Identification and Estimation of Tocotrienols in the Annatto Lipid Fraction by Gas Chromatography–Mass Spectrometry

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ABSTRACT: The lipid fraction of annatto (*Bixa orellana* L.) seeds was extracted with a Soxhlet apparatus with *n*-hexane and isolated by thin-layer chromatography. The fatty-soluble antioxidant fraction contained only tocotrienols, mainly δ-tocotrienol, but no tocopherols. The presence of tocotrienols was confirmed by gas chromatography–mass spectrometry. The quantities of δ-tocotrienol were 140–147 mg/100 g dry seeds and 5.2–5.5% wt/wt of lipid extract, determined by gas chromatography and high-performance liquid chromatography, respectively. Currently no vegetable species seems to contain comparable concentrations of δ-tocotrienol.
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KEY WORDS: Annatto, chemical ionization, mass spectrometry, tocotrienols.

Saturated (tocopherols) and unsaturated (tocoenols) methyl derivatives of tocol [3,4-dihydro-2-methyl-(4′,8′,12′-trimethyltridecyl)-2H-1-benzopyran-6-ol] are lipid-soluble natural antioxidants. Tocopherols are present in vegetable oils and green parts of higher plants (1). In contrast, tocotrienols are found in the bran and germ fraction of various seeds and cereals: wheat (2–6), rye, corn, rice (2,5), triticale, millets (2), oats, barley (2, 4,5), grape seeds (7), almond (5), some *Amaranthus* spp. seeds (8,9), and *Apiaceae* seeds (10). Tocotrienols are also found in oleaginous fruits of coco-tree (4,5) and palm species (5,11–13). Generally, the presence of δ-tocotrienol in nature is rather rare, being found only in few vegetable species: palm (5,12–14), some *Amaranthus* spp. (8,9), rice bran (2,15), barley, millets (2), and *Apiaceae* seeds (10).

Other forms of natural tocols were identified in several vegetable oils: esterified tocopherols and tocotrienols in rice bran oil, soybean oil, sesame oil (16) and palm oils (crude palm oil, palm olein, refined, bleached, and deodorized palm olein) (17); tocodienols in palm oil (10); tocomonoenols in palm, olive, sunflower, safflower, wheat germ, corn, sesame, soybean, and grapeseed oils (10).

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The presence of these compounds is of great importance, because they are able to prevent autoxidation of oil in the seed. There are many studies on the antioxidant and biological properties of tocopherols, both *in vivo* and *in vitro* (1), but the studies on the activity of tocotrienols are relatively few, probably because of the scarcity of these compounds.

Tocotrienols recently received much attention for their important biological activities; in fact they play a fundamental and positive role in the pathologies implicating oxidative stress, especially in the inhibition of cancer development (18–21) and in reducing the risk of cardiovascular disease (19,22–24).

Annatto (*Bixa orellana* L.) seeds contain bixin and orelline, carotenoid pigments present on its outer surface. These pigments are lipid-soluble and are currently used to color butter and margarine. Annatto lipid extracts also have antioxidant properties (25); therefore, the lipid fraction extracted by the seeds has been studied.

EXPERIMENTAL PROCEDURES

Annatto seeds were supplied by Cathay Pacific Multi-Commodities Corporation (Quezon City, Philippines). Sixty grams of seeds were ground and extracted in a Soxhlet apparatus with 200 mL of *n*-hexane for 8 h.

The tocols fraction was analyzed by high-performance liquid chromatography (HPLC) using a Knauer (Berlin, Germany) pump model 64 with an Autochrom M300 gradient/system controller (Milford, MA) and a fluorimetric detector Jasco 821-FP (Tokyo, Japan). A solution of the crude extract in the mobile phase was directly injected into the HPLC system. The operative parameters are in Table 1.

The crude extract was saponified according to the procedures detailed in "Norme Grassi e Derivati" (Method NGD C12-1976) (26). Following the treatment with diazomethane (CH_2N_2) (27) to transform any free fatty acid present in methyl ester, 10% benzene solution of unsaponifiable matter was prepared. After evaporation and treatment with silanizing reagent to transform the hydroxylic groups into trimethylsilyl derivatives (TMS), according to Sweeley *et al.* (28), 50 μL of this solution was analyzed by gas chromatography using a Carlo Erba (Milano, Italy) HRGC 5160 Mega

TABLE 1
Experimental Conditions for Chromatographic Determinations

Analytical parameters	Crude extract	Unsaponifiable matter	Unsaponifiable matter, sterols, alcohols, tocopherols
Type of analysis	HPLC	GC–MS	GC
Column stationary phase	Techsil 5[a]	SPB-5[b]	TAP[c]
Column length (m)	0.25	30	25
Internal diameter (mm)	4.6	0.25	0.32
Film thickness (μm)	—	0.25	0.1
Particle size (μm)	5	—	—
Sample injection system	Rheodyne valve	Splitter	Splitter
Detection system	Fluorimeter λ_{exc} 290, λ_{em} 330 nm	Ion trap	FID.
Carrier	Isopropanol/*n*-hexane 0.5/99.5	He	He
Column flow (mL/min)	1	—	0.8
Column pressure (Kpa)	—	50	—
Solvent flow	Isocratic	—	—
Split ratio	—	1/60	1/80
Oven temperature initial (°C)	—	200 (1 min)	200 (1 min)
Oven temperature final (°C)	—	300 (30 min)	300
Temperature rate (°C/min)	—	3	3
Injector temperature (°C)	—	330	330
Detector temperature (°C)	—	—	330

[a]HPLC Technology Ltd., Macclesfield, Cheshire, United Kingdom. HPLC, high-performance liquid chromatography; GC–MS, gas chromatography–mass spectrometry; FID, flame-ionization detector; $_{exc}$, excitation; $_{em}$, emission.
[b]J&W, Folsom, CA.
[c]Triglyceride analysis phase (Chrompack, Middleburg, The Netherlands).

gas chromatograph interfaced with a Mega 2 computing integrator. The operative parameters are in Table 1.

The unsaponifiable fraction was analyzed, prior and after treatment with TMS reagent, by gas chromatography–mass spectrometry (GC–MS) using a Finnigan Magnum (San Jose, CA) instrument. The operative parameters are in Table 1.

The unsaponifiable matter was fractioned by thin-layer chromatography (TLC) with silica G plate (Stratochrom SI, Carlo Erba), using *n*-hexane/diethyl ether 60/40 (vol/vol) as eluent. Plates were sprayed with 0.2% ethanolic solution of 2,7′-dichlorofluorescein sodium salt, and the bands were scraped off and twice extracted with diethyl ether. Unsaponifiable bands were analyzed with the GC apparatus described above.

Catalytic hydrogenation was performed by bubbling pure hydrogen in a test tube containing a small amount of crude extract dissolved in benzene and a few milligrams of platinum-oxide powder (according to Adams) as catalyst (29). The identification of the compounds was carried out by comparing retention times (RT) with those of pure standards provided by Sigma Chemical Co. (St. Louis, MO) and Supelchem Inc. (Bellefonte, PA), and by comparison with the results published in literature (30). The amount of δ-tocotrienol was determined by GC, employing squalane (2,6,10,15,19,23-hexamethyltetracosane $C_{30}H_{62}$) as internal standard, and by HPLC, using γ-tocopherol as external standard.

RESULTS AND DISCUSSION

In Figure 1 are reported the chromatograms of the whole unsaponifiable fraction (A) and those of the TLC bands of tocopherols/tocotrienols (B), sterols (C), and alcohols (D). The most abundant component of the trace (A) is peak no. 3, accompanied by peaks 7 and 13.

In Figure 2 are reported the GC–MS traces of the whole lipidic extract after treatment with: diazomethane (A), diazomethane and silanizing reagent (B), and of the same extract after catalytic hydrogenation (C). Peak no. 1 shows a RT different from those of the most common tocols (α-, β-, γ-, and δ-tocopherol). The RT remains unaltered after diazomethane treatment, thus excluding the presence of a acid function, while it changes after treatment with a silanizing reagent, showing the presence of a hydroxyl group. The whole extract was submitted to catalytic hydrogenation, and the RT of peak no. 1 changes and equals that of δ-tocopherol.

There are also reported mass spectrums of the most abundant component of unsaponifiable matter (peak no. 1). Mass spectra display a typical fragmentation pattern of tocols: the three most abundant peaks correspond to molecular ion (M^+), an ion derived from the loss of the side chain ($C_{16}H_{33}$ in tocopherols, $C_{16}H_{27}$ in tocotrienols), and an ion originated from the cleavage of the side chain accomplished by the loss of methylacetylene fragment, respectively (4,14). In spectrum 2A molecular ion has an *m/z* ratio corresponding to the molecular weight of δ-tocotrienol. The characteristic fragment at *m/e* 177 derives from the loss of the triunsaturated side chain ($M^+ - 219$). The hydrogenated sample in Figure 2C shows a molecular ion of *m/e* 402 and the typical fragmentation of δ-tocopherol: the loss of the saturated side chain gives the fragment at *m/e* 177 ($M^+ - 225$). Furthermore, the GC–MS analysis of the TMS derivative (Fig. 2B) shows the characteristic

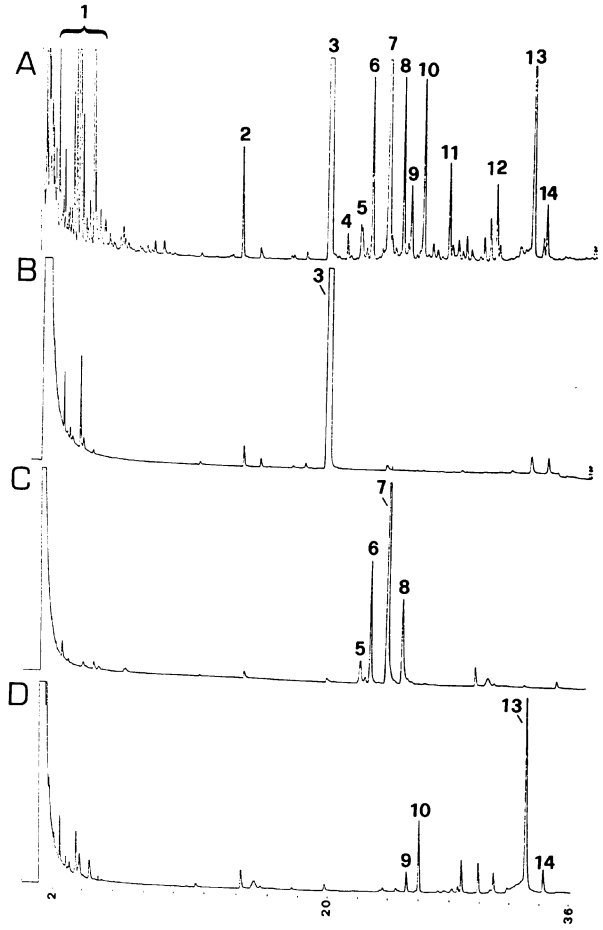

FIG. 1. Chromatograms of the whole unsaponifiable fraction (A) and of thin-layer chromatography bands of tocopherols/tocotrienols (B), sterols (C), and alcohols (D). 1, fatty acids methyl esters; 2, squalene; 3, δ-tocotrienol; 4, not identified; 5, campesterol; 6, stigmasterol; 7, β-sitosterol; 8, Δ^5-avenasterol; 9, cycloartenol, 10, 24-methylenecycloartanol; 11, citrostadienol; 12, not identified; 13, oleanolic acid; and 14, maslinic acid.

fragments with an increase of 72 daltons, typical of a TMS derivative.

On the same traces there is another peak, labeled with no. 2. This peak shows chemical and chromatographic behavior and mass spectra similar to peak no. 1 (Fig. 3), differing only for the presence of a methyl group in the aromatic moiety of the molecule (due to the presence of fragments with mass 151, 191, 223, and 263 instead of 137, 177, 209, and 249, respectively), and it was identified as β-tocotrienol.

The peculiarity of the lipidic fraction extracted from annatto seeds is that it completely lacks in tocopherols, but on the contrary contains a large quantity of δ-tocotrienol and traces of β-tocotrienol. Dosed quantities were 140 mg/100 g dry seeds or 5.2% w/w of lipid extract, determined by GC, and 147 mg/100 g dry seeds or 5.5% w/w of lipid extract, determined by HPLC. Currently, no vegetable species seems to contain comparable concentrations of δ-tocotrienol. This compound was only quantified in few substrata: 69 ppm in palm oil (5); trace–100 ppm in rice bran oil (refined) (15); 0.2 ppm (dry weight) in rice grains; 0.6–0.7 ppm (dry weight) in 2-row barley grains; 2 ppm (dry weight) in millets grains (2); 0.01–0.42 ppm in some *Amaranthus* spp. seeds (8, 99); and 10–360 ppm in fatty oils obtained as by-products of essential oil production from *Apiaceae* seeds (10).

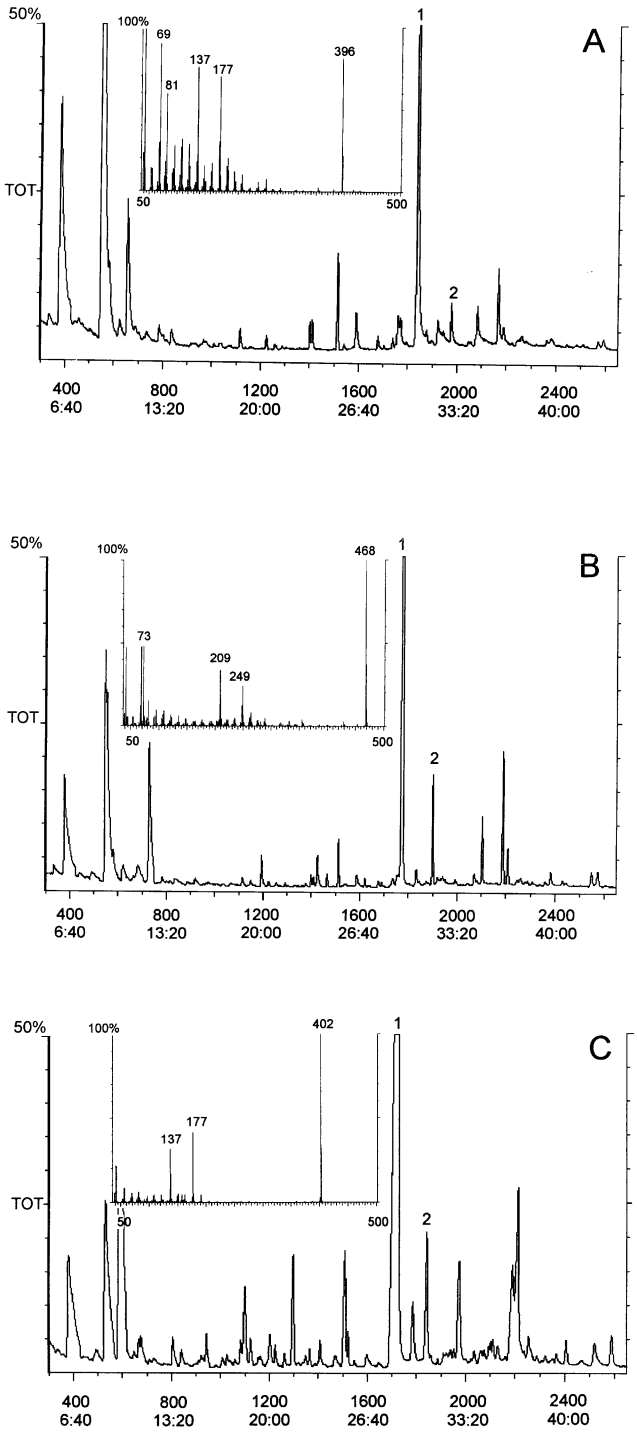

FIG. 2. Gas chromatography–mass spectrometry traces [total ion current (TOT)] of the whole lipidic extract after: (A) methylation, (B) methylation and silanization, and (C) catalytic hydrogenation. Mass spectrums refer to peak no. 1.

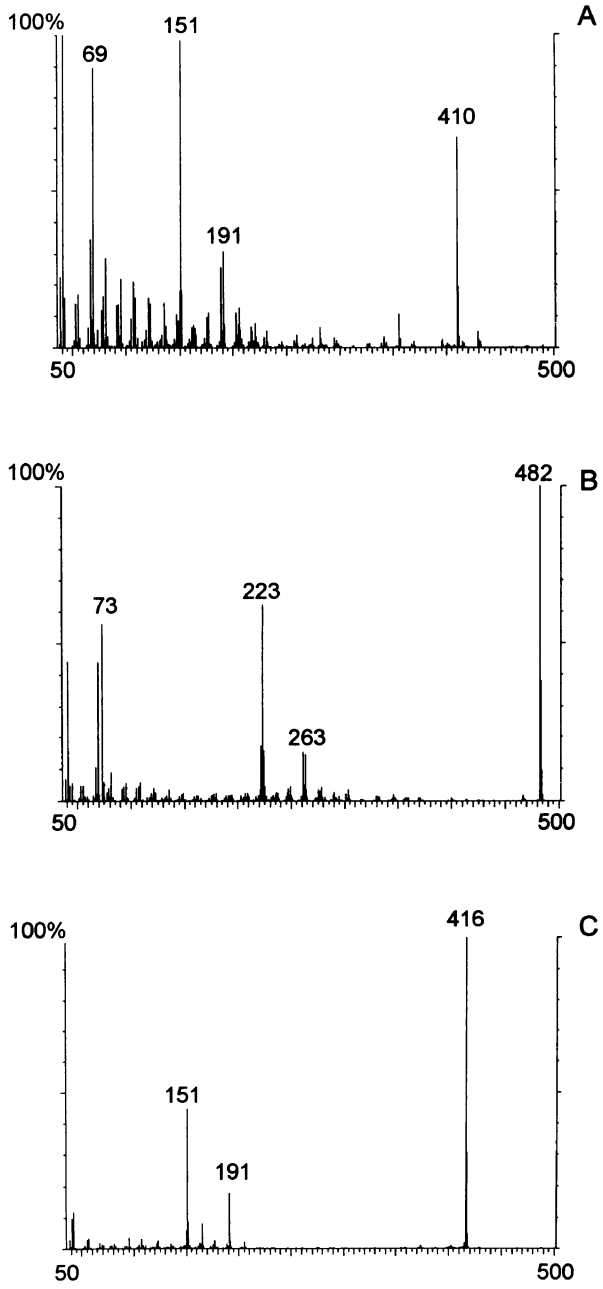

FIG. 3. Mass spectrums of peak no. 2 in Figure 2 after: (A) methylation, (B) methylation and silanization, and (C) catalytic hydrogenation.

REFERENCES

1. Kamal-Eldin, A., and L.A. Appelqvist, The Chemistry and Antioxidant Properties of Tocopherols and Tocotrienols, *Lipids 31*:671–701 (1996).
2. Barnes, P.J., Cereal Tocopherols in *Proceedings of 7th World Cereal and Bread Congress*, Prague, 1982; *Dev. Food Sci. 5B*: 1095–1100 (1983).
3. Telegdy Kovats, L., E. Kraszner-Berndorfer, and E. Hunyadvari, Vitamins and Other Biocatalysts in Hungarian Cereals. I. Tocopherol and Tocotrienol Contents of Some Winter Wheat Varieties, *Elelmezesi Ipar 24*:302–305 (1970).
4. Rao, M.K.G., and E.G. Perkins, Identification and Estimation of Tocopherols and Tocotrienols in Vegetable Oils Using Gas Chromatography–Mass Spectrometry, *J. Agric. Food Chem. 20*:240–245 (1972).
5. Slover, H.T., Tocopherols in Foods and Fats, *Lipids 6*:291–296 (1971).
6. Barnes, P.J., and P.W. Taylor, The Composition of Acyl Lipids and Tocopherols in Wheat Germ Oils from Various Sources, *J. Sci. Food Agric. 31*:997–1006 (1980).
7. Micali, G., and P. Currò, Determinazione dei tocoferoli negli oli vegetali mediante HPLC, *Riv. Ital Sostanze Grasse 61*:95–98 (1984).
8. Budin, J.T., W.M. Breene, and D.H. Putnam, Some Compositional Properties of Seeds and Oils of Eight *Amaranthus* Species, *J. Am. Oil Chem. Soc. 73*:475–481 (1996).
9. Lehmann, J.W., D.H. Putnam, and A.A. Qureshi, Vitamin E Isomers in Grain Amaranths (*Amaranthus* spp.), *Lipids* 29:177–181 (1994).
10. Ivanov, St.A., and K. Aitzetmüller, Untersuchungen über die Tocopherol und Tocotrienolzusammensetzung der Samenöle einiger Vertreter der Familie Apiceae, *Fat Sci. Technol. 97*:24–29 (1995).
11. Lubrano, C., J.R. Robin, and A. Khaiat, Fatty Acid, Sterol and Tocopherol Composition of Oil from the Fruit Mesocarp of Six Palm Species in French Guiana, *Oléagineux 49*:59–65 (1994).
12. Goh, S.H., N.F. Hew, A.S.H. Ong, Y.M. Choo, and S. Brumby, Tocotrienols from Palm Oil: Electron Spin Resonance Spectra of Tocotrienoxyl Radicals, *J. Am. Oil Chem. Soc. 67*:250–254 (1990).
13. Choo, Y.M., S.C. Yap, C.K. Ooi, A.N. Ma, S.H. Goh, and S.H. Ong, Recovered Oil from Palm-Pressed Fiber: A Good Source of Natural Carotenoids, Vitamin E, and Sterols, *Ibid. 73*:599–602 (1996).
14. Mariani, C., and G. Bellan, Content of Tocopherols, Deidrotocopherols, Tocodienols, Tocotrienols in Vegetable Oils, *Riv. Ital. Sostanze Grasse 73*:533–543 (1996).
15. Rogers, E.J., S.M. Rice, R.J. Nicolosi, D.R. Carpenter, C.A. McClelland, and L.J. Romanczyk, Jr., Identification and Quantitation of γ-Oryzanol Components and Simultaneous Assessment of Tocols in Rice Bran Oil, *J. Am. Oil Chem. Soc. 70*:301–307 (1993).
16. Kato, A., K. Tanabe, and M. Yamaoka, Esterified Tocopherols and Tocotrienols in Rice Bran Oil, Soybean Oil, and Sesame Oil, *J. Jpn. Oil Chem. Soc. 30*:515–516 (1981).
17. Kato, A., A. Gapor, K. Tanabe, M. Yamaoka, and H. Mamuro, Esterified Alpha-Tocopherol and Tocotrienols in Palm Oils, *Ibid. 30*:590–591 (1981).
18. He, L., H. Mo, S. Hadisusilo, A.A. Qureshi, and C.E. Elson, Isoprenoids Suppress the Growth of Murine B16 Melanomas *in vitro* and *in vivo*, *J. Nutr. 127*:668–674 (1997).
19. Guthrie, N., A. Gapor, A.F. Chambers, and K.K. Carroll, Inhibition of Proliferation of Estrogen Receptor-negative MDA-MB-435 and Positive MCF-7 Human Breast Cancer Cells by Palm Oil Tocotrienols and Tamoxifen, Alone and in Combination, *J. Nutr. 127*:544S–548S (1997).
20. Makpol, S., N.A. Shamaan, Z. Jarien, A.G. Top, B.A. Khalid, and W.Z. Wan-Ngah, Different Starting Times of Alpha-Tocopherol and Gamma-Tocotrienol Supplementation and Tumor Marker Enzyme Activities in the Rat Chemically Induced with Cancer, *Gen. Pharmacol. 28*:589–592 (1997).
21. Elson, C.E., Suppression of Mevalonate Pathway Activities by Dietary Isoprenoids: Protective Roles in Cancer and Cardiovascular Disease, *J. Nutr. 125*:1666S–1672S (1995).
22. Qureshi, A.A., J.W. Lehmann, and D.M. Peterson, Amaranth and Its Oil Inhibit Cholesterol Biosynthesis in 6-week-old Female Chickens, *Ibid. 126*:1972–1978 (1996).
23. Qureshi, A.A., B.A. Bradlow, L. Brace, J. Manganello, D.M.

Peterson, B.C. Pearce, J.J. Wright, A. Gapor, and C.E. Elson, Response of Hypercholesterolemic Subjects to Administration of Tocotrienols, *Lipids 30*:1171–1177 (1995).
24. Tomeo, A.C., M. Geller, T.R. Watkins, A. Gapor, and M.L. Bierenbaum, Antioxidant Effects of Tocotrienols in Patients with Hyperlipidemia and Carotid Stenosis, *Ibid. 30*:1179–1183 (1995).
25. Haila, K.M., S.M. Lievonen, and M.I. Heinonen, Effects of Lutein, Lycopene, Annatto, and Gamma-Tocopherol on Autoxidation of Triglycerides, *J. Agric. Food. Chem. 44*:2096:2100 (1996).
26. *Norme Grassi e Derivati*, edited by Stazione Sperimentale per le Industrie degli Oli e Grassi, Milano, 1976, Method NGD C12-1976.
27. Fieser, L., and M. Fieser, *Reagents for Organic Synthesis*, John Wiley and Sons Inc., New York, 1967, pp. 191–192.
28. Sweeley, C.C., R. Bentley, M. Makita, and W.W. Wells, Gas–Liquid Chromatography of Trimethylsilyl Derivatives of Sugars and Related Compounds, *J. Am. Chem. Soc. 85*:2497–2504 (1963).
29. Vogel, A.I., *Textbook of Practical Organic Chemistry*, Longman Scientific & Technical, Harlow, England, 1989, pp. 472–473.
30. Frega, N., F. Bocci, and G. Lercker, Direct Gas Chromatographic Analysis of the Unsaponifiable Fraction of Different Oils with a Polar Capillary Column, *J. Am. Oil Chem. Soc. 69*:447–450 (1992).

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