Extraction and Identification of an Antioxidative Component from Peanut Hulls

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Methanol extraction of hulls from Tainan select no. 9 (P-9) and Tainan no. 11 (P-11) peanuts produced a higher yield of a component having stronger antioxidant activity (AOA) than other organic solvents. The AOA of methanolic extracts from peanut hulls was equal to butylated hydroxyanisole and stronger than α-tocopherol. The methanolic extract from peanut (P-11) hulls was separated into 18 fractions by thin-layer chromatography (TLC). High antioxidant activity was found in subfractions with R_fs of 0.20, 0.25, 0.28, 0.31 and 0.37 at 95.6%, 93.8%, 94.7%, 92.0% and 81.6% inhibition of peroxidation of linoleic acid, respectively. Further purification of the subfraction eluting at 0.20 yielded a compound with antioxidant activity of 94.8%. Based on high-performance liquid chromatography (HPLC) analysis, ultraviolet (UV) spectra and ^1H nuclear magnetic resonance (NMR), this compound was identified as luteolin.

KEY WORDS: Antioxidant, extraction, identification, luteolin, peanut hulls.

The addition of antioxidant to foods is one of the most effective means to retard the oxidation of fats. It has become increasing popular as a method for increasing shelf life of food products and improving the stability of lipids and lipids-containing foods, thus preventing loss of sensory and nutritional quality. Synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallate (PG), are used in many foods to prevent the rancidity of lipids. However, the safety of synthetic antioxidants has been questioned in recent years (1-4), stimulating the evaluation of the effectiveness of naturally occurring compounds with antioxidative properties. For these reasons, many studies have been carried out and some antioxidative substances have been found from natural sources (5-15).

Peanut (Spanish-type) is one of the principal agriculture plants in the world. The antioxidative property of peanut has been studied by some researchers. For example, the flavonoid, dihydroquercetin, extracted from peanut kernel exhibited marked antioxidative activity (16). The oxidative stability of peanut oil can be improved by heating the peanut kernels before pressing for oil (17,18). Recently, many research papers have reported the antioxidant activity of hull extracts, such as rice hulls (7) and navy bean hulls (19). Although the relationship between maturity and flavonoid components of peanut hulls has been reported by Daigle *et al.* (20), no attempt has been undertaken to study antioxidative properties of peanut hulls.

The purposes of this study were to investigate the antioxidant activity of various organic solvent extracts, and to identify the major antioxidative component from peanut hulls.

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MATERIALS AND METHODS

Material. Peanuts of Tainan select no. 9 (P-9) and Tainan no. 11 (P-11), Spanish-type, were obtained from the Tainan District Agriculture Improvement Station, Taiwan, Republic of China. After harvesting, the peanuts were dried by sunlight for three days and then the hulls were hand-shelled. Peanut hulls were ground into a fine powder in a mill (Tecator Cemotec 1090 Sample Mill, Hoganas, Sweden), sealed in a plastic bottle and stored at 4°C until used.

Chemical analysis. The percentage of moisture, crude protein, crude fat, crude fiber and ash in peanut hulls was determined by AOAC methods 14.062, 14.067, 14.066, 14.064, and 14.063, respectively (21). All test results are the average of duplicate analyses.

Extraction of antioxidant components from peanut hulls. Each peanut hull powder (2.5 g) was extracted overnight with 50 mL of *n*-hexane, chloroform, acetone, ethanol or methanol, respectively, in a shaking incubator at room temperature. The extracts were filtered with Whatman #1 filter paper. The extraction was repeated twice, and the combined filtrates were evaporated to dryness *in vacuo* and weighed to determine the yield of soluble constituents.

Thin-layer chromatography. An aliquot of methanolic extract (10 μL) was spotted on a precoated silica gel plate (20 × 20 cm, F254, 0.25 mm, E. Merck, Darmstadt, Germany), which had been activated for 15 min at 100°C. The plate was developed in the ascending direction for 17.5 cm with the solvent system benzene:ethyl formate:formic acid (70:25:5, vol/vol/vol) (BEF). After drying, spots on the plate were located by a thin-layer chromatography (TLC) Scanner (CAMAG Ltd., Muttenz, Switzerland) under short-wavelength (254 nm) ultraviolet (UV) radiation.

Purification of the active fractions. To purify and obtain sufficient quantities of the antioxidative components in peanut hulls, the methanolic extract (0.2 mL) was streaked on a precoated silica gel plate and developed with the BEF solvent system. Fractions with the same R_f value were scraped and collected from thirty plates. Each fraction was isolated with methanol; then the combined extracts were filtered through a 0.45 μm Millipore filter (Bedford, MA) and evaporated to near dryness *in vacuo* below 40°C. The residue was weighed to determine the yield of each fraction, and then redissolved in methanol for measuring antioxidant activity.

The active fractions were further purified on a silica gel plate with the following solvent systems: BEF; toluene: ethyl formate:formic acid (66:29:5, vol/vol/vol) (TEF); *n*-butanol:butyl acetate:formic acid (70:20:10, vol/vol/vol) (BBF); toluene:acetone:formic acid (46:44:10, vol/vol/vol) (TAF); and *n*-butanol:glacial acetic acid:water (3:1:1, vol/vol/vol) (BAW).

High-performance liquid chromatography (HPLC). HPLC was performed with a Hitachi Liquid Chromatograph (Hitachi, Ltd., Tokyo, Japan), consisting of a Model

L-6200 pump, a Rheodyne Model 7125 syringe-loading sample injector, a Model L-3000 photo diode array detector set at 254 nm, and a Model D-2000 integrator. A LiChrospher 100 RP-18 reversed-phase column (5 μm, 125 × 4 mm i.d., E. Merck) was used for analysis. The run was performed at ambient temperature. The volume injected was 10 μL and the eluting solvent was methanol: water (25:75, vol/vol) at a flow rate of 0.5 mL/min.

Antioxidant activity determination. The plate was sprayed with carotene-chloroform solution to detect the antioxidant (16). The plate was exposed to daylight until the background color was bleached. Spots in which yellow color persisted were judged to have antioxidant activity, and the intensity of color was related to the extent of activity. The developed TLC plate was also sprayed with aluminum chloride and then observed under long-wavelength UV (366 nm). Yellow fluorescence spots indicated flavonoid-type compounds (10).

Antioxidant activity of all organic solvent extracts and separated fractions was carried out by the thiocyanate method by using 3 mg of extract or 200 μL of each fraction for the assay (22). Each sample was added to a solution mixture of linoleic acid-99.0% ethanol-0.2 M phosphate buffer (pH 7.0). The mixed solution in a conical flask was incubated at 40°C. The peroxide value was determined by reading the absorbance at 500 nm after coloring with $FeCl_2$ and thiocyanate at intervals during incubation. Butylated hydroxyanisole and dl-α-tocopherol (E. Merck) (200 μg) were used as standards for comparison of antioxidant activity. All test data are the average of triplicate analyses.

Melting point determination. The measurement of melting points was performed with a Micro Melting Point Apparatus (Yanaco MP-S3, Yanagimoto Co., Kyoto, Japan).

Ultraviolet (UV) spectrometry. Ultraviolet absorption spectra of the purified active fractions were recorded on a spectrophotometer (Hitachi, U-2000) with a diluted solution in methanol. Shifts in UV absorption were determined with a shift in reagents including NaOMe/MeOH, $AlCl_3$/MeOH, $AlCl_3$/HCl, NaOAC, and NaOAC/H_3BO_3 (7).

^1H nuclear magnetic resonance (NMR) spectrometry. The ^1H NMR (100 MHz) was performed on a Fourier transform (FT)-NMR (Bruker, Karlsruhe, Germany) with tetramethylsilane (TMS), and the chemical shifts are given in δ values.

Statistical analysis. Statistical analysis involved use of the Statistical Analysis System (23) software package. Analyses of variance were performed by ANOVA procedures. Significant differences between means were determined by Duncan's Multiple Range tests.

RESULTS AND DISCUSSION

Table 1 shows the proximate compositions of peanut hulls. Both peanut P-9 and P-11 hulls had similar proximate compositions. The yields and antioxidative activities of different organic solvent extracts from these two peanut hulls are shown in Table 2. The results indicate that the yield of extracts increased with increasing polarity of solvent. The efficiency of the solvents on the extraction was in the order of methanol > ethanol > acetone > chloroform > *n*-hexane. This is in agreement with the report of Economou *et al.* (24) that methanol is a widely used and effective solvent for extraction of antioxidants. The yield of methanolic and ethanolic extracts from peanut P-11 hulls was greater than from peanut P-9 hulls, while the antioxidant activity of extracts from both peanut hulls with different solvents was similar. Among the five organic solvents, methanolic extracts exhibited the highest yield and the strongest antioxidant activity. In addition, the methanolic extracts were easily powdered. Therefore, we focused on the use of methanolic extracts from peanut hulls in the following study.

The antioxidant activities of methanolic extracts from peanut hulls were compared with commercial antioxidants α-tocopherol and BHA. Methanolic extracts of peanut P-9 and P-11 hulls exhibited antioxidant activities of 95.5 ±

TABLE 1

Proximate Analysis of Peanut Hulls[a]

	P-9 (%)	P-11 (%)
Moisture	11.79	10.90
Crude protein	1.30	1.50
Crude fat	0.92	0.83
Ash	4.63	5.58
Crude fiber	51.13	51.02
Nitrogen-free extract[b]	30.23	31.17

[a] Values are means of duplicate analyses.
[b] Calculated by difference.

TABLE 2

Yield and Antioxidant Activity of Peanut Hull Extracts with Various Solvents

Solvents	Yield (mg)[a]		Activity (%)[b]	
	P-9	P-11	P-9	P-11
	(mean ± SD)[c]			
Methanol	104.9 ± 2.19A[d]	120.0 ± 2.02A	91.3 ± 0.82A	92.6 ± 0.48A
Ethanol	49.7 ± 2.05B	61.4 ± 3.00B	90.6 ± 1.18A	91.7 ± 0.99A
Acetone	18.2 ± 1.72C	23.5 ± 1.66C	90.4 ± 1.26A	91.3 ± 0.97A
Chloroform	16.1 ± 1.39D	16.4 ± 2.20D	74.7 ± 0.96B	77.2 ± 0.44B
n-Hexane	9.0 ± 0.70E	8.5 ± 2.27E	31.5 ± 0.87C	33.8 ± 1.21C

[a] Based on 2.5 g of dried peanut hulls for each organic solvent.
[b] The antioxidant activity of extract (3 mg) was determined by the thiocyanate method.
[c] Values are mean ± standard deviation of three replicate analyses.
[d] Means within a column with the same upper case letters are not significantly different at $P < 0.05$.

0.48% and 95.7 ± 0.53%, respectively, based on 8.6 mg of extract. This equaled the 95.1 ± 0.12% antioxidant activity of BHA, but was stronger than the 76.9 ± 1.30% activity of α-tocopherol. As shown in Figure 1, the antioxidant activity of methanolic extracts from peanut hulls increased with increasing concentration up to 100 μL, and then no significant differences ($P < 0.05$) were shown in antioxidant activity with concentration from 100 μL to 500 μL.

With the BEF solvent system, the methanolic extract from peanut hulls separated on TLC into two UV-distinct spots with R_f values of 0.20 and 0.28 that produced a positive reaction to carotene spray. This indicated that the spots with R_f 0.20 and 0.28 contained antioxidant activity. The spot corresponding to R_f 0.20 possessed the yellow fluorescent color with the aluminum chloride spray test, which indicates a flavonoid-type structure (10). Although the compositions and the antioxidant activities of methanolic extracts from peanut P-9 and P-11 hulls were similar, the yield of methanolic extract of P-11 hulls was higher than that of P-9 hulls. Therefore, the methanolic extract from peanut P-11 hulls was used for purification and identification of the antioxidative component.

For this purpose, a large amount of methanolic extract was separated by TLC. Eighteen fractions were observed with the BEF solvent system (Fig. 2). Fractions 5, 7, 8, 9 and 11 with R_f values of 0.20, 0.25, 0.28, 0.31 and 0.37, respectively, inhibited lipid peroxidation to over 80% (Fig. 3). The characteristics of all fractions are shown in Table 3.

The active fractions 5, 7, 8, 9 and 11 were further purified on silica gel plates by using a benzene:ethyl formate:formic acid (70:25:5, vol/vol/vol) solvent system. The number of subfractions for each fraction was two, three, one, two and four, marked 5 (I, II), 7 (I, II, III), 8 (I), 9 (I, II), and 11 (I, II, III, IV), respectively. The main subfractions for these fractions were 5 (II), 7 (II), 8 (I), 9 (II) and 11 (III). These major subfractions were further purified on silica gel with various solvent systems of different polarities, but the bands were not further separated. The antioxidant activities of these subfractions were 94.84 ± 0.09%, 81.11 ± 0.35%, 94.7 ± 0.03%, 11.6 ± 0.1% and 64.38 ± 0.99%, respectively. The activity of subfraction 5 (II) equaled that of subfraction 8 (I), but exhibited considerably more antioxidant activity than did the other subfractions ($P < 0.05$). In addition, subfraction 5 (II) showed the largest amount among all subfractions (data not shown); therefore, the structure of purified subfraction 5 (II) was further identified.

The UV spectra of purified subfraction 5 (II) before and after the addition of shift reagents showed spectra with characteristics of flavonoid components. Bathchromic shifts with reagents were noted due to the presence of phenolic OH groups in their A- and B-ring structures. The spectral λ_{max} values for purified subfraction 5 (II) in MeOH, NaOAC, $AlCl_3$/MeOH and $AlCl_3$ were 253 and 348, 266 and 400, 272 and 402, and 261 and 369, respectively.

The ^1H NMR spectrum of purified subfraction 5 (II) in MeOH showed that the aromatic protons of the B-ring

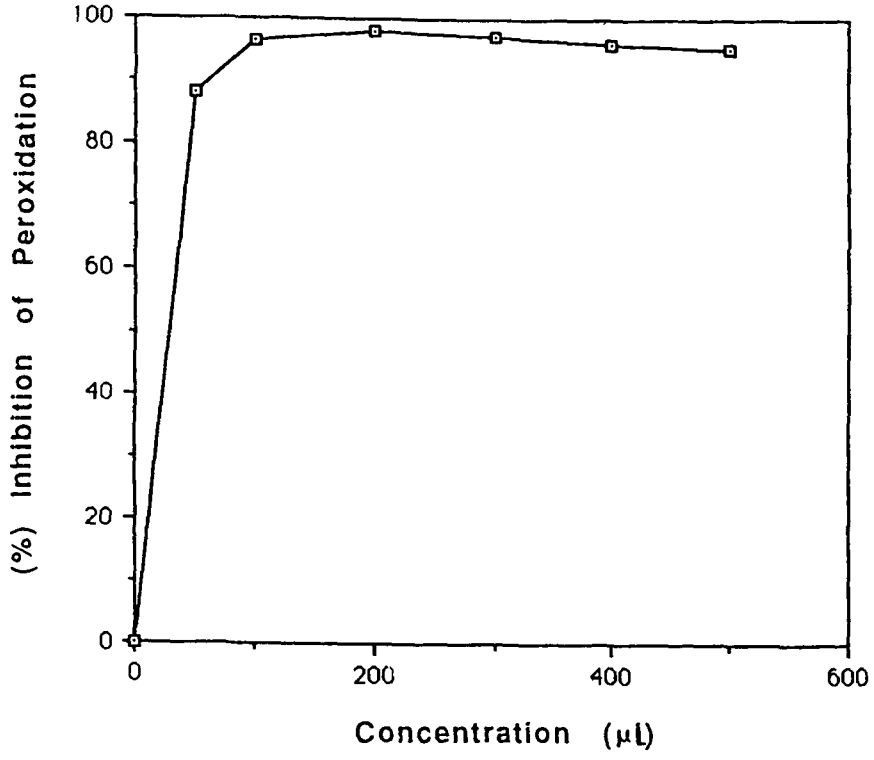

FIG. 1. Antioxidant activity at different concentrations of methanolic extracts from peanut (P-11) hulls.

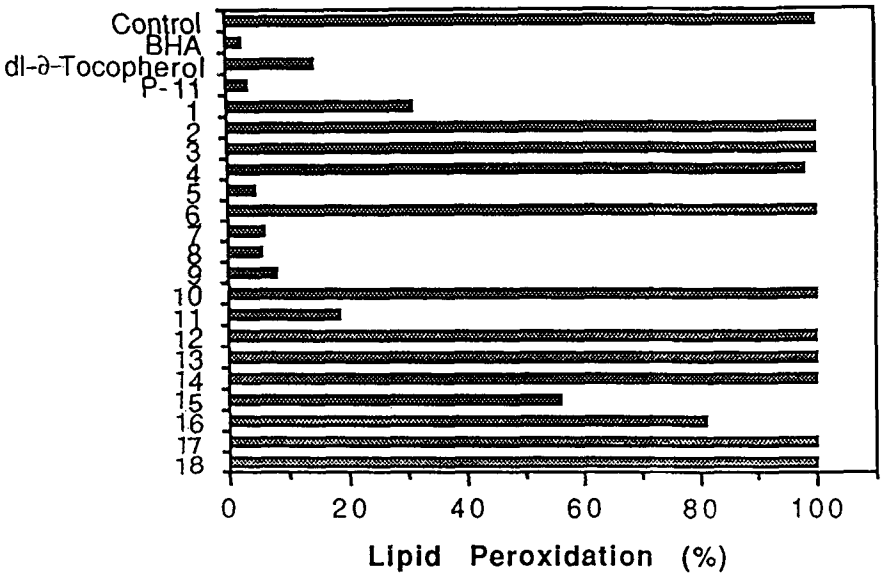

FIG. 2. Thin-layer chromatographic profile of methanolic extracts from peanut (P-11) hulls, as observed by TLC Scanner (254 nm). Solvent system = benzene:ethyl formate:formic acid (70:25:5, vol/vol/vol).

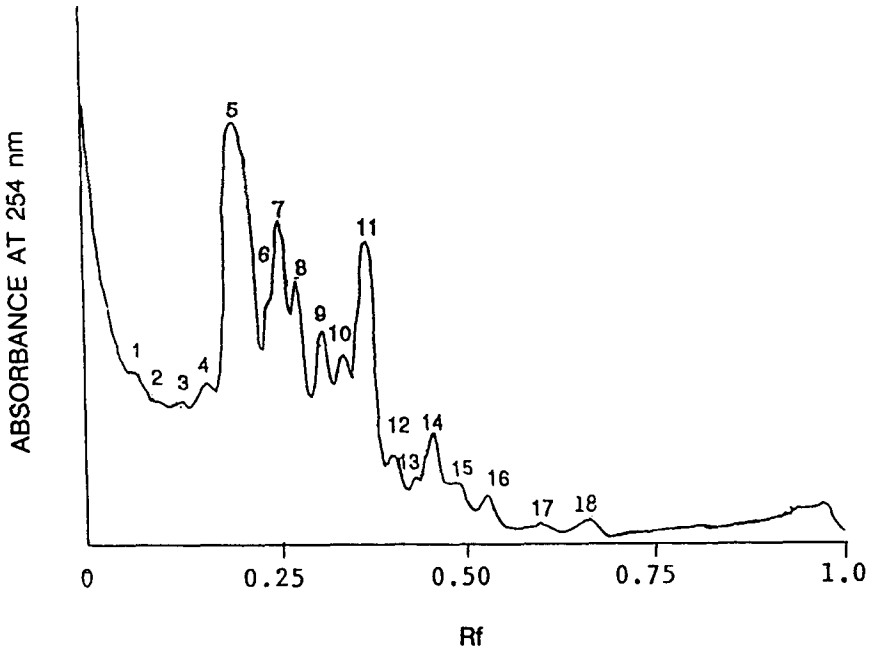

FIG. 3. Antioxidant activity of different fractions of methanolic extracts from peanut (P-11) hulls separated on silica-gel TLC. Antioxidants' concentration: BHA and α-tocopherol, 200 μg; P-11, 200 μL; each fraction, 200 μL.

appeared at δ 7.29 (H-2, *s*), δ 6.77 (H-5, *d*) and δ 7.37 (H-6, *d*), while those of the A-ring appeared at δ 6.48 (H-3, *s*), δ 6.05 (H-6,*s*) and δ 6.33 (H-8, *s*), respectively.

On the basis of the UV and ^{1}H NMR spectra and melting point (>300°C), the component of subfraction 5 (II) was proposed as luteolin, which has been widely reported to be present in plants (25) and is the major flavonoid in hulls from mature peanuts (20). For further confirmation, this component was compared with authentic luteolin on a TLC silica gel plate by developing with

TABLE 3

Some Characteristics of Different Fractions of Methanolic Extracts from Peanut (P-11) Hulls Separated on Silica Gel TLC

Fraction	R_f[a]	UV-254 nm		VIS		Yield (mg)[d]
		Color[b]	Strength[c]	Color[b]	Strength[c]	
1	0.07	SP	++	—	—	n.d.
2	0.09	SP	++	—	—	n.d.
3	0.13	SP	++	—	—	n.d.
4	0.16	SP	++	—	—	n.d.
5	0.20	DBP	+++++	Y	+++	1.67
6	0.24	SB	++	—	—	0.30
7	0.25	BY	+++	SY	+	1.11
8	0.28	BY	++++	Y	++	0.97
9	0.31	BY	+++	Y	++	0.80
10	0.34	SBP	++	—	—	0.33
11	0.37	P	++++	—	—	1.43
12	0.40	SP	+	—	—	n.d.
13	0.43	SP	+	—	—	n.d.
14	0.45	SP	++	—	—	0.73
15	0.49	SP	+	—	—	0.09
16	0.53	SP	+	—	—	0.08
17	0.60	SP	+	—	—	n.d.
18	0.67	SP	+	—	—	n.d.

[a]Solvent system = benzene:ethyl formate:formic acid (70:25:5, vol/vol/vol).

[b]B: brown; P: purple; Y: yellow; D: deep; S: slight.

[c]Strength means the depth of color +++++: very heavy; ++++: heavy; +++: intermediate; ++: slight; +: very slight.

[d]Based on 0.2 mL methanolic extracts, which is equal to 8.6 mg on a dry-weight basis. n.d.: not detected.

different solvent systems, including BEF, TEF, and BAW. The component of subfraction 5 (II) appeared at the same position as authentic luteolin.

The purified subfraction 5 (II) also was compared with authentic luteolin by HPLC analysis, and the retention time of purified subfraction 5 (II) was the same as that of luteolin.

Recently, Das and Pereira (26) indicated that the flavonoid aglycones had more potent antioxidant activity than their corresponding glycosides. Furthermore, flavonoid molecules with polyhydroxylated substitutions on rings A and B, 2-3 double bonds, a free 3-hydroxy substitution and a keto moiety conferred potential antioxidative properties on the compound. Flavonoids also have the ability to chelate metal ions through the cooperation of the -CO- group with either 3- or 5-hydroxy groups, and to reduce the prooxidant activity of trace metals (27). The structure of luteolin, a flavonoid substance, generally fits these structural characteristics. Therefore, the use of luteolin from peanut hulls as an antioxidant in food systems should be investigated.

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REFERENCES

1. Branen, A.L., *J. Am. Oil Chem. Soc. 52*:50 (1975).
2. Imida, K., S. Fukushima., T. Shivai., M. Ohtani., K. Nakanishi and N. Ito, *Carcinogenesis 4*:895 (1983).
3. Van Esch, G.J., *Food Chem. Toxic. 24*:1063 (1986).
4. Van der Heijden, C.A., P.J.C.M. Janssen and T. Strik, *Ibid. 24*:1067 (1986).
5. Rhee, K.S., and K.C. Rhee, *J. Food Protec. 45*:452 (1982).
6. Ramarathnam, N., T. Osawa, M. Namiki and S. Kawakishi, *J. Agric. Food Chem. 36*:732 (1988).
7. Ramarathnam, N., T. Osawa, M. Namiki and S. Kawakishi, *Ibid. 37*:316 (1989).
8. Duve, K.J., and P.J. White, *J. Am. Oil Chem. Soc. 68*:365 (1991).
9. Kramer, R.E., *Ibid. 62*:111 (1985).
10. Huang, S.L., and W.H. Chang, *J. Chinese Agric. Chem. Soc. 24*:199 (1986).
11. Kikuzaki, H., and N. Nakatani, *Agric. Biol. Chem. 53*:519 (1989).
12. Wu, J.W., M.H. Lee, C.T. Ho. and S.S. Cheng, *J. Am. Oil Chem. Soc. 59*:339 (1982).
13. Tsuchiya, T., Japanese patent 7,352,804 (1973).
14. Pratt, D.E., and P.M. Birac, *J. Food Sci. 44*:1720 (1979).
15. Gutfinger, T., *J. Am. Oil Chem. Soc. 58*:966 (1981).
16. Pratt, D.E., and E.E. Miller, *Ibid. 61*:1064 (1984).
17. Huang, J.J., S.L. Shyu and R.L. Chang, *J. Chinese Agric. Chem. Soc. 26*:466 (1988).
18. Yen, G.C., C.K. Wang and P.D. Duh, *Ibid. 28*:285 (1990).
19. Onyeneho, S.N., and N.S. Hettiarachchy, *J. Agric. Food Chem. 39*:1701 (1991).
20. Daigle, D.J., E.J. Conkerton, T.H. Sanders and A.C. Mixon, *Ibid. 36*:1179 (1988).
21. AOAC, *Official Method of Analysis*, 14th edn., Association of Official Analytical Chemists, Washington, DC, 1984.
22. Osawa, T., and M. Namiki, *Agric. Biol. Chem. 45*:735 (1981).
23. SAS, *SAS User's Guide: Statistics*, SAS Institute, Inc., Cary, NC, 1985.
24. Economou, K.D., V. Oreopoulou and C.D. Thomopoulos, *J. Am. Oil Chem. Soc. 68*:109 (1991).
25. Pendes, R., A.V.R. Rao and K. Venkataraman, *Phytochemistry 12*:2033 (1973).
26. Das, N.P., and T.A. Pereira, *J. Am. Oil Chem. Soc. 67*:255 (1990).
27. Hudson, B.J.F., and S.E.O. Mahgoub, *J. Sci. Food Agric. 31*:646 (1980).

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