra were: PG (275 nm), OG (275 nm), DG (275 nm), BHA (290 nm), TBHQ (291 nm), BHT (280 nm), Ionox-100 (276 nm), THBP (280 nm) and NDGA (284 nm).

Tocopherols. α- and δ-Tocopherol and α-tocopherol acetate were baseline separated. β- and γ-Tocopherol, however, are eluted together (Fig. 1). A separation of these two tocopherols may be performed on polar phases [silica, amino or cyano phases (12,17)], but not on reversed phase columns (18). The wavelengths of maximum absorption were: α-tocopherol (295 nm), γ-tocopherol (300 nm), δ-tocopherol (298 nm) and α-tocopherol acetate (289 nm).

Analysis with and without extraction. Chromatograms obtained with acetonitrile for the extraction of a sample dissolved in hexane [according to the AOAC methods 20.009–20.013 (1984)] and without extraction but by dilution with isopropanol/hexane (4:1) only, are shown in Figure 2. The concentrations of the synthetic phenolic antioxidants extracted were somewhat lower, especially in the case of TBHQ. Tocopherols and triglycerides remained, to a large extent, in the hexane solution. This shows that, apart from simplifying the analysis, eliminating the extraction procedure can lead to more accurate determinations.

Triglycerides. UV detection of triglycerides is usually carried out at λ = 215 nm. At this wavelength the absorption is due to the carboxy group. The triglycerides elute according to their partition number (PN), defined as PN = CN − 2n, where CN is the total of fatty acid carbon and n is the number of double bonds in the molecule (Fig. 3). The PN is applicable only to defined analytical conditions (19).

Triglycerides detected at λ = 280 nm, the wavelength used for the detection of antioxidants, reveal unsaturated triglycerides containing conjugated double bonds (Fig. 1). Triglycerides containing conjugated unsaturation differ from their non-conjugated, methylene-interrupted, unsaturated isomers in their UV absorption spectra. Conjugation of double bonds leads to strong absorption at

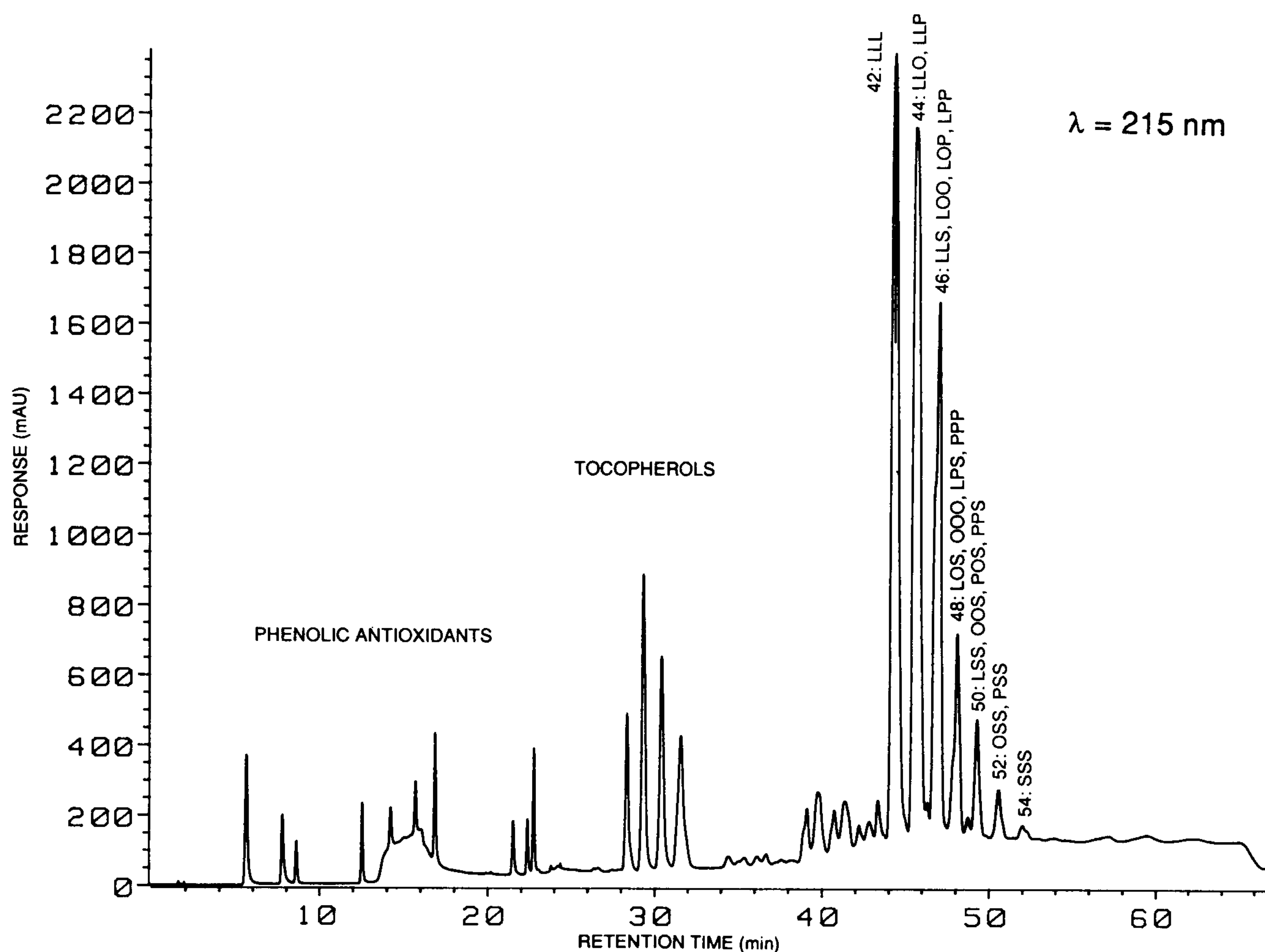

FIG. 3. HPLC of synthetic phenolic antioxidants, tocopherols and triglycerides. For peak identification of phenolic antioxidants and tocopherols, see Figure 1. Triglycerides PN: 42, LLL; 44, LLO, LLP; 46, LLS, LOO, LOP, LPP; 48, LOS, OOO, LPS, OOP, OPP, PPP; 50, LSS, OOS, OPS, PPS; 52, OSS, PSS; 54, SSS. Detection: UV at λ = 215 nm. See text for HPLC conditions.

higher wavelengths. Conjugated dienes absorb at about 232 nm. Conjugated trienes show a characteristic absorption triplet at 258, 268 and 278 nm (maximum at 268 nm), and conjugated tetraenes show a similar absorption triplet at 285, 300 and 315 nm (maximum at 300 nm) (20).

As illustrated in Figure 4, conjugated and oxidized triglyceride derivatives (LLL*, LLO*, LLP*, etc.) elute before the parent triglycerides with methylene-interrupted double bonds (LLL, LLO, LLP, etc.). The same is indicated by the lower retention times of triglycerides detected at 280 nm (Fig. 1) than the respective retention times detected at 215 nm (Fig. 3). The components eluting before the triglycerides are due to oxidation of unsaturated triglycerides in sunflower oil (Figs. 1 and 4). Although alkaline isomerization of linoleic and linolenic acid in inert atmosphere leads to conjugated dienes and trienes, respectively, autoxidation of oils and fats leads to hydroperoxides as primary oxidation products that decompose into a variety of secondary oxidation products (21). Among them, hydroxy derivatives and carbonyl groups conjugated to double bonds of the carbon chain prevail. Also, formation of epoxy and carboxy groups, dimerization and polymerization has been reported (22,23). As a result, secondary oxidation products of trilinolein (LLL*) may absorb in the region of conjugated trienes (Fig. 5) and those of trilinolenin (LnLnLn*) may absorb in the region of conjugated tetraenes (Fig. 5). Schuster (24) obtained similar peaks and UV spectra as shown in Figures 1 and 5. He suggested that they were due to peroxides. However, when the oil was treated with potassium iodide to reduce the peroxides, the peaks and spectra remained. Castor oil, containing ricinoleic acid (C18:1, 9c-12OH) (R) as the main component, gives identical UV spectra. Thus, these peaks may be attributed to triglycerides containing hydroxy fatty acids (Fig. 4).

The fraction of sunflower oil with the retention time between 37 and 42 min (Fig. 1) was also analyzed by gas chromatography/mass spectrometry after hydrolysis and silylation (25). The analysis revealed hydroxy fatty acids, fatty acids, dicarboxylic acids, glycerol, β-sitosterol, stigmasterol and campesterol, indicating coelution of these compounds.

HPLC is considered to be well suited for the separation and analysis of vegetable oils and additives. The

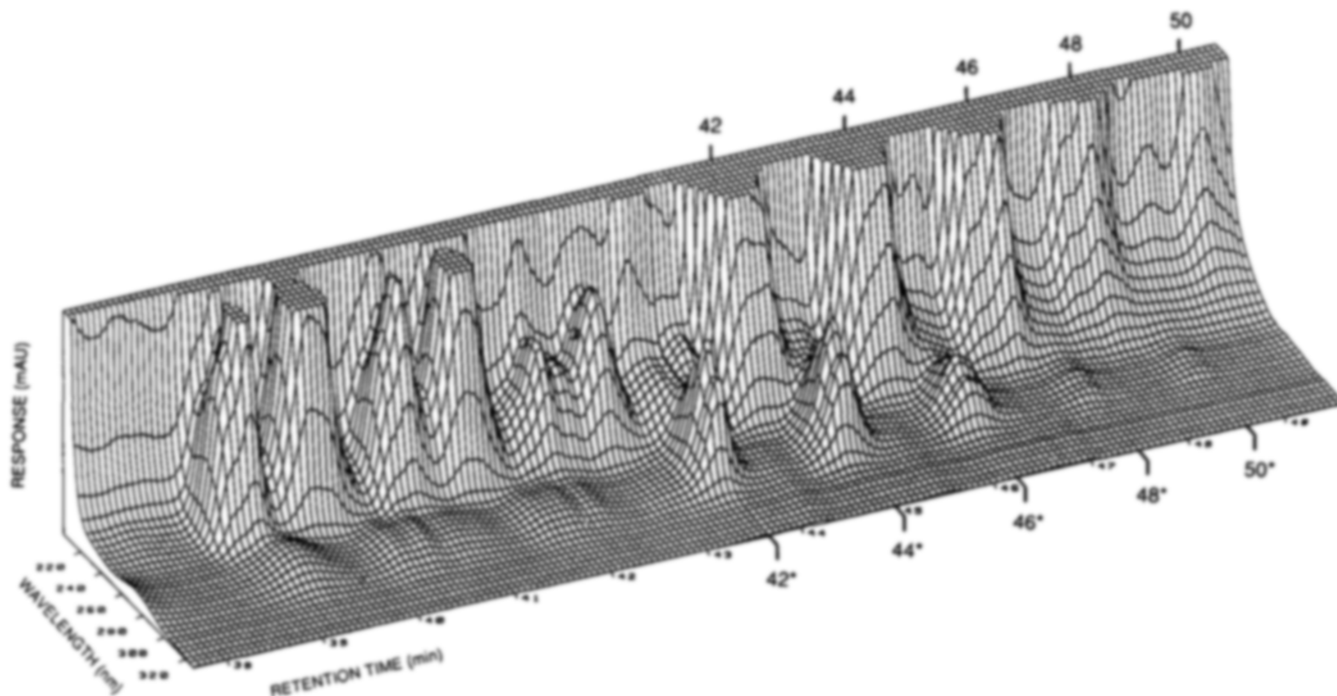

FIG. 4. 3-DChromatogram of the triglyceride fraction (retention time 38–49 min, UV absorption 200–330 nm). Identification of peaks with absorption maxima at 215 nm: PN 42, LLL; 44, LLO, LLP; 46, LLS, LOO, LOP, LPP; 48, LOS, OOO, LPS, OOP, OPP, PPP; 50, LSS, OOS, OPS, PPS; 52, OSS, PSS; 54, SSS. Identification of peaks with absorption maxima at 270 nm: PN 42*, LLL*; 44*, LLO*, LLP*; 46*, LLS*, LOO*, LOP*, LPP*, 48*, LOS*, LPS*; 50*, LSS*. Oxidation products are eluted in the region of 38–43 min. See text for HPLC conditions.

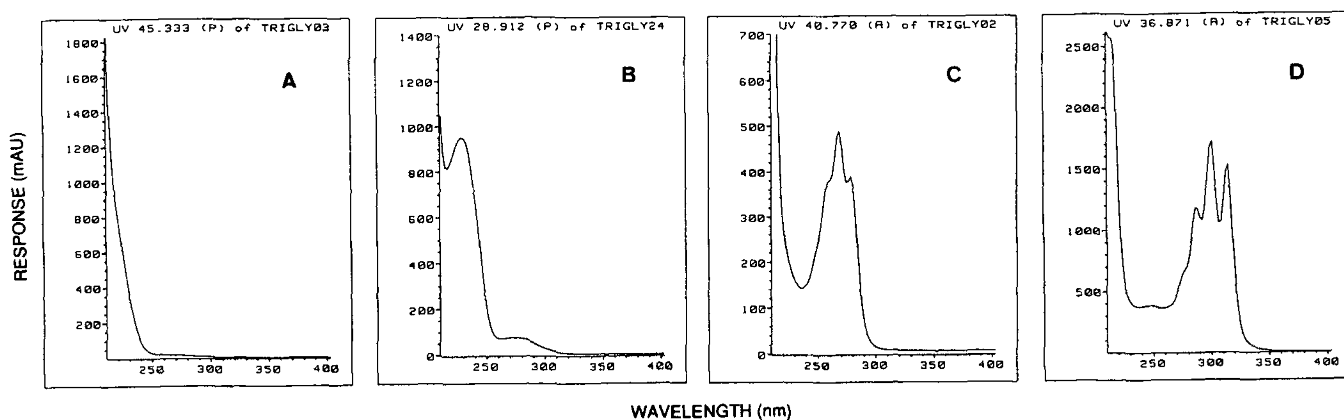

FIG. 5. UV spectra of A, triolein (OOO); B, castor oil (main peak RRR); C, trilinolein (oxidation product LLL*), and D, trilinolenin (oxidation product LnLnLn*). The spectra were recorded from the peak fractions separated by HPLC.

triglyceride elution pattern and the UV spectra allow conclusions about the state of oils, their unsaturation, changes due to refining and heat treatment. Thus, the simultaneous detection of antioxidants and triglycerides may allow study of the inhibition effects of antioxidants.

REFERENCES

1. Labuza, T.P., *Critical Reviews of Food Technology* 2:355 (1971).
2. Page, B.D., *J. Assoc. Off. Anal. Chem.* *66*:727 (1983).
3. Gertz, C., and K. Herrmann, *Z. Lebensm. Unters. Forsch.* *174*:390 (1982).
4. Page, B.D., *J. Assoc. Off. Anal. Chem.* *62*:1239 (1979).
5. King, W.P., K.T. Joseph and P.T. Kissinger, *Ibid.* *63*:137 (1980).
6. Masoud, A.N., and Y.N. Cha, *J. High Resolut. Chromatogr. Commun.* *5*:299 (1982).
7. Gertz, C., and K. Herrmann, *Z. Lebensm. Unters. Forsch.* *177*:168 (1983).
8. Kitada, Y., Y. Veda, M. Yamamoto, K. Shinomiya and H. Nakazawa, *J. Liquid Chromatogr.* *8*:47 (1985).
9. Indik, H., and D.C. Woollard, *J. Chromatogr.* *356*:401 (1986).
10. *Official Methods of Analysis,* Method 20.009–20.013, Association of Official Analytical Chemists, 14th edn., edited by Sidney Williams, 1984.
11. Deschnytere, A., and H. Deelstra, *Fresenius Z. Anal. Chem.* *324*:1

(1986).
12. Pocklington, W.D., and A. Dieffenbacher, *Pure Appl. Chem. 60*:877 (1988).
13. Fiebig, H.J., *Fette Seifen Anstrichmittel 87*:53 (1985).
14. Kapoulas, V.M., and N.K. Andrikopoulos, *J. Chromatogr. 366*:311 (1986).
15. Aitzetmüller, K., H. Wessels and G. Werner, *Fat Sci. Technol. 90*:442 (1988).
16. Wolf, R.L., and A.F.M. Castra, *Revue Française des Corps Gras. 34*:123 (1987).
17. Rammell, J.C., and J.J.L. Hoogenboom, *J. Liquid Chromatogr. 8*:707 (1985).
18. Rammell, G.C., B. Cunliffe and A.J. Kieboom, *J. Liquid Chromatogr. 6*:1123 (1983).
19. Takahashi, K., T. Hirano and K. Zama, *J. Am. Oil Chem. Soc. 61*:1226 (1984).
20. Kapoulas, W.M., and N.K. Andrikopoulos, *Food Chemistry 23*:183 (1987).
21 Galanos, D.S., V.. Kapoulas and E. Voudouris, *Rev. Française des Corps Gras. 15*:291 (1968).
22, Neff, W.E., E.N. Frankel and K. Fujimoto, *J. Am. Oil Chem. Soc. 65*:616 (1988).
23. Gunstone, F.D., *Ibid. 61*:441 (1984).
24. Schuster, R., *Multicomponent Analyses of Fats and Oils Using Diode-Array Detection*, Hewlett-Packard, HPLC Application Note No. 12-5954-6269, 1988.
25. Brueschweiler, H, and A. Hautfenne, *J. Pure and Appl. Chem. 62*:781 (1990).

[Received September 17, 1990; accepted February 20, 1991]