Antioxidant Constituents of Peanut Oil

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The components responsible for increased stability of raw peanut oil at a high relative humidity (RH) of 91% were examined in peanut oil and methyl linoleate systems. Of the constituents, the native gums, which were mostly phospholipids and glycolipids, showed increased antioxidant activity at 91% RH. The isolated components of the gums, namely phospholipids and glycolipids, were prooxidant individually or in combination. Tocopherols did not show increased antioxidant activity at 91% RH.

KEY WORDS: Antioxidant, glycolipids, gums, methyl linoleate, peanut oil, phospholipids, polar lipids, relative humidity, water.

In most Western countries, only degummed, refined, bleached and deodorized vegetable oils are used for edible purposes (the exception being virgin olive oil). In countries such as India, most of the expeller-pressed peanut, mustard and other oils are marketed and consumed without further processing. Generally, the expeller-pressed oil is marketed in packs of 15 kg in tin containers and dispensed loose in retail trade, thus exposing it to air and ambient conditions of humidity and temperature. Considerable information is available on the influence of relative humidity (RH) on the autoxidation of lipids in model systems (1); however, literature on the behavior of vegetable oils under these conditions is sparse (2–4).

We have reported (3,4) that the rate of peroxide development in expeller-pressed peanut oil (raw PNO) decreases with RH, whereas in degummed oil the rate increases with RH. This prompted a study of the components of raw oil that exhibit antioxidant activity at high RH.

MATERIALS AND METHODS

Materials. Raw PNO and alkali-refined and deodorized peanut oil (refined PNO), conforming to Agmark quality–Indian standard (5), were purchased from the local market. Methyl linoleate was prepared from safflower oil (6). DL-α-tocopherol was obtained from E. Merck (Darmstadt, Germany), and silicic acid was from Sigma Chemical Co. (St. Louis, MO). All solvents used were of analytical reagent grade.

Isolation of hydrated gums (HG). Raw PNO was degummed with water (2 mL/100 g oil) to obtain gum setlings as previously described (4). The HG were lyophilized, and the dry HG were stored at 4°C until used for studies.

Isolation of phospholipids from HG. The dry HG (5 g) was placed in a 100-mL beaker, and 10 mL petroleum ether was added and stirred gently to disperse the gums. Then 25 mL acetone was added and stirred well for 5 min. The contents were transferred to centrifuge tubes. The beaker was rinsed with acetone, and the washings were transferred to centrifuge tubes. The tubes were chilled for 15 min in an ice bath at 0–5°C and centrifuged for 5 min at 2000 × *g* in a Remi T-23 centrifuge (Remi Udyog, Bombay, India). The phospholipid sediment was washed with acetone and desolventized under reduced pressure to get acetone-precipitated phospholipids (APP), as described in method Ja 4-46 of the American Oil Chemists' Society (7).

Fractionation of raw PNO into neutral and polar lipids. The raw PNO was fractionated into neutral and polar lipids by the procedure of Galanos and Kapoulas, as described by Christie (8), by using hexane (2 L) equilibrated with 87% ethanol (1.25 L) for every 100 g of oil. The lipid fractions were desolventized under reduced pressure in a rotary flash evaporator held below 30°C. The polar lipids fraction was dissolved in 10 mL chloroform, the neutral lipids fraction was flushed with nitrogen, and the fractions were stored at 0°C until use. The neutral lipids fraction consisted of mainly triacylglycerols (about 98%) and small amounts of mono- and diacylglycerols as determined by thin-layer chromatography (9). The polar lipids fraction consisted of phospholipids (11.2%), glycolipids (7.6%) and neutral lipids (81.2%) (10).

Fractionation of polar lipids. Polar lipids were fractionated into glyco- and phospholipids by the procedure of Rouser *et al.* (10). Activated silicic acid (75 g) was made into a slurry with 500 mL chloroform and poured into a glass column (45 × 4.5 cm), and the column, was allowed to settle by gravity. An aliquot of the polar lipids (500 mg) in chloroform was loaded onto the column, and elution was carried out successively with: (i) chloroform, ten column volumes, approximately 875–1000 mL; (ii) acetone, forty column volumes, approximately 3500–4000 mL; and (iii) methanol, ten column volumes, approximately 875–1000 mL. The glycolipid (acetone eluate) and phospholipid (methanol eluate) fractions were desolventized under reduced pressure in a rotary flash evaporator at room temperature (27°C), taken up in chloroform (10 mL) and stored at 0°C until needed for further studies.

Autoxidation studies. Autoxidation experiments were conducted in refined PNO, a neutral lipid fraction of raw PNO and methyl linoleate. The additives were incorporated into the oil directly and then mixed. For tests with methyl linoleate, an aliquot of the additive in chloroform was added and followed by removal of the solvent under reduced pressure.

Autoxidation of refined PNO with tocopherol or APP was carried out in the apparatus described previously (4). Triplicate samples (100 mL each) were placed in 250-mL Erlenmeyer flasks. For maintaining the RH of the system, fused calcium chloride for 2% RH, or a saturated ammonium dihydrogen phosphate solution for 91% RH, was placed in a 10-mL beaker, and the beaker was hung inside the Erlenmeyer flask (4). The entire assembly was sealed and stored at 40°C. Periodically, samples were withdrawn, and peroxide values (PV) were determined (AOCS Method Cd 8-53) (7). The monomolecular rate constant (K_m) was calculated by the procedure of Labuza *et al.* (11) from the PV data. Autoxidation of the neutral lipids fraction with added polar lipids (triplicate samples) was carried out in a similar manner.

To study the effect of polar lipids on autoxidation of methyl linoleate, the samples (in duplicate) were placed in 100-mL capacity Warburg flasks. The sample (5.0 mL)

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with added polar lipids was placed in the main vessel, and 1.0 mL of saturated ammonium dihydrogen phosphate solution (for 91% RH) was put in the side arm of the flask. The Warburg flasks were stoppered and stored at 37°C. Samples were withdrawn at regular intervals for PV determination. The differences in the rate of autoxidation were read from PV-time plots.

Autoxidation of methyl linoleate, either with addition of polar lipids or with HG, was carried out by using 15-mL Warburg flasks in duplicate, as described in our earlier communication (6), with 2.5 mL of test sample in the main vessel and 0.75 g of fused calcium chloride or 0.5 mL of saturated ammonium dihydrogen phosphate solution in the side arm of the Warburg flask. Experiments with polar lipids were conducted only at 91% RH. The Warburg flasks were connected to respective manometers and humidified to the desired RH by passing air of required RH over the sample. The flask-manometer assembly was transferred to a shaker water bath at 37°C. After equilibrating for 30 min, the assembly was sealed and oxygen uptake was monitored (6). The differences in rate of oxidation were read from the oxygen uptake-time plot.

Statistical analysis of the results in the oxidation studies was carried out by using Duncan's multiple range test (12) by the one-way analysis of variance procedure for segregating differences between means. Whenever a minimum of three replicate values were not available for such data, only average values are given.

Analytical methods. Iron and copper contents in the samples were analyzed according to methods 14.011 and 25.038 of the Association of Official Analytical Chemists (13), respectively, on 500-mg samples of the gums or 50 g of oil. For determining the glycolipids content, the total sugars value, estimated by the procedure of Dubois *et al.* (14), was multiplied by a factor of 4.6. For estimating the total tocopherols content of refined PNO, the oil was saponified, and the unsaponifiable matter (USM) was isolated. Sterols and carotenoids in the USM were removed by following the procedures described in the vitamin E panel method (15), and then the total tocopherol content (further separation and quantitation of individual tocopherols was not attempted) was determined by the colorimetric method (15). Phospholipid content was obtained by determining the phosphorus content of the sample (10-100 mg) by the modified Bartlett micro method (16) and multiplied by a factor of 25. Oil Conductance was measured with a megohmmeter (Tesla BRNO, Czechoslovakia) by dipping the contact terminals in the oil. The oil sample preparation was as follows: Distilled water (0.00, 0.05, 0.15 and 0.25 g) was added to raw, degummed and degummed and refined PNO (100 g) and the mixtures were then stirred magnetically for about 10 min. These samples were then used for conductance measurements. The distance between the two contact terminals was fixed at 0.7 cm throughout the resistance measurements. The conductance (C) of the oil was obtained from resistance (R) by using the relation $C = 1/R$.

RESULTS AND DISCUSSION

The rate of peroxide development at different RH in raw and degummed PNOs, as reported previously (3,4), is graphically represented in Figure 1. These data indicated that the raw oil oxidized slowly at high RH, contrary to the generally accepted trend of higher oxidation rates of lipids with increasing RH (1). However, the degummed oil follows the accepted trend. Therefore, we assumed that the gums had some constituents that were good antioxidants, particularly at a high RH.

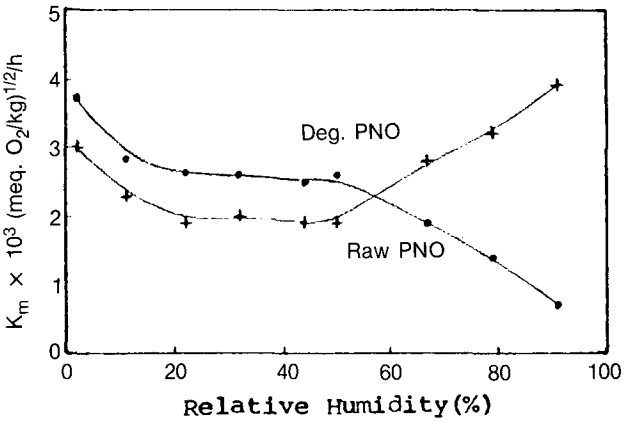

FIG. 1. Effect of relative humidity on rate of autoxidation (Refs. 3,4), as measured by peroxide value of peanut oil at 40°C. Raw PNO, expeller-pressed peanut oil; Deg. PNO, degummed raw PNO.

Antioxidant role of gums. The effect of gums on an autoxidizing system devoid of natural tocopherols was established in methyl linoleate. Refined PNO could not be used for this purpose because it contained tocopherols (23 mg%). When the HG of raw PNO was added to methyl linoleate and autoxidized at 2 and 91% RH, the gums exhibited good antioxidant activity at 91% and a lower antioxidant activity at the 2% RH (Fig. 2A), indicating that the antioxidant activity of the gums increased when hydrated, i.e., at a high RH of 91%.

Among the constituents of PNO, tocopherols (17) and phospholipids (18) have been reported to have antioxidant activity, but sterols do not have antioxidant activity (19). Little is known about the antioxidant efficacy of either tocopherols or phospholipids (constituents of gums) in relation to RH of the autoxidizing lipid system. Hence, the possible antioxidant role of tocopherol in raw PNO was examined.

Effect of tocopherol. PNO is known to contain 22-59 mg of tocopherols (20,21), specifically α-tocopherol, γ-tocopherol, and δ-tocopherol (13, 14 and 7 mg, respectively) per 100 g of oil. The refined PNO used in this study had a total tocopherol content of 23 mg. Data on the effect of 20 mg of added tocopherol (in addition to the 23 mg of naturally present tocopherols) on the rate of peroxide development in refined PNO are given in Table 1. The data show that the PV in the refined PNO, autoxidizing at RHs of 2 and 91%, were similar, which indicates that antioxidant activity of both natural and added tocopherols was unaffected by RH. Added tocopherol showed a slight prooxidant activity in the system, which implies that the low K_m in raw PNO, observed at a high RH in earlier studies (3,4), was possibly an expression of antioxidant activity of other components.

Composition of PNO gums. Silicic acid column chromatography of the gums showed that the HG (7% of the raw PNO) contained 84.8% neutral lipids, 10.2% glycolipids and 5.0% phospholipids (10).

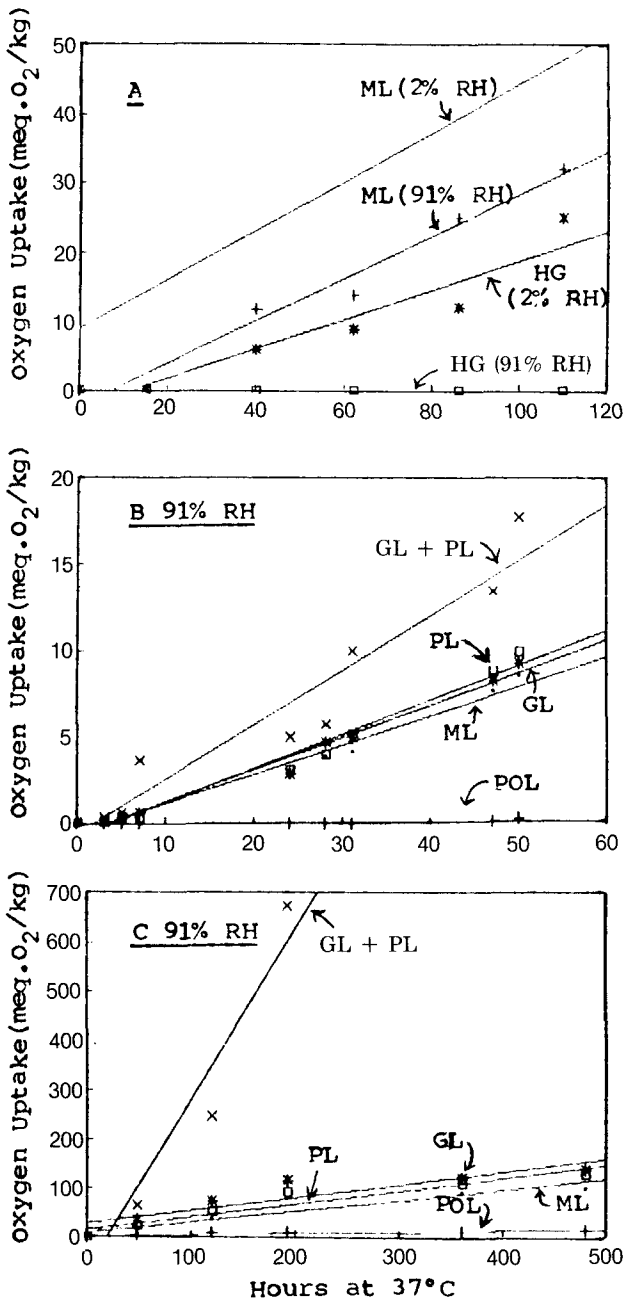

FIG. 2. A. Antioxidant effect of peanut oil gums on autoxidation of methyl linoleate (ML), as measured by oxygen uptake at 37°C, at 2 and 91% relative humidity (RH). ML 2% RH = methyl linoleate (ML) at 2% RH; hydrated gums (HG) 2% RH = ML + 0.5% HG of raw peanut oil (PNO at 2% RH; ML 91% RH = ML at 91% RH; HG 91% RH = ML + 0.5% HG of raw PNO at 91% RH. B. Effect of polar lipids on autoxidation of ML, as measured by O_2 uptake at 37°C and 91% RH. POL = ML + 0.9% polar lipids of raw PNO; GL = ML + 0.5% glycolipids fraction isolated from polar lipids of raw PNO; PL = ML + 0.5% phospholipids fraction isolated from polar lipids of raw PNO; GL + PL = ML + 0.5% phospholipids and 0.5% glycolipids fractions of polar lipids of raw PNO. C. Effect of polar lipids on autoxidation of ML, as measured by peroxide value during storage at 37°C and 91% RH.

Effect of phospholipids and glycolipids. The phospholipids and glycolipids fractionated on the silicic acid column (10) were tested for antioxidant activity in methyl linoleate at 91% RH [Fig. 2 (B and C)]. The data showed

TABLE 1

Effect of Additives on Autoxidation of Peanut Oil (PNO)[a]

Sample	Rate constant[b] at 2% RH	Rate constant[b] at 91% RH
Effect of tocopherol		
1. Refined PNO (control)	3.8[c]	3.9[c]
2. Sample 1 + 0.02% Tocopherol	4.9[d]	5.0[d]
SE_m (df)	±0.02714 (8)	
Effect of APP of gums		
3. Refined PNO (control)	2.5[c]	2.4[c]
4. Refined PNO (sample 3) + 0.5% APP of gums	13.6[d]	16.8[e]
SE_m (df)	±0.0154 (8)	
Effect of total polar lipids		
5. Neutral lipids of PNO (control)	—	4.3[c]
6. Sample 5 + 0.89% POL of PNO	—	2.8[d]
SE_m (df)		±0.003 (4)

[a]Abbreviations: RH, relative humidity; SE_m, standard error of mean; (df), degrees of freedom; APP, acetone-precipitated phospholipids of PNO gums; POL, total polar lipids of raw PNO isolated by hexane–ethanol partition method.
[b]Expressed as $K_m \times 10^3$ (meq O_2/kg oil)$^{1/2}$/h. Any two means of rate constants within the subset with different superscript letters c, d or e are significantly different ($P \leq 0.05$).

that the compounds were prooxidant, both individually and in combination, at the RH studied. The phospholipids isolated from gums by APP were prooxidant (Table 1). However, the total polar lipids (9) showed antioxidant activity in autoxidizing neutral lipids of PNO (Table 1) and methyl linoleate (Fig. 2A). Hence, it is probable that the antioxidant constituents were in the total polar lipids fraction, but the antioxidant activity of phospholipids and glycolipids was somehow lost during the isolation process. To our knowledge, there is no report that indicates any role for glycolipids in autoxidation of lipids. Thus, it seems that the phospholipids have antioxidant activity only in their native state in the oil. The loss of antioxidant activity was possibly due to changes in the state of the phospholipids, Handel and Winters (22) have observed that the phospholipids have a nonpolar character in their native state in the oil, whereas they assume a polar character when isolated. This change of polarity of the native phospholipids might be responsible for loss of antioxidant activity of isolated phospholipids. In fact, prooxidant activity of pure phospholipids in methyl linoleate and fish oil systems has been reported in the literature (23,24).

Mechanisms of antioxidant action of native phospholipid gums. Raw PNO, degummed PNO and degummed and refined PNO were hydrated with 0.00 (control), 0.05, 0.15 and 0.25% water, and the electrical conductance was measured. These oils showed electrical conductance (micromhos $\times 10^5$) values as follows: raw oil, 17, 18, 20, and 22; degummed oil, 9.0, 8.9, 8.8 and 8.7, and degummed and refined oil, 8.0, 7.9, 7.8 and 7.7 for 0.00, 0.05, 0.15 and 0.25% moisture-treated samples, respectively. These data show that the electrical conductance increased with an increase in the concentration of water only in the case of raw peanut oil. Also, raw oil had a higher electrical conductance value than did the degummed and refined oils at the same amounts of moisture. Whether this increased electrical conductance at a high RH had any effect on the antioxidant activity of the phospholipid gums is difficult

to assess from the present study. However, there have been observations of inhibition of autoxidation of hydrocarbons by solvents of high dielectric constant (25). In the present study, a solvent of high dielectric constant, such as water, increased the dielectric constant of the autoxidizing system, possibly resulting in an inhibitory effect on autoxidation, as described by Scott (25).

Metal sequestering action of phospholipids could be possible. For example, raw PNO used in the present studies was analyzed for trace metal contents (data not shown). One oil, with iron and copper contents of 4.2 and 0.2 ppm, respectively, was equilibrated at 91% RH and centrifuged, the supernatant oil (1.55 ppm iron and 0.08 ppm copper) and the sedimented gums (265 ppm iron and 11.90 ppm copper) showed different values for iron and copper content. The sedimented gums contained 63% of the iron and 60% of the copper present in the total oil, indicating that the gums sequester trace metals effectively. The oil used in another study (4,29) had a much lower content of iron and copper (<0.05 ppm), and the autoxidation rates, particularly at 91% RH, were significantly lower than those reported for oil with higher contents of iron and copper (Table 2). Hence, it is possible that the mechanism by which phospholipid gums reduced oxidation was binding trace metals.

Native phospholipid gums are known to scavenge metal ions effectively. Lunde *et al.* (26) showed that phospholipids of soybean oil sequester trace metal ions from an aqueous phase. In studies on oils stored in galvanized iron containers, Kantharaj Urs *et al.* (27) observed higher levels of zinc in crude PNO than in refined PNO with almost the same free fatty acids and moisture contents.

Nevertheless, it is not possible to ascribe the antioxidant activity of gums to the metal sequestering mechanism alone because only about 60% of the iron and copper of the total was found in the gums, and a substantial quantity still remained in the supernatant oil. These trace quantities of the metals were adequate to catalyze the autoxidation reactions (28). Lunde *et al.* (26) have shown isolated phospholipids to sequester metal ions, but the isolated phospholipids, as observed in this study, were devoid of antioxidant activity. Additionally, methyl linoleate used in the present study was virtually free from iron (<0.01 ppm) and copper (<0.5 ppb). Yet, methyl linoleate with added phospholipid gums oxidized very slowly (Fig. 2A) as compared to the control. Hence, mechanisms other than mere metal sequestering could be operating.

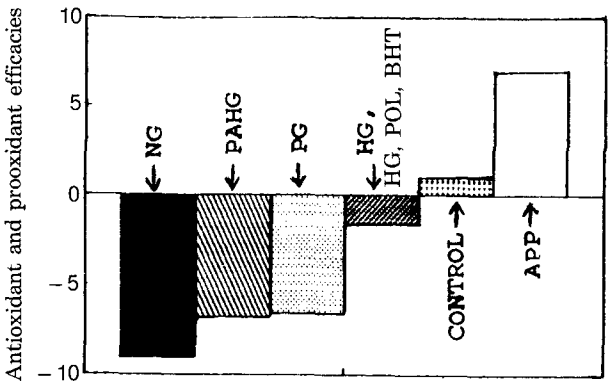

FIG. 3. Antioxidant and prooxidant efficacies of gums isolated by different procedures (current data plus Refs. 4 and 29) compared with synthetic antioxidants at 91% RH in peanut oil. NG = native gums in raw peanut oil (PNO); PAHG = degummed PNO + 3.0% phosphoric acid hydrated gums of raw PNO. PG = refined PNO + 0.02% propyl gallate; POL = neutral lipids of raw PNO + 0.9% polar lipids of raw PNO; BHT = refined PNO + 0.02% butylated hydroxy toluene; HG = degummed raw PNO + 7% hydrated gums of raw PNO; APP = refined PNO + 0.5% acetone-precipitated phospholipids of HG of raw PNO; CONTROL = refined PNO. Antioxidant efficacy = K_m control/K_m with additives [where rate (sample) is < rate (control) and carries a negative sign]; prooxidant efficacy = K_m with additives/K_m control [where rate (sample) > rate (control) and carries a positive sign].

A comparison of published work that shows the efficacy of phospholipid gums isolated by different procedures, such as water degumming (4) and phosphoric acid degumming (29), along with the results of the present study (hexane–ethanol partition) is shown in Figure 3. At 91% RH, the phosphoric acid-hydrated gums (29) showed maximum antioxidant efficacy (comparable to that of propyl gallate), and the HG and the total polar lipids had the lowest antioxidant efficacy (comparable to butylated hydroxy toluene). APP behaved as prooxidants at 91% RH. These results showed that the antioxidant efficacy of the gums was an effect of its constituents.

TABLE 2

Autoxidation Rate Constants of Peanut Oil with Different Trace Metal Contents at Different RH and at 40±1°C (Refs. 4, 29)[a]

RH %	Rate constant[b] of raw PNO	
	Sample 1[c]	Sample 2[c]
2	3.26[d]	6.33[e]
50	2.64[e]	5.90[e]
91	0.77[f]	3.17[f]
SE_m (df)[c]	±0.190(9)	±0.156(6)

[a]Abbreviations as in Table 1.
[b]Expressed as $K_m \times 10^3$ (meq. O_2/kg)$^{1/2}$/h. Any two means having different superscript letters d, e and f in a column are significantly different ($P \leq 0.05$); number of replicates: sample 1, four; sample 2, three (data taken from Refs. 4 and 29, respectively).
[c]Samples 1 and 2 had <0.05 and 4.2 ppm iron and <0.05 and 0.2 ppm copper contents, respectively.

ACKNOWLEDGMENTS

The authors express their gratitude to Dr. S.R. Bhowmik, Director, Central Food Technological Research Institute (Mysore, India), for the keen interest in this investigation. The authors thank Shri S. Dhanaraj, Scientist, of the Institute for help in statistical analysis of the data.

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[Received October 18, 1993; accepted June 19, 1994]