Sugarcane molasses was investigated for antioxidant and prooxidant activity following separation of components by solvent extraction, thin-layer chromatography and gel filtration. Nonfractionated molasses, polar and nonpolar extracts from molasses, and 50ppm Fe$^{++}$ were shown to increase the rate of oxygen uptake by tocopherol-free corn oil. A strong antioxidant was observed in blackstrap molasses. This component inhibited the coupled β-carotene-linoleic acid oxidation 87% relative to controls values. In contrast, weak antioxidant activity was associated with table molasses which reduced oxidation by only 25% relative to control values. The nonpolar extract from blackstrap molasses inhibited the coupled β-carotene-linoleic acid oxidation 97% relative to controls. A medium molecular weight prooxidant was also present in blackstrap but not table molasses. This prooxidant increased linoleic acid oxidation 47% relative to controls. Results from the present study suggested that the antioxidant active compounds isolated from blackstrap molasses were products of the prolonged, high heat treatment of sugarcane processing. Levels of antioxidant
activity were observed to increase 67% during the formation of blackstrap molasses from table molasses. Antioxidant activity appeared to increase with the intensity of browning reaction pigments and was apparently associated with the presence of reductone structures.

When tested in the Ames assay, the compounds showing antioxidant activity from blackstrap molasses did not show mutagenic activity. Moreover, microsomal enzymes did not activate these compounds into mutagenic factors. Blackstrap molasses appears to be a future source of naturally occurring antioxidants for the protection of lipid containing foods.
The Isolation and Characterization of Naturally Occurring Antioxidants in Blackstrap, Sugarcane Molasses

by

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This thesis is dedicated in memory of my grandmother, M. Lona Kovaly whose delightful determination to succeed motivated all around her.
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THE ISOLATION AND CHARACTERIZATION OF NATURALLY OCCURRING ANTIOXIDANTS IN BLACKSTRAP, SUGARCANE MOLASSES

INTRODUCTION

Vegetable oils and fats tend to oxidize rapidly in foods exposed to environmental or internal stress. To alleviate or minimize these effects, food manufacturers routinely add synthetic antioxidants--BHT, BHA, propyl gallate--to products containing unstable fats or oils. These antioxidants maintain lipid quality, retard oxidation and lengthen shelf life. Consumer controversy concerning synthetic antioxidants has created an interest for natural sources of antioxidants to replace the synthetic additives. This interest has led to the examination of many food products as a source of natural antioxidants.

One such food product which has been investigated is molasses. Sugarcane molasses is the concentrated residue obtained during the refining and crystallization of sucrose. Dark, blackstrap molasses is obtained from the final phase of sucrose refining and contains more minerals, solids and organic compounds than light table molasses which is derived from the initial phase of sucrose refining (Meade and Chen, 1977).

Morano (1976) reported antioxidant activity in the methanol extract of blackstrap, sugarcane molasses. The antioxidants in blackstrap molasses were shown to protect lipids against oxidative deterioration more effectively than BHT during an initial 5 day test period. Several other studies have shown substantial antioxidant activity associated with cereal and legume flavonoids (Pratt and Birac, 1979),

The purpose of the present study was to investigate the naturally occurring antioxidant properties of sugarcane molasses. In addition, the oxidative properties and the chemical nature of these components were examined. Separation techniques used to isolate the molasses components included solvent extraction, thin-layer chromatography and gel filtration chromatography. Molasses compounds isolated in this study were measured for antioxidant activity as a function of oxygen uptake in a Gilson respirometer and by the coupled β-carotene-linoleic acid oxidation assay. Oxidative compounds were characterized by their UV spectral absorbance pattern, reaction to organic chemical spot tests, color intensity at Abs\text{470} and response to the Ames Salmonella typhimurium mutagen assay.
LITERATURE REVIEW

Lipid Oxidation

Lipid oxidation and rancidity: Dahl and Richardson (1980) have defined nonenzymatic lipid oxidation as the addition of atmospheric oxygen to allylic double bonds in lipid material. Several studies have shown that the oxidation of unsaturated lipids was associated with unacceptable foods, loss of essential fatty acids, destruction of vitamins, bleaching of pigments, reduction of protein biological value, limited shelf life and the formation of toxic compounds (Labuza, 1971, Dahl and Richardson, 1980). Subsequent lipid oxidation has been reported by Gunstone (1967) to form hydroxy acids, epoxide-free radicals, α,β-unsaturated ketones and dihydroxy esters of varying chain length. Lipid rancidity was defined by Labuza (1971) as the development of off-flavors, off-odors, and off-colors in lipids. Low flavor threshold aldehydes and ketones were reported to form during the early stages of oxidation and to make the lipid inedible. An objectionable odor developed when only 0.00002% of lipid was oxidized (Buttery et al., 1961). Figure 1 shows that rancidity occurs in lipids before peroxides accumulate during oxidation (Labuza, 1971).

Mechanism of free radical oxidation: A free radical chain mechanism has been suggested since 1940 for the initiation of lipid oxidation and the autocatalytic propagation of free radical species (Dahl, 1980). Gunstone (1967) reported that the initial free radical reaction (Figure 2) involved the hydroperoxidation of the methylene group adjacent to
Figure 1. Extent of lipid oxidation as a function of time.
Labuza, 1971

- Oxygen uptake
- Peroxides
- Polymers
- Volatiles

Reaction extent

Induction period

Time (hrs)
Figure 2. Typical pathway for linoleic acid oxidation (Labuza, 1971).
cc-Linoleic acid

\[
\begin{align*}
CH_3-(CH_2)_4-CH=CH-CH_2-CH=CH-CH_2-(CH_2)_6-C-OH
\end{align*}
\]

\[
\begin{align*}
\text{activated oxygen species} \rightarrow H^+
\end{align*}
\]

\[
\begin{align*}
R_1-CH=CH-CH=CH=CH-R_2, \quad C_9, C_{11}, C_{13} \\
\text{Resonance Forms}
\end{align*}
\]

\[
\begin{align*}
R_1-CH=CH-CH=CH-CH-R_2
\end{align*}
\]

\[
\begin{align*}
O_2
\end{align*}
\]

\[
\begin{align*}
R_1-CH=CH-CH=CH=CH-R_2, \quad C_9, C_{11}, C_{13} \\
\text{Peroxy Radicals}
\end{align*}
\]

\[
\begin{align*}
\text{RH}
\end{align*}
\]

\[
\begin{align*}
R_1-CH=CH-CH=CH=CH-R_2, \quad C_9, C_{11}, C_{13} \\
\text{Hydroperoxides}
\end{align*}
\]

\[
\begin{align*}
\text{Products from } C_{13} \\
\text{Cleavage}
\end{align*}
\]

\[
\begin{align*}
&\text{hexanal} \\
&\text{hexanol} \\
&\text{pentane}
\end{align*}
\]

\[
\begin{align*}
R_1 = -(CH_2)_4-CH_3 \quad R_2 = -CH_2-(CH_2)_6-C-OH
\end{align*}
\]
the alkyl double bond. The lipid hydroperoxide was shown to decom-
pose into a peroxide radical which then migrated to neighboring alkyl
double bonds and promoted the propagation of additional free radical
species. Once free radical propagation and hydroperoxide decom-
position occurred, Gunstone (1967) demonstrated that lipid oxidation
accelerated rapidly. At this point, free radical initiation no longer
required an external catalyst and the process became autocatalytic.

Mechanism of activated oxygen species: More recent studies
have implicated the role of activated oxygen species with the actual
attack of oxygen on lipids (Rawl and VanSanten, 1971; Dahl and Richardson,
1980). Dahl and Richardson (1980) have identified the activated oxygen
species in foods which were responsible for initiating lipid oxidation.
The activated oxygen species included singlet oxygen \( ^1\text{O}_2 \), hydroxyl
radical \( \text{HO}^\cdot \), ozone \( \text{O}_3 \), superoxide anion \( \text{O}_2^- \) and hydrogen peroxide
\( \text{H}_2\text{O}_2 \). Singlet oxygen, the hydroxyl radical and ozone were observed
to react directly with food constituents to form peroxides. These
peroxides, subsequently, decomposed and propagated oxidative chain
reactions. Labuza (1971) suggested that ground state, triplet oxygen
(atmospheric oxygen) was unable to react energetically with ground state,
singlet state lipids. He noted that the principle of electron spin
conservation prohibited the direct addition of a triplet species to
a singlet species. Consequently, the formation of hydroperoxides
required a change in electron spin since triplet oxygen could not react
with singlet state lipids to form singlet state peroxides. Conservation
of spin was achieved when singlet oxygen was formed from triplet
oxygen by a photochemical attack of light on triplet oxygen in the presence of a photosensitizer (Rawls and VanSanten, 1971). Several studies have shown that plant photopigments (chlorophyll), trace metals (Fe$^{++}$, Cu$^+$, Co$^+$), UV light and elevated temperatures contributed to the formation of singlet oxygen and thus the initiation of lipid oxidation (Heaton and Uri, 1961; Privit and Blank, 1962; Shelton and Vincent, 1963; Chahine and DeMan, 1971; Ragnarson et al., 1977).

Adam (1975) postulated that singlet oxygen initiated lipid oxidation via the "ene" reaction in which singlet oxygen added directly to the double bond to yield endoperoxides and alkyl hydroperoxides. The oxidation reaction became autocatalytic once singlet oxygen initiated free radical formation and triplet, atmospheric oxygen, was abundant (Adam, 1975; Hanzlik, 1976). Moreover, Labuza (1971) demonstrated that alkyl radicals reacted with triplet oxygen to produce more peroxy radicals and initiate an explosive chain reaction of rapid lipid oxidation.

Many lipid materials were observed to have long induction periods before measurable oxidation rates occurred (Labuza, 1971). The time span of each induction period was dependent on the lipid purity, the degree of lipid unsaturation, temperature, water activity, pH, solvent, neighboring compounds and light (Semenov, 1959; Lundberg, 1962; Labuza, 1971). This phenomena of long induction periods could be explained by low levels of singlet oxygen in the food tissue and the simultaneous protection of triplet oxygen from photosensitizers (Labuza, 1971). However, when foods were held under adverse conditions, singlet oxygen
formed, shelf life decreased and oxidation accelerated (Dahl and Richardson, 1980). Kellogg and Fridovich (1975) have suggested that singlet oxygen and/or the hydroxyl radical were the main activated oxygen species responsible for autocatalytic lipid oxidation. Superoxide anion and hydrogen peroxide, on the other hand, were found to be relatively inert towards organic molecules but were precursors for singlet oxygen and the hydroxyl radical species (Pederson and Aust, 1973; Kellogg and Fridovich, 1975).

Lundberg (1962) has shown that lipid autoxidation could be initiated by metal ions (Fe$^{2+}$, Cu$^+$, Co$^+$) which catalyzed the homolytic cleavage of existing peroxides. In addition, the formation of a charge-transfer complex among two peroxy radicals and an unsaturated lipid bond was reported to initiate lipid oxidation (Shelton and Vincent, 1963; Eriksen et al., 1978).

Dahl and Richardson (1980) have reported that preserving the quality of lipid-containing foods required inhibiting the activated oxygen species from attacking alkyl double bonds in lipids and/or preventing the formation of activated oxygen species. Antioxidants were one method used by the food industry to maintain lipid quality (Scott, 1965).

**Antioxidants**

Labuza (1971) has shown that trace levels of various compounds dramatically accelerated or inhibited lipid oxidation. The most powerful antioxidants (Figure 3) interrupted the free radical chain
mechanism and the formation of activated oxygen species (Scott, 1965; Dahl and Richardson, 1980). Less powerful antioxidants, oxidized before the lipid began to oxidize, chelated metal ions or reduced the primary antioxidant back to the active form (Labuza, 1971). Antioxidants were shown not to improve lipid quality, but rather, to maintain lipid quality by preventing the oxidation of labile constituents (Labuza, 1971). Moreover, once the product was rancid, antioxidants could not reverse the oxidative process.

Labuza (1971) reported that modern food processing methods required the addition of nontoxic antioxidants and/or protective packaging of lipid foods to insure good quality and reasonable shelf life.

Classification of antioxidants: Scott (1965) divided lipid antioxidants into three categories. The first category, Type I antioxidants, donated hydrogen to free radicals and thereby stopped the propagation of free radical chains (Figure 3). Examples of Type I antioxidants were butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate, tocopherol and flavonoids (Scott, 1965; Pratt and Birac, 1979). Type II antioxidants complexed metal ions and thereby inhibited free radicals and the formation of activated oxygen species. Chelating agents EDTA, citric acid, ascorbic acid and reductones were examples of Type II antioxidants. Type III antioxidants included environmental factors which reduced the ability of prooxidative factors to attack susceptible lipid material (Scott, 1965). Low oxygen pressure, controlled moisture levels and light
Figure 3. Mechanism of antioxidant activity on peroxides and free radicals (Labuza, 1971).
\[
\begin{align*}
R' + AH & \rightarrow RH + A' \\
RO' + AH & \rightarrow ROH + A' \\
RO_2' + AH & \rightarrow RO_2H + A' \\
R' + A' & \rightarrow RA \\
RO' + A' & \rightarrow ROA
\end{align*}
\]

RH = unsaturated lipid substrate  
R' = substrate radical  
RO' = lipid oxyl radical  
RO_2' = lipid peroxyl radical  
RO_2H = hydroperoxide  
AH = primary antioxidant  
A' = antioxidant radical
protection were examples of Type III antioxidants.

Scott (1965) has reported that the most effective antioxidants, Type I, showed a delocalization of unpaired electrons after reacting with free radical or singlet oxygen species. The more stable the resonance structure, the more powerful the antioxidant. Consequently, ortho- and para- substituted phenolics contained more resonance forms than meta-substituted phenols, and consequently possessed greater antioxidant activity. Synergistic, Type II antioxidants were observed to enhance the effectiveness of primary, Type I antioxidants (Hill et al., 1969). When Type I and Type II antioxidants were added to a lipid, the resulting induction period was longer than the sum of the induction periods observed with either antioxidant added separately (Hoffman, 1970).

Antioxidant concentration vs. activity: Labuza et al. (1969) has shown that the protective effect of an antioxidant was directly related to its concentration. However, when concentrations exceeded the reaction equilibrium constant, then antioxidant protection decreased with increasing concentration. For example, with tocopherols, if the tocopherol:linoleic acid ratio exceeded $10^{-3}$, then lipid oxidation rapidly increased (Witting, 1969). In fact, Shelton and Vincent (1963) showed that antioxidants often became prooxidants at concentrations greater than optimum. Free radicals were shown to form during the direct attack of oxygen on the antioxidants. These investigators explained the reversal in the direction of the reaction on the huge concentration of antioxidant present in the lipid.
When less than 0.5% atmospheric oxygen existed around the food product, as in vacuum packaging, then the synergistic effect of Type I and Type III antioxidants increased linearly with the concentration of Type I antioxidant (Labuza, 1971). Gooding (1963) has suggested that nitrogen or vacuum packaging is the only practical method to prevent lipid oxidation. However, vacuum packaging is not always feasible for food products.

Naturally occurring antioxidants: Several naturally occurring antioxidants have been reported in a variety of plant and animal sources. Tocopherols (Bunnel et al., 1965), plant phenolics (Pratt and Birac, 1979), flavonoids (Hammerschmidt and Pratt, 1978), browning reaction compounds (Evans et al., 1958) and cysteine (Dahl and Richardson, 1980) were observed to provide varying levels of antioxidant activity in lipid material.

Carlson et al., (1976) has concluded that only the most effective singlet oxygen quenchers could prevent the addition of singlet oxygen to alkyllic double bonds at the 0.01% (lipid basis) level established by the Food and Drug Administration (FDA). For example, β-carotene was considered the most powerful singlet oxygen quencher known, but was not used as an antioxidant in food products due to its orange pigmentation.

Tocopherols: Tocopherols (Vitamin E analogs) were isolated from wheat germ oil (Evans et al., 1938) and later found to protect polyunsaturated fatty acids (PUFA) in plant tissues from oxidation (Horwitt, 1961; Tappel, 1962.)
Hove and Harris (1951) have reported that vegetable oils contained the most important source of tocopherols. The concentration of tocopherol in the various plant oils was directly proportional to the linoleic acid concentrations ($r = +0.79$). Watanbe et al., (1979) have attributed the observed concentration of tocopherols in plants to the protection of PUFAs from autoxidation. However, tocopherols were reported to provide minimal antioxidant activity when added to oils and lipid material.

Plant phenolics and flavonoids: Rhee and Kim (1979) have shown that polyphenol and flavonoid compounds from soybean, cottonseed, wheat and corn flour possessed exceptionally strong antioxidant activity. In addition, Hammerschmidt and Pratt (1978) have isolated isoflavone and chlorogenic isomers from methanolic, soybean meal extracts. These extracted flavonoids possessed substantial antioxidant activity for lipids even when the flavonoids were suspended in the aqueous phase of a lipid-water emulsion. Pratt and Birac (1979) have also shown that ortho-dihydroxy phenolic compounds isolated from plant tissues exhibited antioxidant activity similar to the plant flavonoids. For example, the ortho-dihydroxy phenols, ferulic and caffeic acid were isolated from soy meal and reported to prevent linoleic acid oxidation. Moreover, Watanbe et al. (1979) have shown that 10% (lipid basis) soybean, wheat or corn flour combined with soybean oil and heated to $150^\circ$C, markedly improved the soy oil's shelf life. These investigators demonstrated that the antioxidant activity of the plant flavonoids increased as the level of flour and cooking temperature increased.
Caramelization browning reaction products: Liener (1969) has reported that caramelization browning occurred when polyhydroxycarbonyl compounds, such as sugars, were heated to relatively high temperatures in the absence of amino compounds. Moreover, Rhee and Kim (1975) have shown that the non-nitrogenous, caramelization browning reaction products strongly inhibited lipid oxidation in soybean oil. Their study demonstrated that the acetone extract of a 2.0M glucose solution refluxed 96hrs, 100°C, dramatically inhibited lipid oxidation. Dihydroreductones, produced during the initial reflux period of glucose, were associated with the observed antioxidant activity. Antioxidant concentration in the heated glucose rapidly increased during the initial reflux period and then stabilized as the browning intensified. They concluded that color intensity did not correspond directly with antioxidant activity. Rhee and Kim (1975) emphasized that caramelization browning products could not form melanoidin or melanoidin-like nitrogenous pigments similar to Maillard pigments, but the products did stabilize lipids against oxidation.

Maillard browning reaction pigments: Several studies have shown that Maillard browning reaction products were another source of antioxidants (Evans et al., 1958; Kirigay et al., 1968; Kawashima et al., 1977). Maillard products were formed during the thermal condensation of amino acids and carbonyl compounds. As the Maillard browning reaction progressed, Hodge and Fisher (1963) reported that Amadori and reductone compounds polymerized into melanoidins, browning intensified, and amino acids became deaminated. In addition, reducing
sugars and autoxidizing fats contributed carbonyl groups to the Maillard browning reaction in foods (Evans et al., 1958; Kawashima et al., 1977).

Evans et al. (1958) has shown that anhydroamino- and amino-hexose-reductones were produced during Maillard browning reactions and strongly inhibited peroxide formation in a variety of animal fats, vegetable oils and shortenings. They found that amino-hexose-reductones were stable, colorless, hydrophilic salts. During prolong heating, water was eliminated from amino-hexose-reductones and anhydroamino-hexose-reductones were formed. The deep yellow, anhydroamino-hexose-reductones were reported to be more hydrophobic than their precursors. Evans et al. (1958) and Kawashima et al. (1977) have concluded that the reductone species were highly effective antioxidants by all chemical and oxidative tests. Long induction periods, slow oxygen absorption rates and low peroxide development characterized reductone treated oils (Evans et al., 1958). However, an improvement in the flavor stability of the oil was observed only when reductones were used as synergistic, Type II, antioxidants (Evans et al., 1958; Scott, 1965).

Reductone mechanism of antioxidation: Evans et al. (1958) and Scott (1965) have postulated that if free radicals or activated oxygen species induced lipid oxidation, then antioxidant activity was dependent on the release and/or transfer of hydrogen atoms. The hydrogen atoms from the antioxidant reacted with the fatty acid hydroperoxide radical or activated oxygen species and terminated chain
propagation (Scott, 1965). Each reductone, theoretically, could donate two hydrogen atoms during chain termination in what appeared as a stoichiometric relation. However, they observed that once a free radical chain began to propagate, several thousand moles of hydroperoxides developed. Moreover, reductone antioxidant activity increased as a linear function of concentration from 0% to 0.02% (lipid basis) (Evans et al., 1958). They conclude that the reductones, like other antioxidants, were exhausted by the time rapid lipid oxidation occurred. Consequently, the best antioxidants prevented the initiation of lipid oxidation rather than the propagation of free radical and activated oxygen species (Scott, 1965).

Screening for Potential Molasses Toxicants

Setliff and Mower (1977) have reported that a variety of carbohydrates, when heated to their decomposition point, tested positive with Ames Salmonella typhimurium strains TA98 and TA100 for mutagenicity. Their results indicated the presence of mutagens with both point and frameshift activity in heated sugars. Caramelization browning and Maillard browning reactions were examples of the thermal decomposition reactions formed during high heat treatments. Moreover, a diversity of commercial products derived from carbohydrates showed similar mutagenicity results. Such mutagen containing products included molasses, honey, brown sugar, and soy sauce. In addition, Setliff and Mower's (1977) study showed that microsomal enzymes were not required to activate these thermally produced mutagens.
Application of Maillard Pigments to Foods

Lingnert (1980) has shown that the formation of antioxidant active Maillard reaction products could be optimized in many foods by adjusting the processing parameters and the food recipe. Consequently, he added protein hydrolysates, amino acids or sugars to food recipes which normally received sufficient heat treatment and observed improved shelf lifes of the treated products. Lingnert (1980) reported that the storage stability of cookies was greatly improved when histidine and glucose were added to the cookie dough. He attributed the observed antioxidant effect to the Maillard reaction products formed in the cookies during the baking process.

In addition to cookies, the use of browning reaction products as antioxidants has also been used to reduce the warmed over flavors in meat and poultry (Reineccius, 1979).

Currently, the application of naturally occurring antioxidants to food systems remains in the experimental phase. More research and characterization of these naturally occurring antioxidants is needed before either Maillard or caramelization browning reaction products can be used as normal food additives.
MATERIALS AND METHODS

Materials

Blackstrap and table molasses used in these studies were a gift of Ingredient Technology Corporation, Woodbridge, NJ. All samples were stored at 4°C upon arrival to the laboratory. Tocopherol-free corn oil (Eastman, Rochester, NY), β-carotene (Sigma, St. Louis, MO) and purified linoleic acid (NU CHECK, Elyson, MN) were stored at 0°C in nitrogen-flushed, brown glass containers. All chemicals used in this study were reagent grade.

Separation of Molasses Fractions

Polar and nonpolar soluble compounds were extracted from molasses following a modified Bligh and Dyer (1959) procedure. A 10g fraction of molasses was homogenized 10min in 30ml chloroform:methanol (1:2 v/v) and filtered with a Buchner funnel through Whatman #1 filter paper. The residue was resuspended in 30ml CHCl₃:MeOH (1:2 v/v) and 8ml distilled water, stirred 5min, and filtered with a Buchner funnel through Whatman #1 filter paper with 15ml CHCl₃:MeOH (1:2 v/v). Filtrate from both filtering steps was combined in a 500ml separatory funnel, mixed with 25ml CHCl₃ and 29ml water, agitated vigorously and allowed to partition 24hrs at 4°C. The chloroform, nonpolar phase was removed and stored at 4°C. The polar phase was reduced to 20mls by rotary vacuum evaporation at 40°C and stored at 4°C. The residue from each filtration step was discarded since preliminary oxidation tests were not effected by its presence.
Measurement of Antioxidation by Spectrophotometry

Antioxidant activity was determined by the coupled β-carotene-linoleic acid oxidation assay described by Hammerschmidt and Pratt (1978).

The β-carotene stock solution consisted of 1g β-carotene dissolved in 50ml chloroform. All stock solutions were protected from light with an aluminum foil wrap and stored at 0°C. For each assay, 1.0ml stock solution, 20mg purified linoleic acid and 200mg Tween 40 (Sigma, St. Louis, MO) were pipetted into a round bottom flask. After removing the chloroform by rotary vacuum evaporation at 40°C, 50ml aerated-distilled water was added to the β-carotene residue, the solution was vigorously agitated 1min and then 5.0ml fractions were pipetted into screw-cap tubes containing 0.2ml aqueous test solution. Test samples were read against a blank containing the emulsion minus β-carotene. Controls consisted of 5.0ml β-carotene emulsion.

All spectral readings were recorded at time zero and every 15min for 120min at 470nm on a Beckman Spectronic 20 spectrophotometer. During analysis the capped tubes were held at 39°C in a water bath. Each experiment was repeated three times.

The rate of β-carotene bleaching, which was a function of the rate of linoleic acid oxidation, was measured by the difference in the spectral absorbance at 470nm from time zero to 120min. Antioxidant activity was defined as the ratio of β-carotene bleaching with a test compound to the rate of β-carotene bleaching in the absence of a test compound.
Analysis of each molasses fraction collected from the Sephadex G-100 gel filtration column required slight modifications in the procedure. First, the stock solution contained 10mg β-carotene dissolved in 50ml chloroform. Second, a 0.15ml aliquot of stock solution was added to the round bottom flask. Third, screw-capped tubes contained 2.5ml gel-eluted molasses fractions with 2.5ml diluted stock solution. The antioxidant activity of each test sample was measured as previously described.

Measurement of Antioxidation by Oxygen Uptake

Antioxidant activity as a function of oxygen uptake was measured with a Gilson respirometer following a modification of methods developed separately by Labuza (1975) and Einerson (1977). Oxygen uptake rates were determined manometrically from the change in pressure observed within the closed system during a specific time period. The μl/hr oxygen absorbed in each reaction flask corresponded to the rate and magnitude of oxidation in tocopherol-free corn oil. Samples were tested in three separate experiments.

A basic reaction mixture for the assay was prepared with 40g microcrystalline cellulose (MCC Avicel RC-591, FMC, Marcus Hook, PA) impregnated with 6.4g distilled water. Water activity (Aw) was maintained in all samples at Aw0.75, the Aw associated with maximum lipid oxidation, by storing samples in a desiccator of saturated NaCl (Labuza, 1971). All mixtures and samples were equilibrated to Aw0.75 for 24hrs prior to each experiment.
For each assay, 2.0g fractions of test reaction mixture and 2.0g of
tocopherol-free corn oil were placed in a Gilson reaction flask, mixed
thoroughly and randomly sealed onto a manometer with lanolin. Sodium
chloride crystals (10mg) and 0.5ml saturated NaCl were added to the
side arm of each Gilson flask to maintain a Aw0.75 headspace. Control
flasks contained 2.0g MCC, Aw0.75, and 2.0g tocopherol-free corn oil.
Iron catalyzed lipid oxidation flasks contained 2.0g MCC impregnated
with 50ppm Fe\(^{++}\)(FeSO\(_4\)·7H\(_2\)O) (lipid basis) and 2.0g tocopherol-free
corn oil. Blank Gilson flasks contained 2.0ml saturated NaCl.

Molasses reaction flasks were prepared as follows: 40g MCC was
impregnated with 6.4g molasses fractions, the solvent was evaporated,
6.4g distilled water was added and the molasses-MCC mixture stored
24hrs over saturated NaCl, Aw0.75. A 2.0g fraction of this molasses
mixture was mixed with 2.0g tocopherol-free corn oil in a Gilson
reaction flask and subsequently measured for oxygen uptake influence.

At the start of each assay, the Gilson flasks were open to
atmospheric conditions. The flasks were lowered into a 39\(^{0}\)C water
bath, allowed to equilibrate 10min and then sealed from the atmosphere.
The change in internal pressure was measured at time zero and every
4-6hrs for 52hrs. The concentration and rate of oxygen absorbed by
the oil was calculated from the difference in the oxygen absorbed by
the test flask and the oxygen absorbed by the blank flask. The in-
fluence of environmental stress was measured by the concentration
of oxygen absorbed by the untreated tocopherol-free corn oil during
oxidation. An oxidation ratio was calculated from the ratio of \(\mu l O\_2\)
absorbed/hr in the test flask to the $\mu l$ $O_2$ absorbed/hr in the control vessel. Oxidation rates were measured in $\mu l$ $O_2$/hr after the induction of lipid oxidation. Lipid oxidation was assumed to occur when a marked increase in oxygen uptake was observed.

Microliters of oxygen absorbed in each Gilson reaction flask was calculated from the average micrometer value measured in replicate samples minus the average micrometer value measured in the blank. Oxygen uptake measurements were then converted to standard conditions by the following formula:

$$\frac{(273^0K)(P_b)}{(273^0K + 0^\circ C)(760\text{mm Hg})}$$

where $P_b$ = atmospheric pressure in mm mercury and $0^\circ C$ = water bath temperature ($39^\circ C$) (Umbreit et al., 1964). The average atmospheric pressure during the 3 day assay was 760mm Hg. Thus, all measured oxygen uptake values were multiplied by 0.9 to obtain the microliters of dry oxygen absorbed at 760mm Hg.

**Thin-layer Chromatography**

Polar and nonpolar molasses extracts were chromatographed on Silica Gel-G precoated plates, 20cm x 20cm (E. Merck, Darmstadt, Germany). Plates were conditioned prior to each separation by eluting them in solvent, activating 45min at $110^\circ C$, cooling 10min and then spotting with the sample. A 20mg sample of molasses was spotted on each plate, developed 15cm in the appropriate solvent and then dried in a $20^\circ C$ CuSO$_4$ desiccator. Developed spots were observed under short and long UV light.
For flavonoid investigation of molasses extracts, a 20mg sample was developed in ethyl acetate:formic acid:water (1:2:3 v/v/v). The solvent front and fluorescent bands were scraped from each plate, extracted from the silica with methanol, evaporated to dryness under nitrogen gas and redissolved in 1.0ml methanol. The compounds scraped from the solvent front were then rechromatographed in 1-butanol:acetic acid:water (4:1:1 v/v/v). Fluorescent bands were scraped from the plate and extracted as previously described. Each band of compounds was tested for antioxidant activity by the coupled β-carotene-linoleic acid oxidation assay. Three separate sets of Sephadex G-100 separated samples from blackstrap and table molasses were examined.

A 10mg sample of polar extract from blackstrap and table molasses were cochromatographed with 5.0mg caffeic acid and 5.0mg ferulic acid (1.0mg/1.0ml MeOH) in ethyl acetate:acetic acid:formic acid:water (5:3:1:1 v/v/v/v). After developing, the plates were observed under short and long wave UV light.

Model Browning Reaction Solution

0.08M fructose-0.8M aspartic acid solution was refluxed for 6hrs at 110°C to produce Maillard browning reaction pigments. The reaction products were tested for antioxidant effectiveness by the coupled β-carotene-linoleic acid oxidation assay. Fructose was the most common monosaccharide and aspartic acid was the most common amino acid in blackstrap molasses (Mee,1979). Thus, the two compounds were selected for the production of Maillard browning reaction products.
Gel Filtration Separation of Molasses Compounds

Blackstrap and table molasses components were separated by molecular weight through a Sephadex G-100, 29cm x 3cm, gel filtration column (Pharmacia Co., Denmark) attached to an ISCO Model 272 fraction collector. Sephadex G-100 was allowed to swell overnight at 4°C in excess 0.1M Tris buffer pH8.0 before being poured into the glass column. The void volume was determined by the volume of fluid necessary to elute 0.15ml 0.05% Dextran-Blue 2000. Prior to each gel separation of molasses, the column was flushed with 400ml distilled water. A 1.0ml aqueous fraction of 10% blackstrap or 12% table molasses was pipetted onto the gel surface and eluted with deionized-distilled water. Initially, molasses samples were eluted through a Sephadex G-100 column with 0.1M Tris buffer pH8.0. Tris buffer and the alkaline pH, however, interfered with lipid oxidation and the β-carotene-linoleic acid oxidation assay. Therefore, the eluting solvent was changed to distilled water, a solvent which did not interfere with the assay or resolution of the compounds.

Approximately 35, 2.5ml molasses fractions were collected from the gel column during each filtration. The flow rate was maintained at 0.5ml solvent/min. Each fraction was spectrophotometrically scanned from 750nm-190nm on a Beckman DB Acta CIII recording spectrophotometer. The antioxidant effects of each gel-eluted molasses fraction was measured by the coupled β-carotene-linoleic acid oxidation assay.
Chemical Characterization of Molasses Antioxidants

Chemical spot tests for reductone structures were performed on each gel fraction possessing antioxidant activity.

Methyl ketones: One drop of test solution, 1 drop of 5.0% sodium nitroferric cyanide and 1 drop of 30% NaOH were placed in a 3cm watch glass. After 2min, 2.0 drops of glacial acetic acid were added. A red to blue solution indicated the presence of methyl ketones (Feigl, 1966).

Aromatic and aliphatic α,β-unsaturated aldhydes or ketones: Three drops test solution, 20mg thiobarbaturic acid and 2 drops concentrated phosphoric acid were added to a micro test tube. Tubes were heated in a glycerol bath to 120°C. A yellow-orange reaction within 1-3min indicated the presence of aromatic and aliphatic α,β-unsaturated aldehydes or ketones (Feigl, 1966).

Mutagen Assay of Gel-eluted Molasses Fractions

A cross section of molasses samples separated through Sephadex G-100 were screened for potential mutagenic activity following the procedure developed by Ames (1975). Salmonella typhimurium mutants were selected for their extreme sensitivity and specificity in being reverted from a histidine requirement to a prototrophy by a variety of mutagens. The bacteria possessed two additional mutations: loss of their excision repair system and loss of their surface lipopolysaccharide barrier.

Two S. typhimurium strains were used for the mutagenicity test: 1) TA1535 and TA100 which detected base-pair substitutions and 2)
TA1537 and TA98 which detected frame shift mutations. The TA100 and TA98 strains contained resistance transfer factor which increased their sensitivity to mutagenic compounds.

All *S. typhimurium* cultures were obtained from Ames Laboratory, University of California, Berkley, CA, tested for purity, and stored at -40°C in glass vials. Rat liver homogenate (S-9) was prepared as described from polychlorinated biphenyl (PCB) induced Sprague rats (Ames 1975). Plates were prepared as follows: 2.0ml molten top agar at 45°C, 0.1ml bacterial tester strain in nutrient culture broth, 1.0ml test solution and 0.5ml S-9 liver homogenate (if used) were added in the given order. The contents were mixed thoroughly, uniformly poured on minimal glucose agar plates, promptly covered to avoid the effects light on photosensitive chemicals and allowed to harden several minutes. After 1hr, plates were transfered to a dark, 37°C incubator for 48hrs. Colonies in both the test and the control plates were counted after 48hrs. An Ames index was determined for each triplicate set of plates from the ratio of the number of revertant colonies in the presence of the test solution to the number of revertant colonies in the control. An Ames Index greater than 2.0 was considered significant for the presence of a mutagenic compounds.
RESULTS AND DISCUSSION

Effect of Nonfractionated Molasses on Oxygen Uptake

Control experiments with 5%, 10%, and 25% nonfractionated table molasses (lipid basis) in tocopherol-free corn oil promoted oxygen uptake at rates equal to or greater than 50ppm Fe^{++} (lipid basis) catalyzed corn oil oxidation (Figure 4). As shown in Table 1, oxygen uptake rates in tocopherol-free corn oil were similar with 5% and 10% molasses or 50ppm Fe^{++} additions, but oxygen uptake rates were considerably greater with 25% molasses treated corn oil. Molasses in excess of 10% formed a crumbly mixture in the reaction flasks containing microcrystalline cellulose (MCC) and tocopherol-free corn oil. The coarse mix had a greater surface area than the syrupy control flasks. These results suggested that the greater exposure of the coarse 25% molasses mix to atmospheric oxygen allowed for greater oxygen uptake. Karel (1960) also showed that an increase in surface area was directly related to an increase in the relative rates of lipid oxidation. The results of the present study showed that tocopherol-free corn oil mixed with more than 25% molasses absorbed oxygen at greater rates than with lower percentages of molasses.

Factors Affecting Oxygen Uptake

The induction period, oxygen uptake rate and total µls of oxygen absorbed in similar lipid samples varied among test runs as a possible function of environmental conditions and susceptibility of the lipid to oxidation (Table 1). Labuza (1971) also showed that environmental
Figure 4. Influence of nonfractionated table molasses on the rate of oxygen uptake by tocopherol-free corn oil. Each point represents the mean of three samples. 5% molasses (☆), 10% molasses (●), 25% molasses (★), 50ppm Fe^{++} (○), control (☉).
Table 1. The effects of table molasses concentration on the oxygen uptake by tocopherol-free corn oil.

<table>
<thead>
<tr>
<th>Experiment #</th>
<th>Conditions</th>
<th>Number</th>
<th>Induction Time hrs</th>
<th>( \mu l \text{O}_2/hr )</th>
<th>Total(^a) ( \mu l \text{O}_2)</th>
<th>Test:Control(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>control</td>
<td>3</td>
<td>35+</td>
<td>0.43</td>
<td>49.6±14.4</td>
<td>1.00</td>
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<tr>
<td></td>
<td>5% molasses</td>
<td>2</td>
<td>5</td>
<td>6.30</td>
<td>174.5±37.5</td>
<td>3.48</td>
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<td></td>
<td>10% molasses</td>
<td>2</td>
<td>5</td>
<td>6.50</td>
<td>170.0±25.0</td>
<td>3.40</td>
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<td></td>
<td>25% molasses</td>
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<td>5</td>
<td>6.60</td>
<td>212.0±10.0</td>
<td>4.24</td>
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<td></td>
<td>50ppm Fe(^{++})</td>
<td>2</td>
<td>5</td>
<td>6.00</td>
<td>148.5±14.8</td>
<td>2.97</td>
</tr>
<tr>
<td>2</td>
<td>control</td>
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<td>28</td>
<td>0.32</td>
<td>93.0±25.0</td>
<td>1.00</td>
</tr>
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<td></td>
<td>5% molasses</td>
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<td>25</td>
<td>3.12</td>
<td>147.6±17.0</td>
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<td></td>
<td>25% molasses</td>
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<td>5.78</td>
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<td>1.98</td>
</tr>
<tr>
<td></td>
<td>50ppm Fe(^{++})</td>
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<td>27</td>
<td>3.42</td>
<td>145.6±28.9</td>
<td>1.71</td>
</tr>
</tbody>
</table>

\(^a\) Results are expressed as mean ± SD (n).

\(^b\) Test:Control is the ratio of the total \( \mu l \text{O}_2 \) absorbed by the test sample to the total \( \mu l \text{O}_2 \) absorbed by the control.
factors greatly influenced oxygen uptake data in pure lipid systems. Among replicate samples in each experiment, oxidation rates were also inconsistent (Table 1). Environmental parameters which may have affected oxygen uptake during the 50hr assay included fluctuating atmospheric pressure, temperature, fluorescent light intensity and moisture content. Labuza (1971) has also reported that the purity of the lipid material may influence the length of the induction period. In real food systems, where fat was a minor component, other food constituents had a strong influence on the mechanism of oxidation. Consequently, this made the analysis of oxidation reactions difficult since one could not account for all the interactions which effected the lipid's stability (Labuza, 1971). The complex nature of molasses and molasses extracts may have caused the tocopherol-free corn oil to oxidize in an unpredictable manner, as shown by the wide deviation in oxygen uptake values (Table 1).

When 500ppm Fe$^{++}$ were added to tocopherol-free oil, no induction period was observed and rapid oxygen uptake occurred. Lipid oxidation in the presence of 50ppm Fe$^{++}$ showed an induction period and provided a better picture of controlled oxygen uptake rates. Oxygen uptake values recorded from tocopherol-free corn oil mixed with microcrystalline cellulose represented a normal lipid oxidation pattern initiated solely by environmental conditions. Thus, the control and iron catalyzed lipid oxidation values were used to relate the strength of molasses prooxidants and antioxidants.
Solvent Extraction and Examination of Molasses Compounds

Methanolic (polar) and chloroform (nonpolar) soluble compounds from blackstrap molasses promoted oxygen uptake in tocopherol-free corn oil (Table 2). Figure 5 shows the oxygen uptake patterns observed with nonpolar and polar extracts from blackstrap molasses added to tocopherol-free corn oil. The presence of the solvent extracts from molasses increased oxygen uptake about 50%. However, 50ppm Fe²⁺ increased the relative oxygen uptake by tocopherol-free corn oil about 100%. Although a variance in absolute oxygen uptake values was observed, the relative oxygen uptake values were consistent among replicate tests. Nonpolar extracts from blackstrap molasses, on the other hand, strongly inhibited lipid oxidation in the coupled β-carotene-linoleic acid oxidation assay (Figure 6). The results of the present study suggested that oxygen was absorbed by the lipid system but 1) was not converted to an activated oxygen species or 2) the activated oxygen species formed were quenched by the nonpolar extracts before initiating lipid oxidation.

Nonpolar compounds from blackstrap molasses possessed substantially greater antioxidant activity than nonpolar compounds from table molasses when measured by the coupled β-carotene-linoleic acid oxidation assay (Figure 6). The difference of antioxidant activity in blackstrap and table molasses indicated that the antioxidant compounds were products of thermal processing and formed during the subsequent high heat treatment of table molasses. Likewise, Kirigay et al. (1968) have demonstrated that antioxidant activity formed during
Table 2. The effect of solvent extracts from blackstrap molasses on oxygen uptake in tocopherol-free corn oil.

<table>
<thead>
<tr>
<th>Experiment #</th>
<th>Conditions</th>
<th>Number</th>
<th>Induction Time (hrs)</th>
<th>$\mu l \text{O}_2$/hr</th>
<th>Total $\mu l \text{O}_2$</th>
<th>Test:Control $^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>control</td>
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<td>24</td>
<td>1.65</td>
<td>58.5±23.0</td>
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<tr>
<td></td>
<td>polar</td>
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<td>103.5±10.9</td>
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</tr>
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<td>nonpolar</td>
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</tr>
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<td>50ppm Fe$^{++}$</td>
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<td>24</td>
<td>2.89</td>
<td>136.8±13.2</td>
<td>2.41</td>
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a) Results are expressed as mean ± SD (n).

b) Test:Control is the ratio of the total $\mu l \text{O}_2$ absorbed by the test sample to the total $\mu l \text{O}_2$ absorbed by the control.
Figure 5. Effect of polar and nonpolar extracts from blackstrap molasses on the rate of oxygen uptake in tocopherol-free corn oil. 50ppm Fe^{2+} (•), polar extract (◦), nonpolar extract (○), control (⊙).
Figure 6. Influence of solvent extracts from blackstrap molasses on the coupled oxidation of \( \beta \)-carotene and linoleic acid. A decrease in absorbance indicates oxidation. Browning reaction pigments from refluxed 0.8M fructose-0.8M aspartic acid, ferulic acid or caffeic acid (\( \circ \)), nonpolar extract (\( \bullet \)), polar extract (\( \circ \)), control (\( \star \)), nonpolar extract from table molasses (\( \square \)).
the Maillard browning process increased with increased high heat
treatment. The nonpolar extracts from blackstrap molasses continued
to prevent lipid oxidation well over 2wks. Morano (1976), on the
other hand, reported that the polar extract of blackstrap molasses
reduced peroxide development in prime steamed lard more effectively
than butylated hydroxytoluene (BHT) during an initial 5 day test period.

Characterization of Solvent Extracts from Molasses

Polar and nonpolar extracts from molasses were investigated for
conjugated structures. Evans et al. (1958) suggested that conjugated
species were associated with reductones and antioxidant activity.
A single UV absorption peak, $\text{Abs}_{266}$, was observed in the nonpolar
extract; whereas, a broad UV absorption peak, $\text{Abs}_{220}$ to $\text{Abs}_{351}$, and
at $\text{Abs}_{404}$ was observed in the polar extract of blackstrap molasses.
These results suggested that the polar extracts from molasses contained
a complex mix of conjugated species, while the nonpolar extracts from
molasses contained a single conjugated species.

Upon visual examination, browning reaction pigments separated
into the polar extract. Evans et al. (1958) suggested that browning
reaction pigments were products of the Maillard browning reaction.
Several studies have shown that Maillard browning reaction pigments
were associated with increased antioxidant activity (Evans et al.,
1958, Kirigay et al., 1968; Labuza, 1971). On the other hand, the
nonpolar extract contained deep yellow pigments which have been
associated with anhydroamino-carbonyl reductones (Evans et al., 1958).
These compounds have been shown by Evans et al. (1957) to increase
with thermal processing. This may have explained the more intense yellow associated with the nonpolar extract from blackstrap molasses. In contrast, the nonpolar extract from table molasses received less heat treatment and appeared lighter yellow.

Thin-layer Chromatography

Blackstrap and table molasses compounds developed on Silica-Gel- G with ethyl acetate:formic acid:water (10:2:3 v/v/v) and re-chromatographed in 1-butanol:acetic acid:water (4:1:1 v/v/v) separated into two distinct bands (Table 3). Blackstrap polar extracts resolved into a thin, bright fluorescing band at Rf 0.26 and a wide, faint band at Rf 0.06-0.66. Polar extracts of table molasses resolved into similar bands: a wide, faint band at Rf 0.2-0.29 and a wide, bright fluorescing band at Rf 0.58-0.64. Hammerschmidt and Pratt (1978) reported 4 fluorescing bands at Rf 0.21, 0.44, 0.56, and 0.62 in soy meal extracts. They found strong antioxidant activity associated with the latter three bands when measured with the coupled β-carotene-linoleic acid oxidation assay. In the present study, however, the developed molasses bands did not affect linoleic acid oxidation and were not associated with antioxidant activity.

Ten phenolic compounds and benzoic acid have been identified in blackstrap molasses using gas chromatography: anisole, phenetole, phenol, m-cresol, salicylic acid, resorcinol, vanillic acid, syringic acid, p-coumaric acid, and vanillin (Hashizume et al., 1967). However, Scott (1965) determined that the most effective phenolic antioxidants
Table 3. Thin-layer chromatography separation of molasses on Silica Gel-G.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Compound</th>
<th>Rf</th>
<th>UV Fluorescent Response</th>
<th>Antioxidant&lt;sup&gt;a&lt;/sup&gt; Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Blackstrap</td>
<td>0.26</td>
<td>thin, bright</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.60-0.66</td>
<td>wide, faint</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>Table</td>
<td>0.20-0.29</td>
<td>wide, faint</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.58-0.64</td>
<td>wide, faint</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>Soy&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.21</td>
<td>none</td>
<td>moderate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.44</td>
<td>moderate</td>
<td>moderate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.56</td>
<td></td>
<td>strong</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Blackstrap</td>
<td>0.11</td>
<td>yellow-green</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.88</td>
<td>light green</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>Table</td>
<td>0.87</td>
<td>light yellow-green</td>
<td>none</td>
</tr>
<tr>
<td></td>
<td>Caffeic Acid</td>
<td>0.92</td>
<td>dark purple</td>
<td>strong</td>
</tr>
<tr>
<td></td>
<td>Ferulic Acid</td>
<td>0.91</td>
<td>purple ring</td>
<td>strong</td>
</tr>
</tbody>
</table>

<sup>a</sup> Measured by the coupled β-carotene-linoleic acid oxidation assay.


<sup>c</sup> Reported by Hammerschmidt and Pratt (1978).

<sup>d</sup> Developed in ethyl acetate:acetone:formic acid:water (5:3:1:1 v/v/v/v).
were ortho- or para- substituted hydrogen donors. These antioxidants have an effective delocalization of unpaired electrons. Phenolic compounds which have been reported by Hashizume et al. (1967) in molasses were not dihydroxy species, had not been previously associated with antioxidant activity and were, thus, not further investigated. In the present study molasses was investigated for the known phenolic antioxidants, ferulic and caffeic acid. Pratt and Birac (1979) have isolated these antioxidants from soy meal. Table 3 lists the Rf values and UV fluorescent response of the molasses fractions and the plant flavonoids developed in ethyl acetate:acetone:formic acid:water (5:3:1:1 v/v/v/v). As expected, molasses and molasses extracts did not show any spots which corresponded to the Rf values of codeveloped ferulic and caffeic acid.

**Gel Filtration Chromatography**

Separation of molasses pigments: As shown in Figure 7, Sephadex G-100 separated the pigments in blackstrap molasses into two distinct components. The first component of brown pigments occurred at the solvent front (32mls), as determined by the elution volume of Dextran-Blue 2000. Like Dextran-Blue 2000, these large molecular weight, colloidal pigments from molasses were not absorbed by the gel matrix during the separation process. Preliminary lipid oxidation tests with the first band of molasses pigments were negative and thus, these pigments were discarded.

The second component of molasses pigments occurred between 75mls
Figure 7. Elution pattern of pigments from blackstrap molasses separated through Sephadex G-100 with distilled water. Dextran-Blue 2000 (○), Blackstrap molasses (●).
and 100mls of elutant with the maximum concentration observed at 87mls (Figure 7). Studies with table molasses showed a similar, less intense absorbance pattern with the pattern in Figure 7 for blackstrap molasses.

Kirigay et al. (1968) reported that browning intensity was directly related to antioxidant activity. Rhee and Kim (1975) have also shown that high molecular weight browning reaction pigments produced in the latter stages of browning possessed some antioxidant activity. However, these investigators found that the major antioxidant compounds were colorless, lower molecular weight intermediates from the browning reaction. These intermediates were possibly reductones and dihydro-reductones produced during the earlier stages of Maillard or caramelization browning reactions. Evans et al. (1958) also reported an increase in antioxidant activity associated with lower molecular weight amino-hexose reductones that formed during Maillard browning reactions.

Analysis of antioxidant activity: As shown in Figure 8, blackstrap molasses contained a strong, low molecular weight antioxidant. These compounds eluted from Sephadex G-100 between 85mls and 100mls with peak antioxidant activity observed at 91mls. When assayed by coupled β-carotene-linoleic acid oxidation assay, the antioxidants from blackstrap molasses reduced lipid oxidation 87% more efficiently than the control (Figure 9). Results from the present study suggested that the antioxidant compounds and browning reaction pigments in blackstrap molasses eluted within a similar range of solvent and may
Figure 8. Elution pattern of molasses compounds separated by Sephadex G-100 and their effect on the coupled oxidation of β-carotene and linoleic acid. Positive values indicate antioxidant activity, negative values indicate prooxidant activity. Blackstrap molasses (☉), table molasses (●).
Figure 9. Effect of fractions from blackstrap molasses separated by Sephadex G-100 on the coupled oxidation of β-carotene and linoleic acid as a function of time. A decrease in absorbance indicates oxidation. 45.0ml (○), 87.0ml (□), 94.0ml (⊗), control (★).
be related (Figure 7 and 8). Similar observations relating antioxidant compounds and browning reaction compounds have been reported (Evans et al., 1958; Kirigay et al., 1968; Rhee and Kim, 1975).

In contrast with blackstrap molasses, table molasses showed weak antioxidant activity and inhibited linoleic acid oxidation only 25% relative to the control (Figure 10). These compounds eluted from Sephadex G-100 between 92mls with maximum activity observed at 96mls.

The results of the present study demonstrated that antioxidants from blackstrap molasses were 62% more efficient than antioxidants from table molasses at preventing linoleic acid oxidation. As suggested from this observation, the compounds in molasses responsible for inhibiting lipid oxidation were formed during the high heat processing of sugarcane. Most likely, these antioxidant compounds increased dramatically during the subsequent thermal processing of table molasses into blackstrap molasses.

Analysis of prooxidant activity: As shown in Figure 9, blackstrap molasses also contained a moderated prooxidant which eluted between 48mls and 64mls with peak activity observed at 54mls. These medium molecular weight prooxidants promoted linoleic acid oxidation 47% greater than the controls. In contrast with blackstrap molasses, compounds in table molasses were not associated with prooxidant activity (Figure 10). Consequently, this relation again suggested that prooxidants in molasses were produced during the high heat, prolonged processing of sugarcane.
Figure 10. Effect of fractions from table molasses separated by Sephadex G-100 on the coupled oxidation of \( \beta \)-carotene and linoleic acid as a function of time. A decrease in absorbance indicates oxidation 54mls (⊙), 94mls (●), 98mls (□), control (★).
Analysis of Antioxidant Properties in Molasses

Spectrophotometric properties of molasses fractions: UV spectral scans of the aqueous antioxidants in table and blackstrap molasses were similar. As shown in Table 4, the position of each absorption peak in molasses fractions with antioxidant activity corresponded but, the magnitude of each peak observed in table molasses was greater. Conversely, antioxidant activity was observed to be substantially greater in blackstrap molasses than in table molasses. This suggested that the compounds in molasses responsible for antioxidant activity contained similar conjugated structures, but that the conjugated structures provided markedly different levels of lipid protection.

Results of organic spot tests: The intensity of the thiobarbaturic-phosphoric acid reaction for α,β-unsaturated aromatic or aliphatic aldehydes or ketones was greater in table molasses than in blackstrap molasses fractions, but, as noted before, the antioxidants in blackstrap molasses were more active than the antioxidants in table molasses. This suggested that the reaction intensity of the assay was not a direct function of antioxidant activity. Antioxidant compounds in both types of molasses reacted positive for methyl ketones. These organic configurations have been associated with reductones and antioxidant activity (Scott, 1965; Pratt and Birac, 1979). The tests in this study strongly suggested the presence of reductones in molasses and their contribution to molasses antioxidant activity.

Competitive oxidation in molasses: Polar extracts from blackstrap molasses did not effect the oxidation of linoleic acid.
Table 4. Position and magnitude of UV spectral peaks observed in the antioxidant fractions of blackstrap and table molasses.

<table>
<thead>
<tr>
<th>Molasses</th>
<th>Absorption Peak (nm)</th>
<th>Peak Intensity (O.D.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blackstrap</td>
<td>312</td>
<td>1.52</td>
</tr>
<tr>
<td></td>
<td>214-225</td>
<td>2.90</td>
</tr>
<tr>
<td>Table</td>
<td>307</td>
<td>2.00</td>
</tr>
<tr>
<td></td>
<td>213-221</td>
<td>2.96</td>
</tr>
</tbody>
</table>
However, when these polar compounds were separated by molecular weight, a prooxidant and an antioxidant were observed. These prooxidant and antioxidant compounds apparently counteracted each other in the polar extract and therefore, they neither promoted nor inhibited linoleic acid oxidation. However, when each compound acted alone, as after separation with Sephadex G-100, their individual effects on linoleic acid was observed. Consequently, the neutral effect from these competitive compounds may have explained why the polar extract from blackstrap molasses did not influence linoleic acid oxidation, whereas, the gel-separated fractions did. Labuza (1971) has reported that competitive oxidation in pure lipids, such as linoleic acid, was complicated and hard to analyze. Moreover, he found that competitive oxidation in real food systems was almost impossible to analyze. Too many environmental factors affected the stability of a food product for one to predict the influence of one parameter on lipid oxidation (Labuza, 1971).

Flavonoids vs. antioxidant activity in molasses: If antioxidant active flavonoid and phenolic compounds were present in molasses, then their ability to inhibit lipid oxidation should have been similar in blackstrap and table molasses. However, the antioxidants contained in blackstrap molasses inhibited linoleic acid oxidation more efficiently than those compounds in table molasses. Apparently, the phenolic and flavonoid compounds in sugarcane molasses were either 1) not responsible for measurable antioxidant activity, 2) destroyed during thermal processing or 3) converted during the thermal processing to antioxidant active flavonoid derivatives.
Ames Mutagen Screening Assay

Table 5 lists the Ames index values for blackstrap molasses fractions tested with Ames *Salmonella typhimurium* strains TA98, TA100, TA1535 and TA1537. The results from this assay indicated that all molasses compounds were nonmutagenic and were not activated by microsomal enzymes to mutagenic compounds. Claims by Setliff and Mower (1977) that browning reaction compounds in molasses were mutagenic to *S. typhimurium* were unsupported. All Ames index values calculated for molasses fractions separated by Sephadex G-100 in the present study were less than 2.0 and therefore insignificant for mutagenic activity.
Table 5. Ames Index\textsuperscript{b} values for blackstrap molasses fractions tested with Ame's Salmonella typhimurium cultures.

<table>
<thead>
<tr>
<th>Total mls solvent\textsuperscript{c}</th>
<th>Ames cultures without liver homogenate (S-9)</th>
<th>Ames cultures with liver homogenate (S-9)\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TA98  TA100  TA1535  TA1537</td>
<td>TA98  TA100  TA1535  TA1537</td>
</tr>
<tr>
<td>27</td>
<td>1.33  0.87  0.95  1.07</td>
<td>0.43  0.90  0.86  0.75</td>
</tr>
<tr>
<td>47</td>
<td>0.80  0.95  1.10  0.90</td>
<td>0.82  1.20  0.60  0.80</td>
</tr>
<tr>
<td>65</td>
<td>0.80  0.85  1.20  1.40</td>
<td>0.97  0.95  0.55  0.60</td>
</tr>
<tr>
<td>83</td>
<td>0.94  1.10  1.60  0.76</td>
<td>1.10  0.36  0.95  1.13</td>
</tr>
<tr>
<td>94</td>
<td>1.57  0.82  0.60  1.00</td>
<td>1.34  1.01  0.73  1.00</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Poly chlorinated biphenyl (PCB) induced rat liver.

\textsuperscript{b} Ames Index \( A/I = \frac{\text{number of revertant colonies}}{\text{number of control colonies}} \) (average of 3 replicates)

\( A/I \) must be greater than 2.0 to be significant.

\textsuperscript{c} Total mls of solvent eluted through Sephadex G-100 prior to 1.0 ml molasses sample.
SUMMARY AND CONCLUSIONS

The following conclusions were observed from this study on the isolation and characterization of naturally occurring antioxidants in sugar-cane molasses.

1. Polar and nonpolar fractions from blackstrap and table molasses increased oxygen uptake in tocopherol-free corn oil.

2. Nonpolar fractions from blackstrap molasses inhibited the coupled β-carotene-linoleic acid oxidation rate.

3. Compounds in nonpolar fractions from blackstrap molasses increased oxygen absorption in tocopherol-free corn oil, but dramatically inhibited linoleic acid oxidation. The nonpolar compounds either prevented the formation, inhibited the reactivity or quenched the activated oxygen species and free radicals associated with lipid oxidation.

4. Polar molasses fractions contained browning reaction pigments and a complex mixture of conjugated compounds.

5. Nonpolar fractions from molasses contained deep yellow pigments, that suggested the presence of anhydroamino-hexose-carbonyl reductones and a single conjugated mixture of compounds.

6. The rate of oxygen uptake observed in tocopherol-free corn oil was similar in the presence of 5% molasses, 10% molasses or 50ppm Fe^{++}. However, the rate of oxygen uptake increased markedly when more than 25% molasses or 50ppm Fe^{++} was combined with tocopherol-free corn oil.
7. Molasses fractions separated by thin-layer chromatography (TLC) did not affect linoleic acid oxidation. Antioxidant activity from phenolic or flavonoid compounds in sugarcane molasses was not detected in molasses fractions separated by TLC.

8. Molasses fractions separated by Sephadex G-100 and eluted with distilled water contained a prooxidant and an antioxidant.

9. The compounds with prooxidant activity increased linoleic acid oxidation by 47% and were observed only in blackstrap molasses.

10. The low molecular weight components from blackstrap molasses inhibited linoleic acid oxidation 87%. These components were found to be 62% more effective as antioxidants than those isolated from table molasses.

11. Blackstrap and table molasses fraction which showed antioxidant activity, tested positive for potential reductone structures. In addition, the antioxidant compounds showed similar UV absorption patterns with substantially different peak intensity values. Table molasses absorption peaks were more intense than blackstrap molasses absorption peaks.

12. Molasses fractions separated by Sephadex Gel-100 showed a negative responses with Ames Salmonella typhimurium strains TA98, TA100, TA1535, and TA1537 for both point and frameshift mutagen activity. Moreover, microsomal enzymes did not activate any of the molasses compounds into mutagenic compounds.
13. Antioxidant and prooxidant compounds, which were isolated from blackstrap molasses, were products of the prolong, high processing of sugarcane into sucrose crystals. Consequently, fractions from blackstrap molasses contained substantially more compounds which influenced linoleic acid oxidation.

In summary, the isolation of antioxidant active compounds from molasses was successful. The results of the present study showed that molasses contained a strong group of natural antioxidants. Moreover blackstrap, sugarcane molasses appears to be a promising source of naturally occurring antioxidants for protecting lipid-containing food products.
REFERENCES


