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by

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INTRODUCTION

Deep-fat frying is a favorite method for food preparation. However, the procedure exposes the frying medium to light, elevated temperatures and atmospheric oxygen. This results in a very complex pattern of both thermolytic and oxidative reactions in the medium (Fritsch, 1981). The quality of the frying medium is reduced and the food fried in the used material absorbs more fat and has an overall decrease in quality. There is also some suggestion that ingestion of high quantities of heat-abused fats and oils can be harmful (Alexander, 1983). High temperature and atmospheric oxygen also accelerate cholesterol oxidation when animal fats are used for deep-fat frying. Some of the cholesterol oxidation products (COPs), e.g., 3β, 5, 6β-hydroxycholestanol and 25-hydroxycholesterol, are very cytotoxic and angiotoxic to laboratory animals. Therefore, many researchers are studying the role of the COPs in the development of human atherosclerosis. The presence of such oxidation products can be detected in processed foods such as spray-dried egg products which are incorporated in many prepared foods and mixes. Therefore, there is a great need for analytical methods that can accurately identify and quantify common cholesterol oxidation products that are found in foods. In addition, antioxidants that are effective in reducing triglyceride and cholesterol oxidation at frying temperature need to be developed to minimize human consumption of these potentially harmful compounds.

The purpose of this research was to study the effectiveness at 180 C of linalyl acetate and polydimethyl siloxane (MS) in reducing triglyceride oxidation in soybean oil and in reducing triglyceride and

cholesterol oxidation in lard with added cholesterol. The method of Park and Addis (1985, 1986a, b) was used and modified in the identification and quantification of COPs.

LITERATURE REVIEW

Atherosclerosis is a major cause of death and disability in industrial nations. Its relation to diet has been extensively documented (McGill, 1979). In a recent article, Jacobson et al. (1985) summarized two major hypotheses for causes of atherosclerosis, including 1) the response-to-injury hypothesis and 2) the lipid hypothesis. The response-to-injury hypothesis states that the fundamental lesion is endothelial in nature. As a result of repeated endothelial injury and platelet-vessel interaction, macrophage, smooth muscle cell, lipid and connective tissue infiltrate the arterial wall. The lipid hypothesis states that dietary and genetic factors interact to produce hyperlipemia which, combined with contributing factors such as hypertension or smoking, results in arterial lesions. Regardless of the theories regarding the disease, a number of risk factors including hypertension, smoking, family history, dietary cholesterol intake and serum cholesterol level, appear to influence atherosclerosis. Control of these factors has proven to be problematic. Therefore, other risk factors which can be modified must be found to reduce the toll in morbidity and mortality from this disease.

Atherogenic Effect of Cholesterol Oxidation Products

The autoxidation of cholesterol and the cytotoxic and angiotoxic properties of several cholesterol oxidation products (COPs) have been widely recognized (Sevanian and Peterson, 1986; Addis, 1986; Imai et al., 1980; Kandutsch et al., 1978). In general, the approach to studying the atherogenic effects of COPs can be grouped into in vivo and in vitro studies. Peng et al. (1985) used New Zealand male white rabbits as their model. They investigated the effects of 25-hydroxycholesterol (25-OH) and

cholestane-3β, 5α, 6β-triol (triol) on intimal surfaces as observed by scanning and transmission electron microscopy when acute doses (2.5mg/kg) of these COPs were infused intravenously. The triol was shown to be more atherogenic than the 25-OH but both showed significiantly more craters and balloon-like lesions than from the control diet. Similar balloon-like protrusions and crater-like defects have described in rats, rabbits and Rhesus monkeys subjected to ischemia following arterial occlusions, in mice fed high-fat diets and in the arterial endothelium of rabbits that were chronically maintained on an atherogenic diet. Peng et al. (1985) proposed that such arterial defects are non-specific reactions of the endothelial cells to recurring insults and repair eventually results in the development of atherosclerotic plaque.

Jacobson et al. (1985) looked at low-level triol feeding (amounts estimated to be similar to U.S. dietary intake levels) in White Carnean pigeons. No significant differences were seen in plasma triglyceride, total cholesterol or high density lipoprotein (HDL) cholesterol between the cholesterol-fed control group and the triol fed treatment group. Yet coronary artery atherosclerosis, as measured by percent mean lumenal stenosis was 87% higher in the triol groups. This increase was accompanied by a 42% increase in aortic calcium accumulation. These observations suggest that the atherogenic effects seen are not mediated through changes in absorption or metabolism of lipids or lipoproteins but through a direct effect on the arterial wall.

Observations from in vitro experiments help to support hypothesis drawn by researchers from in vivo experiments. One such hypotheses pertains to the possible mechanism of cell death which leads to eventual

arterial lesions. It is hypothesized that the ability of COPs to suppress 3-hydroxy-3-methylglutaryl Coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of cholesterol biosynthesis, and to suppress DNA synthesis leads to dysfunctioning of cell membranes, resulting in increased fragility and permeability of the cells which then causes cell death. Kandutsch et al. (1974) and Peng et al. (1979) showed in cultured mouse cells and aortic smooth muscle cells, respectively, the effect of COPs and not cholesterol in suppressing HMG-CoA reductase. Sevanian and Peterson (1984) studied cholesterol α-epoxide in V79 Chinese hamster cells and found that it is a direct-acting mutagen and it inhibits DNA synthesis.

Tissue studies provide further evidence of a link between COPs and atherosclerosis. Gray et al. (1971) found 25-hydroxycholesterol (25-OH) and cholesterol 5α, 6α-epoxide, (α-epoxide) in serum of human hyperlipemics but not in normal volunteers. van Lier and Smith (1970) observed the presence of 6-hydroxycholesterol in fatty streaks and atheromas of adult males compared to children. When Peng et al. (1982) fed 25-OH to squirrel monkeys, they found that the majority of 25-OH was transported to peripheral tissues, including vascular tissue, by very low density lipoprotein (VLDL) and low density lipoprotein (LDL). In contrast, HDL carried only a minute amount of 25-OH. The presence of COPs in body tissues lead scientists to speculate that the cytotoxicity, angiotoxicity and mutagenicity could injure the artery, thus resulting in atherosclerosis.

Mutagenic and Carcinogenic Effects of COPs

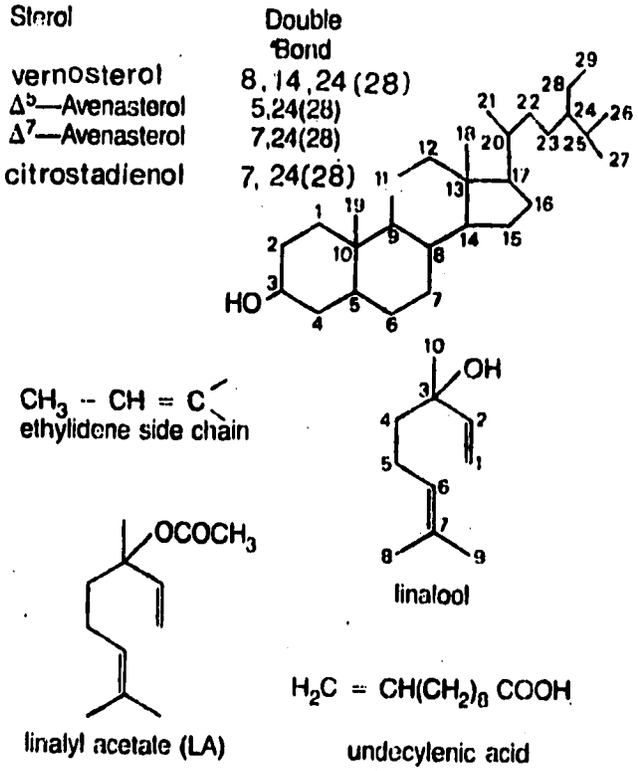
Since cholesterol epoxides possess an electrophilic oxirane group, they could be expected to be genotoxic and carcinogenic. The detection of cholesterol epoxide (Black and Douglas, 1972) in UV-induced skin cancer and triol in human colon cancer (Reddy and Wynder, 1977) suggests that the etiology of these cancers may be associated with COPs.

The Food and Drug Administration (FDA), as one of the Federal Government's primary consumer protection agencies, has recognized the pathological effects of COPs as a potential public health issue (Sheppard and Shen, 1980). Within the FDA, research is being conducted to develop appropriate methodology for the analysis of COPs to be used for monitoring the food supply and for possible regulatory purposes.

Analysis of COPs in foods is made difficult by their low concentrations and by the complexities of the food matrices. Maerker (1987) briefly summaried the current knowledge of the chemistry of cholesterol oxidation that aids in the interpretation of the analytical findings of research reports. The monograph by Smith (1981) provides an in-depth discussion on cholesterol oxidation. Information from both above sources is summarized below.

Chemistry of Cholesterol Oxidation

The chemical structure of cholesterol is seen in Figure 1. The molecule has a polycyclic nucleus with four fused rings, a branched aliphatic side chain attached to the D ring at C-17, a hydroxyl group that is attached to C-3 of the A-ring and is in β configuration, and a Δ^5 double bond in the B ring. Sites that are sensitive to autoxidation include the B



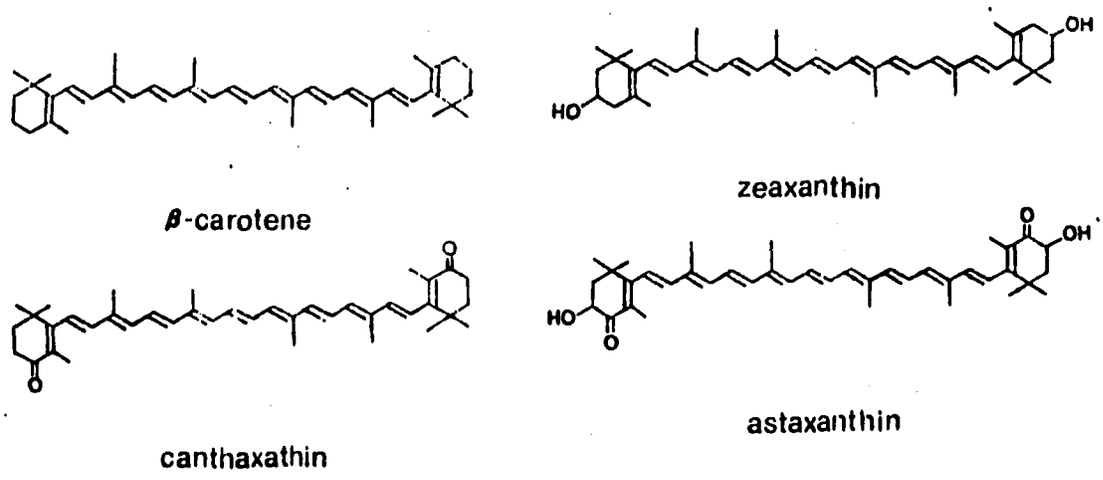


Figure 1. Chemical structures of compounds containing an ethylidene group and carotenoids.

ring unsaturation, positions allylic to it and the two tertiary carbons in the side chain.

Cholesterol oxidation is initiated by hydrogen abstraction, predominantly at C-7. In solid phase oxidation (i.e., oxidation of crystalline cholesterol), oxidation of the side chain occurs at the tertiary C-25 and sometimes at C-20, resulting in hydroperoxide formation. By contrast, side chain oxidation is not observed in autoxidations carried out in solution or in aqueous dispersions. Oxidation in solution provides a better model for cholesterol in foods than do those of solid phase cholesterol oxidation.

One would expect the two allylic carbons, C-4 and C-7, to be attacked equally by oxygen. Yet, attack at C-4 rarely occurs. Abstraction of a hydrogen atom at C-7 followed by attack by molecular oxygen gives rise to two epimeric hydroperoxides. The β-isomer is thermodynamically more stable than the α-form, and ready interconversion occurs. The thermal instability of the 7-hydroperoxides causes the formation of the principal stable products of 7-ketocholesterol (7-keto) and the epimeric 7-OHs. Interconversion also occurs between the 7-OHs, favoring the equatorial β-epimer. The epimeric 5,6-epoxides are found in autoxidation mixtures in the crystalline state or in solution or aqueous dispersion. It was shown by different researchers that 5,6-epoxides are the products of attack by various hydroperoxides on the 5,6-double bond of cholesterol and, therefore, are secondary oxidation products. Hydration of either epimer of 5,6-epoxide results in the formation of the same 3β, 5α, 6β-triol, the most toxic of COPs tested to date.

It has been common practice to isolate cholesterol and its oxidation products from lipid matrices by hot alkaline saponification and subsequent separation from the unsaponifiable residue (Higley et al., 1986; Finocchiaro et al., 1984). Yet, 7-keto, a major product of cholesterol autoxidation, is highly sensitive to hot alkali, resulting in the formation of 3, 5-cholestadien-7-one and several other products.

This briefly summarizes the most commonly encountered COPs in an autoxidation mixture. Methods for analyzing COPs in various food systems follow.

Analytical Methods for Cholesterol Oxidation Products

Thin-layer chromatograpy (TLC)

The advent of TLC in the early 1960s helped the detection and the separation of cholesterol and its oxidation products. Chicoye et al. were able to identify COPs in an aerated cholesterol solution (1968a) and in spray-dried yolk solids irradiated with fluorescent or summer sunlight (1968b). Analytical TLC plates were used to determine the extent of oxidation. Diethyl ether was the developing solvent. Tentative identification of COPs was carried out by charring the TLC plates sprayed with 50% H_2SO_4 and noting both R_c (defined as distance of sample spot from the origin/distance of cholesterol spot from the origin) and spot colorations. They also employed preparative TLC plates to isolate unknown compounds for mass spectral (MS) and infrared spectral (IR) analysis. Band positions were detected by applying 50% H_2SO_4 to a confined channel or by spraying the plates with 0.2% dichlorofluorescein and locating with UV lights. Dichlorofluorescein was removed by another preparative TLC run. Another way to utilize UV light for COP detection was described by

Finnocchiaro et al. (1984). They ran the unsaponifiable fractions on silica gel UV_{254} plates and illuminated with 254 nm light to detect fluorescence-quenching compounds.

Even with the advent of more powerful analytical techniques, TLC is still used because it is simple and fast. Kimura et al. (1979) used TLC to follow the progress of cholesterol oxidation in an aqueous stearate dispersion model system. Their solvent system was benzene-ethyl acetate (3:2). Hydroperoxides were visualized by spraying with Wurster dyes (Smith and Hill, 1972). Other compounds were located by 50% H_2SO_4 spray as mentioned above. With this time-course study, they discovered that the major product in the early period was 7-hydroperoxides. The level of 7-keto and 7-OHs increased as 7-hydroperoxides decreased. More importantly, they discovered that cholesterol oxidation ceased when more than 70% of the initial cholesterol was consumed. Luby et al. (1986) also used TLC to follow the progression of photooxidation of cholesterol in butter. de Vore (1988) used TLC to evaluate the eluates from disposable silica columns to determine separation efficiency of 7-keto from major lipid components in meat lipid extracts. In addition to using TLC as a screening tool for samples that contain COPs, it can be used to purify an individual COP to be used as standards for quantitation. Maerker and Bunick (1986) prepared β-epoxide by the method of Chicoye et al. (1968a) and purified it by preparative-TLC before use. The TLC can also be used as a quantitative tool. Finocchiaro et al. (1984) were able to quantify cholesterol, epoxides and triol from oxidized cheese lipids by establishing standard curves for each of the oxides. A spot area was measured with a digital

planimeter and they were able to obtain r-values of at least 0.937 from their regression curves.

There are a few shortcomings of TLC on COP identification and quantification. Spot colorations fade rapidly; COPs such as 7-keto and 5,6-epoxides are poorly resolved from each other by TLC; and some COPs may be unstable under lengthy TLC runs.

High-performance liquid chromatography (HPLC)

HPLC has been used by a number of workers to analyze COPs. Ansari and Smith (1979) demonstrated the powerful capability of HPLC for resolving isomers. Tsai and Hudson (1981) studied such resolving power more systematically by examining the retention times of twenty-four oxygenated cholesterols and structurally-related compounds by normal phase HPLC. Hexane/2-propanol was found to be superior to hexane/tetrahydrofuran (THF) and hexane/ethyl acetate as the mobile phase. Elution was monitored by a variable wavelength absorption detector set at 210 nm and a differential refractometer connected in series. The two detectors were necessary because the absorption detector does not respond to α- and β-epoxide, whereas the differential refractometer is not as sensitive a detector as the absorption detector.

Both reversed phase and normal phase HPLC systems have been used but the latter have been more successful. Silicic acid absorbs compounds through the free election pair of the oxygen bridges and the hydrogen bonding of the hydroxyl group. Therefore, both the relative elution power of the eluting solvents and the retention volumes of COPs should correlate to the hydrogen bonding capabilities of their functional groups. The position and the spatial orientation of these functional groups were also

found to affect the retention volumes. In general, the separation coefficient, k', was affected in decreasing order by: hydroxy on the ring, carbonyl on the ring, epoxy on the ring, hydroxy on side chain and carbonyl on side chain. The excellent resolving power of HPLC on epimers and isomers was apparently brought on by its sensitivity to the steric effect. Therefore, the configurations of the functional group in relation to the whole molecule and the synergistic effects to its neighboring functional group created by each configuration together determine the k' for the compound. This work by Tsai and Hudson also revealed a major shortcoming of HPLC, i.e., triol was strongly retained on the normal phase column and was never eluted. When they attempted to increase the polarity of the eluting solvent, triol did elute faster; yet the rest of the compounds were poorly resolved. Therefore, triol, the most toxic COP, often is not determined by adsorptive HPLC. In one of their later studies, Tsai and Hudson (1984 and 1985) tested their HPLC method on the isolation and identification of α- and β-epoxide in a variety of commercial dry egg products. Due to the presence of large amount of phospholipids in yolk lipids, acetone was chosen as the extracting lipid. With the help of radioactive α-epoxide, they found that the extract recovered all of the α-epoxide while it excluded the phospholipid when they chromatographed the extract on a silicic acid HPLC column. The mobile phase was changed linearly from 1% THF in hexane to 100% THF. The radioactive fractions were collected and pooled. Further chromatography of the pooled fraction on some yolk samples using their 1981 method resolved an unknown compound co-eluting with the spiked radioactive α-epoxide. Spectrometric determinations (MS, NMR and IR) identified that the unknown compound was β-

epoxide. Tsai and Hudson followed up their method development by testing it on familiar food items such as fresh yolk and dried yolk products. Their method worked well for screening food for the presence of COPs. Its ability to resolve the epimeric epoxides is of added importance since the pathological effects of α-epoxide are well-documented. Therefore, knowing the actual level of α-epoxide in foods is important.

Herian and Lee (1985) used the method of Tsai and Hudson (1981) to measure the formation of 7α- and 7β-OH in a dry egg nog mix under fluorescent light. Prior to HPLC analysis, they added a purification step that involved hot saponification and passing the unsaponifiables through an arrestant column to remove remaining long chain fatty acids and an argentated column to retain carotenoids. In this way, they were able to resolve and quantitate 7α- and 7β-OH in just 8 minutes. These data provide additional confidence that the method by Tsai and Hudson (1981) is useful.

The method by Kou and Holmes (1985) involved a very elaborate clean-up scheme before the actual cholesterol oxide analysis. Lipid extraction was carried out by HPLC-grade hexane to eliminate UV absorbing contaminants at 205nm. Samples were saponified overnight at room temperature to avoid the occurrence of contaminants that appeared with hot saponification. Acetonitrile extracts of the unsaponifiables were loaded on a C_{18}-silicic acid Sep-Pak cartridge that removed neutral lipids, including cholesterol, from the extract. They found that the use of two HPLC columns (C_{18} reversed-phase and silicic acid normal phase) was necessary to obtain consistent baseline resolution. Quantitation was done by an external standard method. They tried quantitating 25-OH on a wide array of samples, including rat plasma, muscle and liver tissue, egg and egg products,

adipose tissue and vegetable oil. Likely, because this method is a multi-step procedure, its cumulative recovery was reported to be only 58%.

Most COP determination methods include multi-step sample clean-up procedures and, to properly quantify for losses along each step, an internal standard or spiking the sample with radioactive COP should be involved. An example of the latter was discussed earlier in the work of Tsai and Hudson (1984). To incorporate an appropriate internal standard at the start of sample preparation is more frequently done. In the work of Park and Addis (1985a), 7-ketopregnenolone was added as the internal standard (IS). Sugino et al. (1986) and Missler et al. (1985) employed 6-ketocholestanol instead. In choosing an appropriate IS for the quantitation of cholesterol and COPs, attention should be given to whether the compound has polarity in between all compounds determined and has the same polycyclic nucleus with four fused rings. The appropriateness of the IS can be tested by performing response linearity. It is determined by plotting ratios of peak areas against ratios of weights with various amounts of COPs and cholesterol with a fixed amount of internal standard. Ketocholesterols were found to be structurally altered under harsh conditions such as hot saponification. Therefore, if 6-ketocholestanol were used as an IS in methods involving hot saponification, the quantitation would be grossly distorted.

Reversed phase HPLC systems are sometimes used for COP determinations. For example, Sugino et al. (1986) cleaned up their lipid extract by applying it to a disposable silica packed column and collected the cholesterol and cholesterol epoxides fraction. In this way, triglycerides were removed first and discarded, the cholesterol and cholesterol epoxides

were removed together in the second fraction and the phospholipids were retained on the column. The cholesterol and cholesterol epoxides were dried and derivatized with p-nitrobenzoyl chloride before resolving them on a reversed phase HPLC column. Solvent programming was necessary, and drift of the baseline was accounted for by a data processor.

Few HPLC procedures attempt to measure more than two to three COPs in an actual food sample. In a recent method by Csallany et al. (1989), they quantitated 7-keto, 7α-OH, 7β-OH, 25-OH and cholesterol in various tissues without an elaborate clean-up step. Their method involved only lipid extraction and filtration of lipid extract through a membrane, although accurate control of mobile phase composition and volume and absorbance wavelength was needed. Their method was still unable to measure other biologically significant COPs such as α-epoxide and triol.

Missler et al. (1985) and Maerker and Unruh (1986) took advantage of HPLC (non-destructive detection and high loading capacity) and capillary GLC (sensitive detection) and utilized a semi-preparative HPLC system to enrich the COPs before analyzing them by capillary GLC.

The HPLC methods allow chances of artifact formation during sample analysis, and the conditions encountered are not harsh enough to cause the degradation of ketocholesterols and the ring opening of epoxides. Detection methods are non-destructive, usually relying on absorption wavelengths (e.g., 233nm for 7-keto and 206nm for 7-OHs, 25-OH and cholesterol) or changes in refractive index. Therefore, fractions can be collected to isolate pure compounds for spectrometric identifications. However, more than one detection device is needed to detect all the commonly encountered COPs. Therefore, despite very significant advances of

HPLC techniques, they are somewhat cumbersome in the separation and quantification of complex mixtures of COPs. The application of GLC methods has been somewhat more successful.

Gas-liquid chromatography (GLC)

GLC has been used in the analysis of cholesterol and COPs for some time (Chicoye et al., 1968a), but resolution on packed columns has not been satisfactory. Chicoye et al. (1968a) determined the presence of COPs in fresh, unirradiated and irradiated spray-dried egg yolk. A glass column with 1% SE-30 was used and retention times were calculated relative to 5α-cholestane. Tentative identification of COPs was done by comparing the relative retention times and steroid number (a procedure to characterize steroids on non-polar columns as described by Fumagalli, 1969). Cholesterol and five COPs were reasonably resolved but the peak shapes were not symmetrical and a complete run took 70 min. In a subsequent paper, Chicoye et al. (1968b) studied α-epoxide in an aqueous dispersion and they silylated the COPs before analyzing them on a packed GLC column. It was discovered that when sterols are analyzed in the free form, they may undergo irreversible adsorption and/or thermal decomposition because of interactions between active sites on the columns and functional groups of the compounds (Fumagalli, 1969). Derivatization usually improves peak shapes and reduces peak tailings. Other problems associated with packed GLC included the incapability of resolving α- and β-isomers in a COP mixture (Tsai et al., 1980). The method they described used a combination of GLC (to quantitate total epoxides) and HPLC (to resolve and determine the ratio of α- and β-epoxide) to determine the amount of α-epoxide in dry egg products.

With the advent of open-tubular chromatography, analytical methods that are capable of isolating and quantifying a wide range of angiotoxic sterols in foods produced by the oxidation of cholesterol were developed. Missler et al. (1985) described a method that utilized semi-preparative HPLC to enrich the COP fraction and TMS-derivatization of the COPs to improve peak shapes. Samples were injected directly onto a bonded phase fused silica capillary column through an on-column injector. Quantitation was based on a peak area comparison with 6-ketocholestanol, the internal standard. In this way, the compounds, including the α- and β-epoxides, were successfully resolved and, because of the cool on-column injector, 7-OHs did not undergo dehydration in heated injection ports to form 7-dehydrocholesterol. Other advantages include reduced column bleeding associated with bonded phase columns, increased sensitivity due to improved peak shapes and injection of less concentrated samples since there is no splitting of the solvent with the on-column injection mode. By coupling a mass selective detector (MSD) or mass spectrometer (MS) to a capillary GLC column, structural information of each resolved COP can be obtained and reduced column bleed can increase sensitivity of GC-MS determinations and reduce ion source contamination of the MS or the MSD. The method of Maerker and Unruh (1986) followed a similar approach of semi-preparative enrichment and direct on-column capillary GLC chromatography for the analysis of a wide array of COPs. They experimented with injecting the underivatized COP mixture. Twelve compounds were resolved except 25-OH which was poorly separated from the isomeric 7-OHs. This problem was solved by derivatizing the mixture prior to injection. Because their method does not have a saponification step, oxidation of cholesteryl esters

was not detected. Subsequently, Zubillaga and Maerker (1988) reported a method involving transesterification of cholesteryl esters that liberates cholesterol from its esters, thereby allowing the quantitation of total cholesterol without saponification. Saponification is known to cause artifact formation.

It is not often possible to adapt an older GLC injection port to do direct on-column injections. Therefore, the method of Park and Addis (1985b) that utilized the split injection mode has some advantages. To enhance the stereo- and positional-isomeric differences, they evaluated three columns of increasing polarity, DB-1, DB-5 and DB-1701. Degradation reactions of all diol oxidation products were detected during GLC by the appearance of many small peaks. Therefore, TMS derivatization was performed, and resolution of TMS-ethers was best on DB-1. Among the ten compounds tested, baseline resolution was achieved between each major oxidation product except for 7α-OH and 4α-OH, and the triol and 7-keto. Oxides within each pair were slightly fused to each other. They tested the applicability of capillary GLC for quantification of major COPs by checking the response linearity with 5α-cholestane as the IS. They discovered excellent linear response when a fixed amount of IS was tested against various amounts of each sterol after derivatization. In two subsequent studies, Park and Addis (1986a,b) adapted this method with slight modifications to determine the rate of COP formation in tallow heated under several conditions. Overnight room-temperature saponification was used to remove triglycerides and recovery studies showed that the alkali-sensitive COPs, 7-keto and epoxides, were not affected.

The work done by Nourooz-Zadeh and Appelqvist (1987, 1988, 1989) also employed a similar polarity capillary GLC column except their sample clean-up and enrichment method did not allow them to quantify residual cholesterol in food samples. Briefly, 5α-cholestane was used as the IS and food samples were extracted by hexane/isopropanol. The lipid extract was applied onto a Sep-Pak silica cartridge to remove cholesterol and triacylglycerols. The more polar lipids were applied to a gel filtration column (Lipidex 5000) and fractions of different lipid classes were collected. The fraction that contained COPs was passed through another ion-exchange column (TEAP-Lipidex). Some COP fractions were analyzed after TMS-derivatization. Others, such as the cholesteryl esters and the triol fractions, were saponified before derivatization and GLC analysis. Because of the extensive clean-up and enrichment done to the samples, GLC time was reduced and much less interference was observed when compared to the chromatogram of Park and Addis (1985b). However, a small amount of base fusion was still seen between 7-keto and 25-OH.

Both Park and Addis and Nourooz-Zadeh and Appelqvist mentioned that complete separation by capillary GLC of all relevant COPs is not easily achieved. Yet, both methods, with careful manipulation of GLC operating conditions and capillary GLC column parameters, separated eight important COPs. These COPs included the epimeric 7-OHs, the isomeric 5,6-epoxides, 20- and 25-OH, triol and 7-keto. In both methods, the authors performed recovery and response linearity studies on each COP. To confirm the identity of the resolved TMS-ethers, mass spectrometric analyses were done and the data reported. The authors of both methods also subsequently

applied their methods to measure COPs in many different foods. In this way, their methods were thoroughly tested in their laboratories.

In studies by Nourooz-Zadeh and Appelqvist (1987, 1988, 1989) and Kou and Holmes (1985), it was found that most COPs are found in foods that have been dried or extensively heat treated. Exposure of both cholesterol and triglycerides to frying and deep-fat frying, for example, has been shown to cause extensive changes, including oxidation, in both compounds (Bascoul, 1986; Park and Addis, 1986a, b). In the United States, a large portion of fats and oils is used in the preparation of fried foods. Due to consumer demands, the fast food industry has taken steps to switch from animal to vegetable fats in its frying operation (Haumann, 1987). McDonald's Corporation and Burger King Corporation changed from an animal-vegetable oil shortening blend to an all-vegetable blend during 1986 (Haumann, 1987). Both food chains, however, continue to fry french fries in a shortening containing some tallow to produce a beefy flavor. Consequently, information is needed regarding lipid oxidation (cholesterol and/or triglyceride) in frying media containing either vegetable or animal fats and on how oxidation can be delayed.

Triglyceride Oxidation at High Temperatures and its Detection

The chemistry of lipid oxidation at high temperature is complex since both thermolytic and oxidative reactions are simultaneously involved. These reactions change the physical (Thompson, 1967), chemical (Gere, 1982) and sensory (Frankel et al., 1985) properties of fats and oils. Frankel (1980) reviewed analytical methods used in the study of autoxidation processes and the mechanisms for hydroperoxide formation and their subsequent decomposition into complex mixtures of volatile and non-volatile

secondary products. Nawar and Witchwoot (1980) and Nawar (1985) reviewed autoxidation of fats and oils at elevated temperatures. They reported data obtained from thermal decomposition of saturated and unsaturated model systems of fatty acid esters and glycerides. They found that the formation and destruction of hydroperoxides was extremely rapid at high temperatures and that the resulting primary decomposition products were unstable at those high temperatures and rapidly underwent further oxidative decomposition. As a result, although the primary oxidative events appear to be the same over a wide temperature range, correlations between the pattern of end products and classic carbon-carbon cleavage of the expected hydroperoxide intermediates diminishes as the temperature of oxidation or the degree of unsaturation increases. The number of compounds that have been identified in thermally oxidized lipids is in the hundreds (Chang et al., 1978; Frankel, 1982). They include hydrocarbons, aldehydes, ketone, acids, esters, lactones, dimers and polymers.

No one analytical technique can separate all the compounds present in oxidized oils. Some researchers have studied one type of secondary oxidation product. Waltking et al. (1975) studied polymeric products in oils heated at 185 C for up to 40 hr. They examined a gel permeation chromatography (GPC) procedure, a GLC procedure, and an iodine value procedure and found that the iodine value of the heated fat and the amount of GLC-retained material provide a good estimation of polymeric products measured directly by the gel filtration chromatography procedure. Rojo and Perkins (1987) examined the formation of monomeric cyclic fatty acids in a model system in which partially hydrogenated soybean oil was heated intermittently for 80 hr at 195 C. An elaborate scheme involving

fractionation of methyl esters by GLC, preparation of fatty acid methyl esters (FAMEs), microhydrogeneration of FAMEs, urea fractionation of hydrogenerated FAMEs, analysis by capillary GLC and structural characterization by GC-MS were developed. Their results showed that a confirmatory step such as MS is needed in methods that study the structure of cyclic monomers due to the diversity of interfering substances present.

If the objective of a study were to determine a rejection point for used frying fats, a procedure that compares the fresh frying fat to the heat abused fat is needed. Dobarganes and Perez-Camino (1988) devised a procedure for quantitating oxidized material in heated fats. They fractionated fresh and heated oils into polar and non-polar triglyceride, and quantitated the methyl esters from polar triglycerides (indicative of hydrolytic degradation), non-polar dimers (indicative of thermal degradation) and total methyl esters of fatty acids (indicative of oxidative degradation). They concluded that criterion for discarding heated fat drawn from these analyses is more suitable than the accepted criterion that recommends replacing the fat when its level of polar compounds is higher than 27%. Such criterion is based on a nonlinear correlation with the percentage of oxidized fatty acids and is not objective enough.

Perkins and Pinter (1988) attempted to "profile" the oxidized products of heated fat on a reverse-phase HPLC column, by investigating various methods of extraction and concentration. They found that a batch type counter current distribution method using acetonitrile/hexane was the most effective in concentrating the polar products and removing of palmitate and stearate from heated fat. The HPLC procedure then partially separated the

concentrated oxidized material. The resultant chromatogram could be useful in assessing the quality of the used fat.

Frequently, triglyceride oxidation is studied for the purpose of evaluating the effectiveness of certain additives as antioxidants. Therefore, indirect measuring methods are sufficient. Peled et al. (1975) studied the effect of water and BHT on the stability of cottonseed oil during frying. Changes caused by heating were evaluated through determination of peroxide, acid and iodine values by using the standard American Oil Chemists' Society (AOCS) methods. They also examined the extinction at 232nm (reflects an increase in the formation of conjugated dienes as the methylene interrupted double bonds of unsaturated fatty acids isomerize upon heating) and 460nm (reflects a darkening of heated fat), and a change in the fatty acid composition. They found that all changes were less pronounced when the frying was carried out in the presence of either nitrogen or water, whereas BHT had no delaying effect on the determination of the oil. Ishikawa et al. (1984) studied the antioxidant and synergistic effect of a natural compound, flavoglaucin. This compound was shown to be stable at 180 C and it reduced the total carbonyl and acid values of the heated oil.

All in all, the complexity of triglyceride oxidation in foods precludes its evaluation by any one single method. Therefore, the method(s) of choice largely depends on the purpose of the study.

Antioxidants

Sherwin (1976, 1978) and Buck (1981) described most antioxidants used by the food industry. A few of the phenolic compounds such as BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), propyl gallates

(PG), tertiary butylhydroxy quinone (TBHQ) and tocoperols have the ability to lengthen the oxidation induction period by inhibiting the free radical chain reactions. They are permitted for use alone or in combination with each other as antioxidants in foods. These antioxidants are very effective at room temperature; however, at deep frying conditions, they can be steam distilled or destroyed and have little carry-over effect (Peled et al., 1975). Martin (1953) and Freeman et al. (1973) studied the antioxidative effects of polydimethyl siloxane (MS). It is known to suppress foaming in aqueous solutions. During frying, MS is thought to indirectly inhibit oxidation by suppressing the accumulation of foam-promoting oxidation products such as free fatty acids and food exudates. Freeman et al. (1973) tested sunflower seed oil heated to 180 C with up to 1ppm MS. Between 0.02 and 1ppm of MS, the oxidation rates of the protected oil were very similar, and significantly lower than in the control. They concluded that MS gave its full protective effect at 0.02ppm. They also discovered that the solubility of MS in oil is low and that any excess adheres onto the surface of fried food. More importantly, Rock and Roth (1967) showed that MS can be both an antioxidant and a prooxidant at 2ppm in frying fats, depending upon the method of heating. When fat was heated in a fryer at 375 F, MS acted as an antioxidant. When fat was heated and maintained at 375 F in the oven, MS was a prooxidant. Consequently, MS should be added and treated at a controlled manner.

Most approved antioxidants that are added to foods are synthetic. The intense interest of consumers in natural food products, as well as the interest of researchers to study how lipids are protected in their natural environment lead to a great deal of research in the area of natural

antioxidants. Houlihan and Ho (1985) recently prepared an extensive summary on the identification and application of natural antioxidants, but most of those mentioned are useful mainly at room temperature. Among the antioxidants not reviewed by Houlihan and Ho is a group of plant sterols studied by Sims et al. (1972), Boskou and Morton (1976), Gordon and Magos (1983) and White and Armstrong (1986). Sims et al. (1972) found that the unsaponiables from olive, corn, wheat germ and Vernonia anthelmintica oils could protect safflower oil from oxidative polymerization during heating at frying temperature. Specifically, vernosterol, Δ^7-avenasterol and citrostadienol (Figure 1) were the effective agents. Boskou and Morton also found that pure Δ^5-avenasterol reduced oxidation in cottonseed oil at frying temperatures. When heated in the oil, α-sitosterol was ineffective initially, but became slightly prooxidant after prolonged heating. Gordon and Magos (1983) also found Δ^5-avenasterol and fucosterol to be effective antioxidants in a triglyceride mixture similar in composition to olive oil.

All the sterols shown to be effective at preventing oxidation at frying temperatures have an ethylidene group in their side chain. Gordon and Magos (1983) proposed that the ethylidene side chain (Figure 1) reacts rapidly with lipid free radicals to form stable allylic tertiary free radicals that interrupt the oxidation chain. The ethylidene side chain forms free radicals rapidly due to the presence of unhindered hydrogen atoms on an allylic carbon atom.

Burton and Ingold (1984) studied β-carotene as an unusual type of antioxidant. They reviewed the mechanisms of lipid autoxidation and the ways in which conventional antioxidants work. The peroxide-decomposing type of antioxidants reduce hydroperoxides to the corresponding alcohol or

catalytically decompose it to nonradical products. Enzymes such as catalase and peroxidase belong to this catagory. The chain-breaking antioxidants, generally phenols or aromatic amines, are able to trap peroxy radicals. The resultant phenoxyl radical is resonance stabilized and relatively unreactive. Burton and Ingold suggested that there is a third type of antioxidant class to which β-carotene belongs and called it a radical-trapping type. In in vitro experiments, β-carotene was very effective in reducing the oxidation rate of substrates like methyl linoleate at very low oxygen pressures (e.g., 15 torr). At higher oxygen pressures, β-carotene lost its antioxidant activity and had an autocatalytic, prooxidant effect, particularly at relatively high concentrations. They believe that the mechanism of its antioxidant action through the formation of a resonance-stabilized, carbon-centered radical which is probably formed by the addition of a peroxyl radical to the conjugated system of β-carotene. Terao (1989) looked at the antioxidant activities of four carotenoids including β-carotene. His results suggest that the presence of oxo groups at the 4 and 4′ position of the β-ionone rings enhances the antioxidant activities of carotenoids. It is possible that the electron-withdrawing character of the oxygen atoms substantially reduces the unpaired electron density on the carbon skeleton, resulting in the decreased reactivity of the carbon-centered radical toward molecular oxygen.

The theory of Gordon and Magos (1983) and the theory of Burton and Ingold (1984) are similar. Compounds involved in both theories do not have phenolic structures. Instead, their antioxidant activities are believed to be the result of the formation of a stable carbon-centered radical which

interrupts the chain reaction. However, the plant sterols are effective at high temperatures and the carotenoids are effective at low oxygen pressure. Although Burton and Ingold (1984) did not test the antioxidant effect of β-carotene at high temperatures, they did note that triphenylmethane had antioxidant activity via mode of action similar to β-carotene. Those data were from a study by Hendry and Russell (1964) who noted triphenylmethane was more effective at higher temperature (90 vs 60 C) and lower oxygen pressure (300 vs 710mm Hg). Finally, carotenoids have a conjugated double bond system to delocalize the free radical. This is paralleled by a similar observation of Gordon and Magos (1983). They found that sterols having one or more endocyclic double bonds in addition to the ethylidene group are the most effective antioxidants, possibly because those double bonds create other sites for free radical formation and delocalization.

The present study was undertaken to further explore the high-temperature antioxidant activity of compounds with an ethylidene-like group. Compounds containing the ethylidene group but without the sterol moiety were chosen to further pinpoint the structure responsible for the antioxidant activity. Compounds fulfilling these requirements include a group of monoterpene alcohols. Plant sterols and β-carotene are all terpenoids. In the current study, linalool and linalyl acetate were selected. A literature review showed that these compounds are used mainly in the perfume and flavoring industry (Kogami et al., 1967; Morin and Richard, 1985). The high-temperature antioxidant effect of linalyl acetate and MS on SBO was presented and published as an abstract in the 1987 American Oil Chemists' Society Annual Meeting (Yan and White, 1987). In 1989, J. M. Willemse of the Lever Brothers Company, N.Y. received a patent

on the effectiveness of silicones, linalool or linalyl acetate (or their mixtures) in lowering the development of unpleasant flavor when fat and oil products were subjected to prolonged heat treatment above 120 C. Their methods of evaluation included sensory evaluation of the odor intensity of the heated fat, and chemical evaluations (peroxide value, anisidine value, percentage free fatty acid and decomposition point) of fresh and heated fat. Therefore, their data and the data from the present study describing the effect of linalyl acetate on changes in the fatty acid composition and conjugated diene percentage of SBO heated at 180 C, will be looked at.

SECTION 1. LINALYL ACETATE AND OTHER COMPOUNDS WITH AN ETHYLIDENE GROUP AS ANTIOXIDANTS IN HEATED SOYBEAN OIL

Abstract

Researchers have theorized that the antioxidant activity at high temperatures of some plant sterols is caused by the presence of an ethylidene side chain. In the present study, linalyl acetate and undecylenic acid were studied to determine the feasibility of this theory. All compounds to be tested were added to soybean oil and heated to 180 C for 56 to 70 hr. Fatty acid changes and conjugated diene formation were monitored. Acetylation of linalool to linalyl acetate (LA) caused the formation of many by-products, which were partly chromatographed into three bands. The materials isolated from the bands were tested and found to be equally effective antioxidants. Purchased LA had a similar effect. The LA materials from the bands were further purified and identified by GC-MS and by NMR. All the effective compounds were similar in structure to LA. Undecylenic acid provided some protective effect but less than that of LA, which had less antioxidant effect than Δ^7 avenasterol and polydimethyl siloxane.

Introduction

A large portion of fats and oils consumed in the United States each year is used in the preparation of fried foods. During deep-fat frying, the fat is exposed to light, elevated temperature and atmospheric oxygen. Fritsch (1981) described the complex decomposition pattern that is formed as a result of superimposing both thermolytic and oxidative reactions. Sherwin (1978) reported many of the aspects of thermal

oxidation of lipids. It is widely accepted that unsaturated fatty acids are much more susceptible to oxidation than their saturated analogs as summarized by Gere (1982). The principal reactions are via the formation and decomposition of hydroperoxide intermediates through a free-radical process. Certain phenolic compounds are able to inhibit such free-radical chain reactions and thus lengthen the oxidation-induction period. Sherwin (1976) and Buck (1981) described antioxidants used by the food industry. A few of the phenolic compounds such as BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), propyl gallates, and tocopherols are permitted for use alone or in combination with each other as antioxidants in foods. These antioxidants are very effective at room temperature; however, at deep-frying conditions, they can be steam-distilled or destroyed and have little carry-over effect (Peled et al., 1975). Martin (1953) and Freeman et al. (1973) studied the antioxidative effects of polydimethyl siloxane (MS). It is known to suppress foaming in aqueous solutions. During frying, MS is thought to indirectly inhibit oxidation by suppressing the accumulation of foam-promoting oxidation products such as free fatty acids and food exudates.

Most approved antioxidants added to foods are synthetic. The intense interest of consumers in natural food products has broadened the market for naturally derived antioxidants. Houlihan and Ho (1985) recently prepared an extensive summary on the identification and application of natural antioxidants, but most of those mentioned are useful mainly at room temperature. Sims et al. (1972), Gordon and Magos (1983), and White and Armstrong (1986) demonstrated the effects at frying temperatures of plant-sterol antioxidants. Gordon and Magos

(1983) proposed that an ethylidene side chain (Figure 2) on the effective sterols reacts rapidly with lipid free-radicals to form stable allylic tertiary free-radicals that interrupt the oxidation chain. The ethylidene side chain forms free-radicals rapidly due to the presence of unhindered hydrogen atoms on an allylic carbon atom.

The purpose of the present study was to determine whether the high-temperature antioxidative activity of the sterols is caused by the presence of the ethylidene side chain. Compounds containing the ethylidene group (linalool and products of its acetylation) or a similar structure with an unhindered allylic carbon atom (undecylenic acid) were tested for antioxidant activity in heated soybean oils.

Experimental Procedures

Materials

Oils. Two separate batches of refined, bleached, and deodorized, soybean oil (SBO) were obtained from a commercial refining operation. Citric acid (CA) was used during the processing, but no additives were included.

Before the heating tests, peroxide values (PVs) for all the batches of oil were determined according to the AOCS method Cd 8-53 (1983). PVs of 0.0-0.3 were obtained.

Antioxidants. Linalool and MS were purchased from Sigma Chemical Co., St. Louis, MO. Linalyl acetate (LA, Figure 2) was purchased from Aldrich Chemical Co. (Milwaukee, WI). Delta-7 (Δ^7, Figure 2) avenasterol was a gift from Dr. J. Fioriti and had been prepared according to his reported procedure (Fioriti et al., 1971).

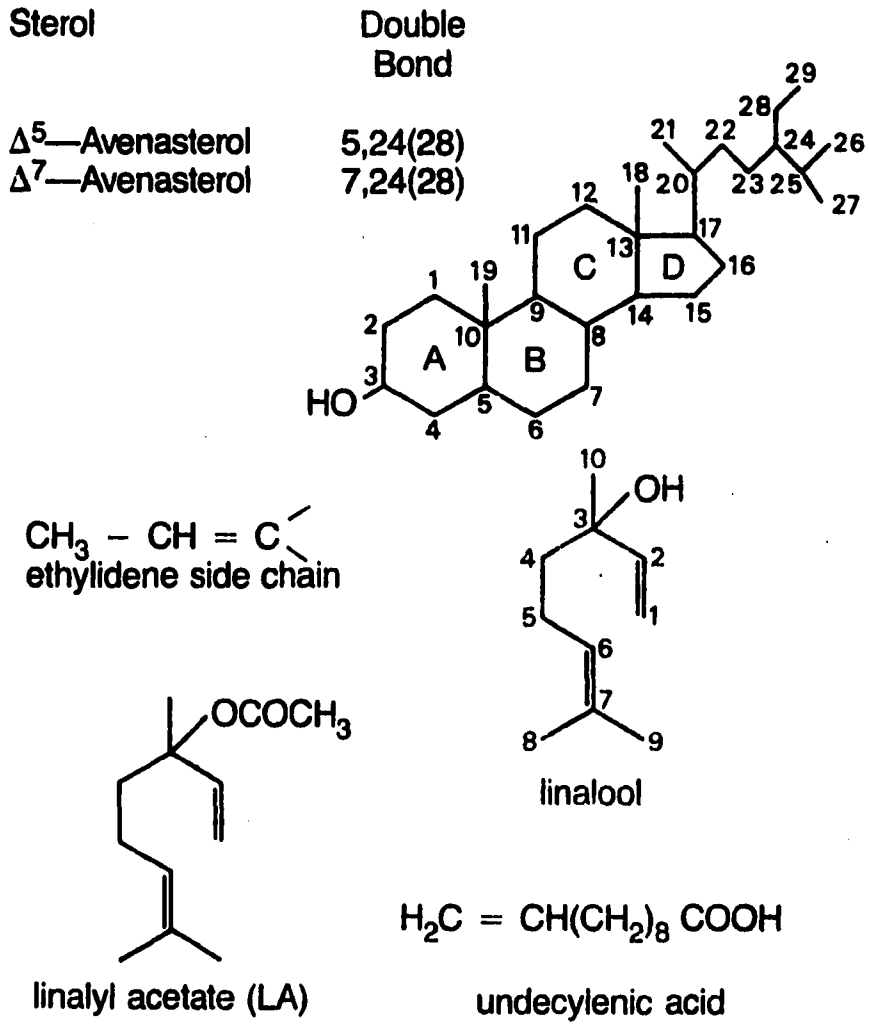


Figure 2. Chemical structures of compounds containing an ethylidene group.

Linalyl acetate also was synthesized in the laboratory by adapting the acetylation procedure from the AOCS method Cd 4-40 (1983). Thin-layer chromatograpy (TLC) revealed four major spots with some minor spots after the completion of the acetylation step. The labile nature of linalool and LA under acidic conditions and elevated temperatures as reported by Morin and Richard (1985) likely contributed to the number of spots. The material later was chromatographed by preparative TLC (0.5 mm Silica G Uniplates, Alltech Associates, Newark, DE) by using hexane/diethyl ether (95:5) as the developing solvent. The spots were designated as the lower, middle and upper band. The fourth major spot was very close to the origin and had an Rf similar to that of linalool. Undecylenic acid was purchased (Sigma Chemical Co., St. Louis, MO) and used without additional purification.

Heating tests

SBO samples (60 g), with and without the various additives, were heated in 100-ml Pyrex beakers at 180±5 C for 7 hr each day for five to ten days. Samples were cooled to room temperature between days. Aliquots were removed at 4 hr on day 1 and then at the end of each day of heating and stored under nitrogen at -18 C until analyzed.

In Test I, the materials isolated from the lower, middle and upper bands from TLC of the laboratory-prepared linalyl acetate (LA-LP) were heated in SBO at a level of 0.05% each. Linalool alone (97% pure), added to SBO at 0.05%, and a control SBO containing no additives also were tested. The breakdown of LA was followed by monitoring the GC-MS and GC of a commercial source of LA (LA-C) heated in naphthalene. In Test II, undecylenic acid was heated in SBO at levels of 0.0 (control),

0.5, 1.0, 2.0, and 4.0%. The same batch of oil was used in Tests I and II.

Several different antioxidants were added to SBO, heated and compared in Test III. A known antioxidant (Δ7 avenasterol) was tested at 0.02%, MS was tested at 0.3 ppm, and LA-C was tested at 0.02 and 0.04%. A control SBO with no additives was heated for comparison. The lower band from LA-LP of Test I was further purified by gravity-flow column chromatograhy (CC) (Silica gel 60-100 mesh, Davisil, Aldrich Chemical Co., Milwaukee, WI), and the resulting material tested at 0.02%. Its purity was determined by using analytical TLC. A second batch of refined, bleached and deodorized SBO from the same source as in Tests I and II was used in Test III.

Analysis of heated oils

Gas-Liquid Chromatography. A Varian Aerograph series 3700 Gas-Liquid Chromatograph (GC) equipped with a flame ionization detector was used. The method of Metcalfe et al. (1966) was followed for the preparation of fatty acid methyl esters (FAMEs). The GC contained a stainless-steel packed column (100/120 Gas Chrom Q II with 10% Silar 10C coating; Alltech Associates, Deerfield, IL) of 6.0ft x 0.085in. Peak areas were measured with the internal standard procedure of a Hewlett Packard (HP) 3390A reporting integrator. Triheptadecanoin was added to all the samples as an internal standard (IS). This method of measurement was suggested by Waltking and Zmachinski (1970) to be the preferred method in determining total polyunsaturated fatty acids. The fatty acid data reported in the current study list the percentage

retention for each fatty acid over the heating time, based on the amount at time zero. All test results are the average of duplicate samples.

The same GC also was used for following the degradation of LA-C heated in naphthalene. A DB-5 capillary column (30m x 0.25mm, 1.0 micron film thickness; J&W Scientific, Inc., Rancho Cordova, CA) was used. The column was temperature programmed at 10 C/min from 80-250 C. The injection port temperature was set at 200 C and later at 250 C to compare LA degradation at two injection port temperatures.

Ultra-violet Spectrometry. Conjugated dienoic acids (CD) were measured by using AOCS method Ti 1a-64 (1983). All test results are the average of duplicate samples. A Gilford model 240 Spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, OH) was used.

Gas Chromatography-Mass Spectrometry (GC-MS). A Finnigan 4500 GC-MS with a DB-1 fused silica capillary column (30m x 0.25mm, 1.0 micron film thickness) was used to identify the chemical components of LA-LP fractions. The mass spectrometer was linked to a 1984 Revision A NBS library of more than 38,000 chemical compounds. The injection port temperature was set at 250 C. The column was temperature programmed at 10 C/min from 80-250 C, with helium as the carrier gas.

Nuclear-Magnetic Resonance (NMR). The structures of the synthesized compounds were either determined or partially characterized by 300 MHz ^{1}H NMR, 75.46 MHz ^{13}C broad-band (BB) decoupled and ^{13}C gate-decoupled NMR in $CDCl_3$. The instrument used was a NMC-1280 with a NIC 293C Programmable Pulser from Nicolet Magnetics Corp., Fremont, CA.

Results and Discussion

Test I

Linalool is a nonsterol compound containing an ethylidene group (Fig. 2), and it is found in large amounts in herbs such as basil and coriander (Heath, 1985). Herbs and spices are known sources of natural antioxidants. The ethylidene group in linalool is present at C6 and C7. The allylic proton in linalool, like that in effective plant-sterols, is relatively unhindered and thus accessible for oxidation. When linalool was tested at 0.05% in heated SBO, it was shown to be slightly prooxidative when compared with the control. The data are not presented here. This prooxidative effect possibly was caused by the reactive tertiary alcohol group at C3. Its reactivity stems from the stable tertiary carbocation that is formed upon heterolysis of the protonated alcohol that is formed during an acid/base reaction. The carbocation can readily lose a proton to a base that then yields an alkene, which can also undergo other reactions to form a host of hydrocarbon byproducts. To reduce the reactivity at C3, a protective group (acetate) was added to produce LA by using the AOCS method Cd 4-40 (1983). Adding an acetyl group to linalool also increased the b.p. of linalool from 196 C to 220 C, which could have an advantage in a frying oil.

The products resulting from acetylation of linalool were separated by TLC into four major spots with some closely eluting minor spots. The intensity of the spots varied according to the reaction conditions. By preparative TLC, the mixture eluted into four major bands, and they were referred to as the upper, middle, lower and lower-lower (LL) bands. The

materials eluted from the top three bands were tested at 0.05% in SBO heated to 180±5 C for 56 to 70 hr. The LL band was not tested because its Rf was similar to that of linalool and it was presumed to be such.

The percentage of CD and the percentage retention of 18:1, 18:2,and 18:3 determined in the heated oils over the heating period are shown in Figures 3 and 4, respectively. The three fractions were similarly effective in minimizing the deterioration processes of SBO at frying temperature. The SBOs containing the LA fractions produced considerably less %CD and retained much more 18:2 and 18:3 over the heating period when compared with the control SBO. Even retention of 18:1 was better in the treated SBOs.

Attempts were made to separate the LA bands into purer compounds for testing in oils by using additional preparative TLC, but closely eluting spots still were not separated. In addition, purification of the compounds was tried by using high-vacuum fractional distillation, but the compounds were too close to each other in b.p. and their viscous nature caused cross contamination.

GC-MS identification. By repeatedly rechromatographing the LA bands on preparative plates (0.50mm in thickness), the purity of the bands was somewhat improved. These were used in GC-MS and NMR determinations of LA-LP fractions. The fragmentation pattern of the lower-band material came closest to that of geranyl acetate with a fit of 0.73. Geranyl acetate and LA are allylic isomers. In geranyl acetate, the double bond is shifted to C2, and the alcohol group is moved from C3 to C1. It is likely that such allylic rearrangement occurs during GC-MS analysis. The library search on the middle-band

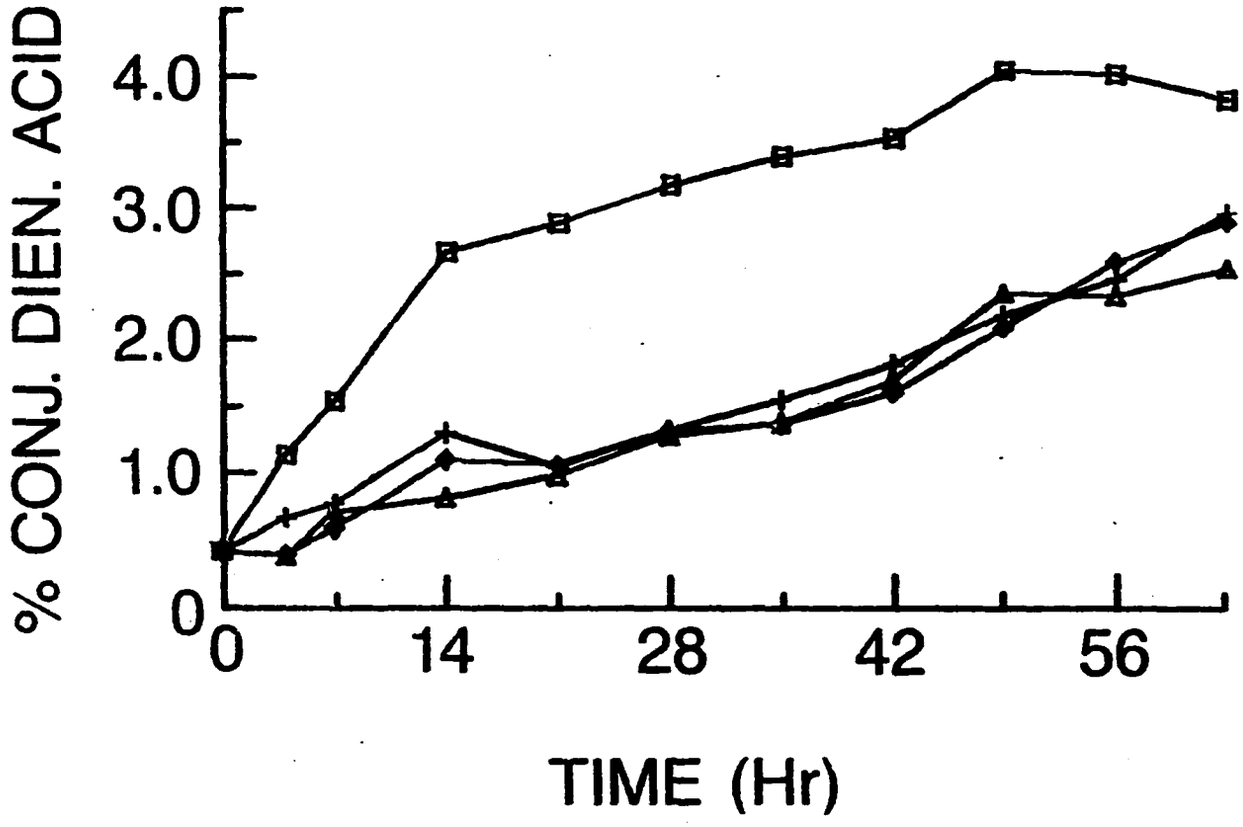


Figure 3. Percentage of conjugated dienoic acid in SBO protected with 0.05% of LA-LP fractions: (□) control, (+) lower, (◇) middle, (△) upper, (TestI).

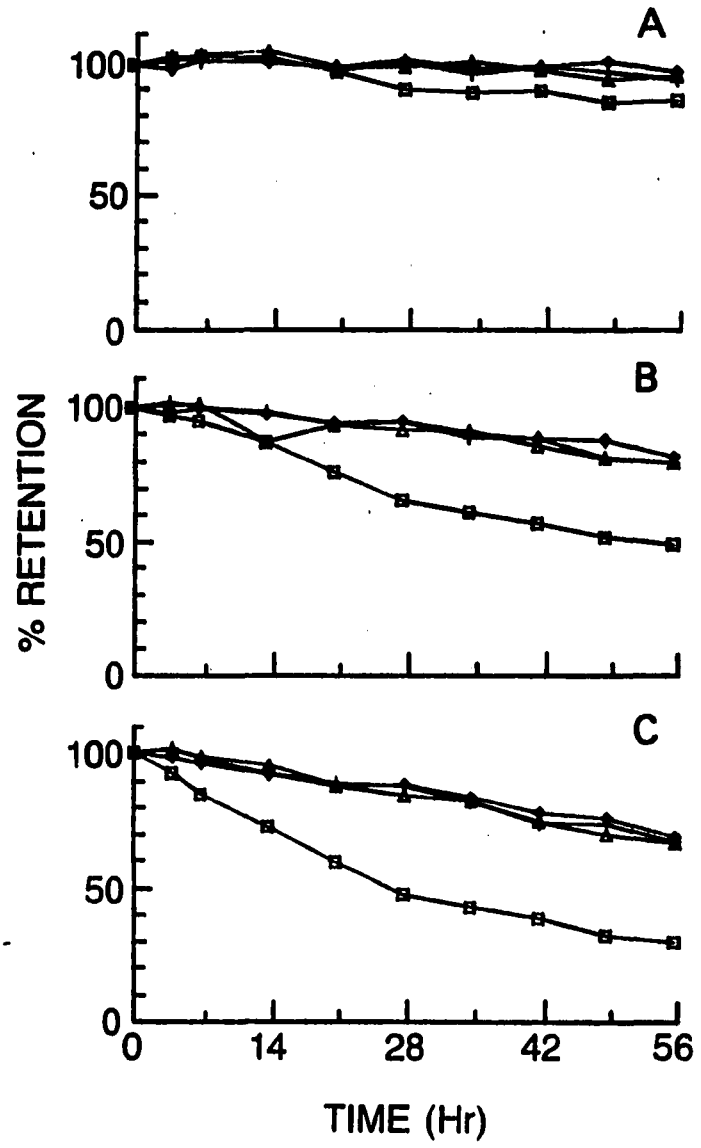


Figure 4. Percentage retention of 18:1 (A), 18:2 (B), and 18:3 (C) in SBO protected with 0.05% of LA-LP fractions: (□) control, (◇) lower, (+) middle, (△) upper, (Test I).

material did not reveal any closely fitting compounds. The upper-band material was not pure enough for interpretation of GC-MS results. The MS spectra of the eluting peaks from both the middle and upper bands had molecular ions higher than that of LA. That they also eluted higher on the TLC plates suggests that the compounds may be dehydrated and/or dimerized.

Morin and Richard (1985), Valenzuela and Cori (1967) and von Rudloff (1961) reported that terpene alcohols and esters, undergo elimination and rearrangement reactions when they are subjected to intense heat, steam distillation and/or acidic conditions. To test the stability of LA at deep-frying temperatures and at GC injection-port temperatures, 5 g of LA-C was added to 60 g of naphthalene. Naphthalene was chosen as the heating medium because it has a high b.p. and because the LA-C/naphthalene mixture could be directly injected onto the GC column. The mixture of LA-C and naphthalene was heated to 180±5 C. Samples were collected at 0, 4, and 7 hr of heating. These heated samples plus unheated LA-C were chromatographed by GC on a DB-5 capillary column as described earlier. The unheated LA-C sample was run at injection port temperatures of 200 and 250 C. At 200 C, there were one major peak (LA) and six minor peaks. At 250 C, the major peak decreased in size, the six minor peaks increased in size, and two new ones were observed. This shows that even a slight increase in injection-port temperature causes an increase in LA degradation. For LA heated in naphthalene, the injection-port temperature was kept at 200 C to reduce degradation during GC analysis. As heating time increased, the LA peak decreased while the minor peaks grew steadily. By 7 hr of

heating, the major peak was no longer LA. From these results, it is evident that LA does degrade with time and temperature.

To gain some insight into the nature of the compounds formed from LA during heating, the unheated LA-C and the 7-hr sample were subjected to GC-MS in the Finnigan 4500 under operating conditions described previously. Interestingly, the unheated LA-C was not matched to LA by a library search. The first choice fit for LA-C was geranyl acetate with a fit of 0.73, again indicating that LA isomerizes to form geranyl acetate in GC injection ports. The heated LA-C sample yielded eight peaks (not including the naphthalene peak) and six of these were library searched. Three peaks were matched to compounds that had MWs that were one acetate group less than LA and their fits ranged from 0.88-0.89. The LA peak and two other peaks were all matched by the search to geranyl acetate. Their fits ranged from 0.73-0.81. Finally, all the compounds that were matched to the peaks resulting from LA breakdown had an ethylidene side chain and at least one other double bond. Therefore, one can postulate that although LA is not stable at the heating conditions described here, its beginning degradation products may still possess high-temperature antioxidant activity.

NMR identification. Proton magnetic resonance (PMR) gives information on the chemical environment of the proton. The ^{13}C BB decoupling technique removes any coupling between protons and carbon atoms and thus gives a sharp singlet for each carbon atom. Therefore, the number of peaks present and their chemical shifts suggest the number of symmetrical carbons in the compound as well as the sp^1 and sp^2 characteristics of the carbon nuclei. The gated decoupling technique

helps in determining the number of protons on each of the carbons when the resultant splitting pattern is compared with that of the BB decoupling spectrum.

The BB decoupling spectra for the material isolated from LL and the lower bands showed four carbons in the region of 110-150 ppm, thus signifying the presence of two double bonds. The gate-decoupled spectra indicated that only one of the singlets remained. Two of the singlets became doublets and one became a triplet. The singlet that remained was thus the only tertiary carbon. This, together with the presence of two doublets and one triplet in the olefinic regions signified the presence of one ethylidene group at C6 and a double bond at C1. This structure is similar to that of linalool and not geraniol. If the compound were geraniol, the same region would show two singlets and two doublets. The presence of the ethylidene group in these two fractions was further substantiated by the presence of signals at 1.6 and 1.7ppm in the PMR spectra (Figure 5), which matched very well to the LA spectrum shown in the Handbook of Proton-NMR Spectra and Data (1985). The lower-band material had an acetate group indicated by the presence of a sharp singlet at 2.0ppm and an uncoupled ^{13}C singlet at 170ppm. The LL material did not show such absorption in its spectra. Its ^{13}C BB-decoupled spectrum showed ten peaks, which indicated that the compound in this band is the starting material, linalool. It was not possible to obtain pure enough compounds from the middle and the upper bands to obtain NMR spectra with first-order splitting patterns. However, it is clear from their spectra that they did not have an acetate group.

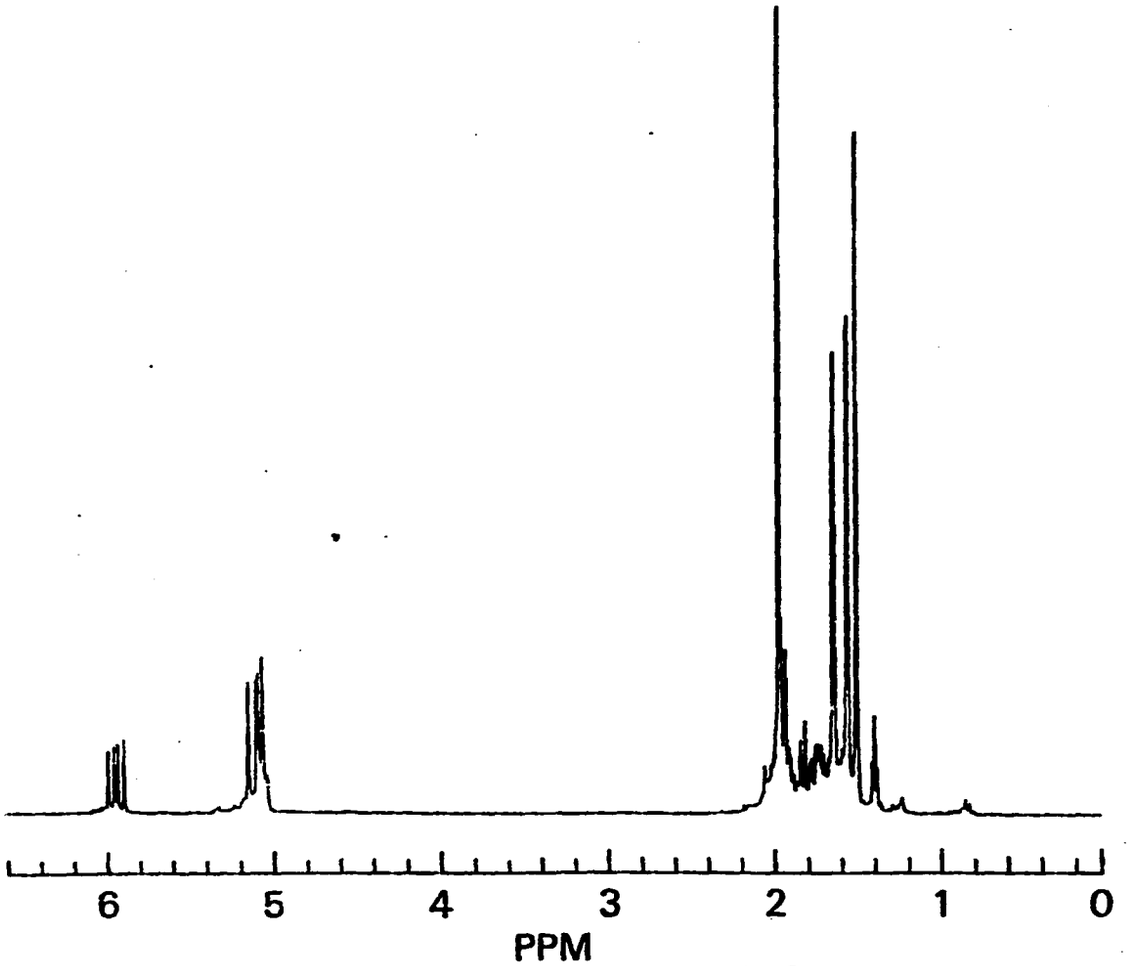


Figure 5. PMR spectrum of material isolated from the LA-LP lower band.

Test II

Undecylenic acid has a double-bond system similar to the ethylidene group in LA in that it is close to one end of the structure and thus easily accessible for possible proton abstraction. See Figure 2. It differs from LA in that the carbon involved is a secondary carbon, which in theory, should be less effective in free radical dispersal during oxidative processes. The free radical forms should be less stable; thus, undecylenic acid should show less effect than LA in SBO heated to frying temperatures. Undecylenic acid was heated in SBO at levels of 0.0 (control), 0.5, 1.0, 2.0, and 4.0%. The CD values and data on the percentage retention of 18:1, 18:2, and 18:3 are shown in Figures 6 and 7, respectively. Clearly, the antioxidant effect was concentration dependent within the range tested. The SBO had the highest %CD and least retention of 18:1, 18:2, and 18:3 when compared with SBO containing undecylenic acid. Undecylenic acid was more effective at 2.0% than at 1.0 or 0.5%. When it was tested at 4.0%, the effect was similar to that of 2.0% so those data are not shown. At 0.5%, undecylenic acid was not as effective as the LA-LP fractions from Test I. At 56 hr of heating, only 50% of the 18:3 was left in oil protected with 0.5% of undecylenic acid, whereas 70% of the 18:3 was left in oils containing only 0.05% of any one of LA-LP fractions. The effectiveness of undecylenic acid at frying temperature and the observed differences in effectiveness between it and the LA-LP fractions further point to the feasibility of the ethylidene theory in plant sterol high-temperature antioxidants.

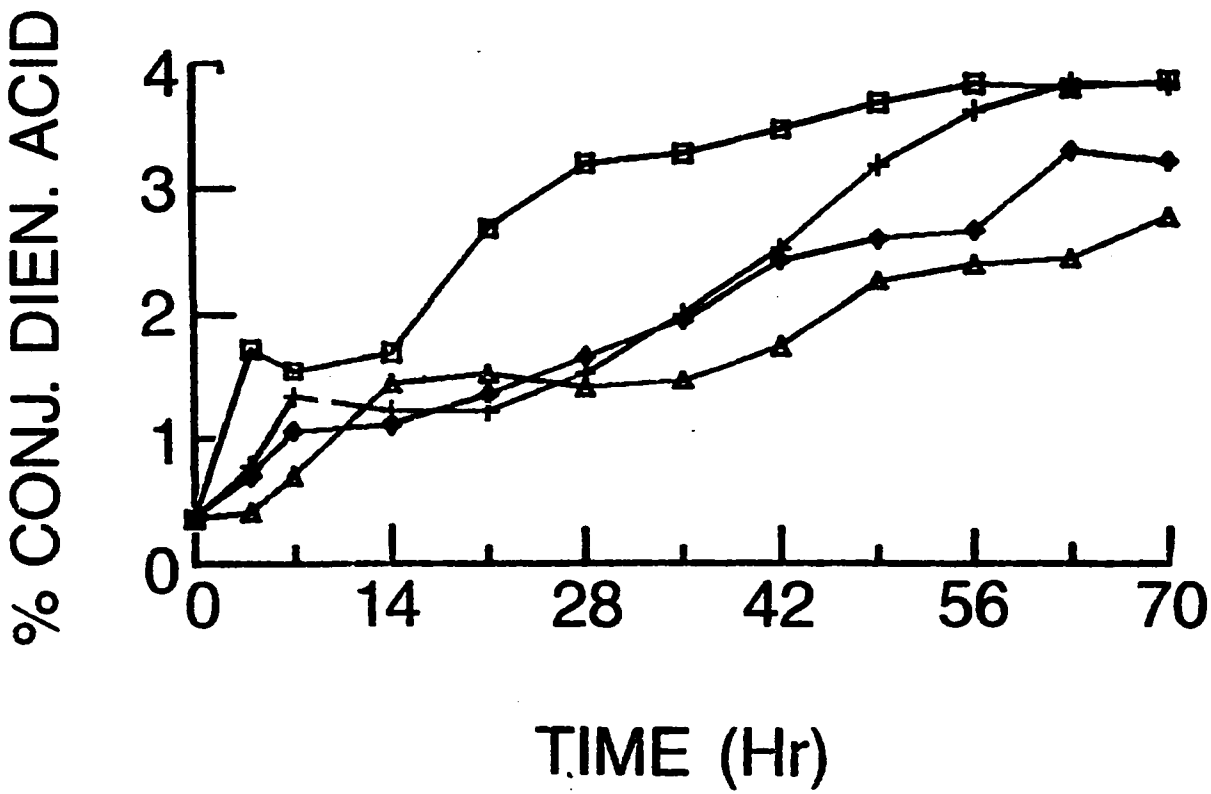


Figure 6. Percentage of conjugated dienoic acid in SBO protected with different levels of undecylenic acid: (□) control, (+) 0.5%, (◇) 1.0%, (△) 2.0%, (Test II).

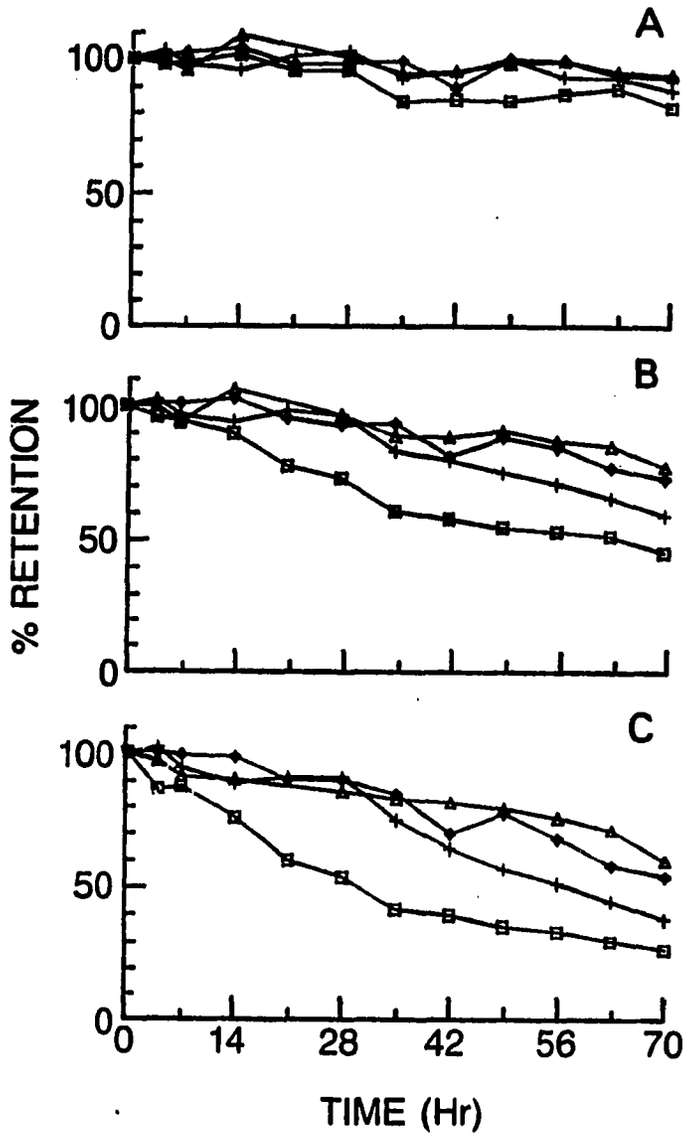


Figure 7. Percentage retention of 18:1 (A), 18:2 (B), and 18:3 (C) in SBO protected with different levels of undecylenic acid: (□) control, (+) 0.5%, (◇) 1.0%, (△) 2.0%, (Test II).

Test III

The LA-C was tested in SBO at 0.02% and 0.04% without further purification. When chromatographed by TLC, the Rf of the major spot in LA-C had the same Rf value as that of the lower band from the LA-LP. The less intense spot of LA-C had the same Rf value as that of the upper band of LA-LP. A column-purified fraction from the lower band of LA-LP was tested at 0.02%. The purification procedure was described earlier. Its PMR spectrum was close to that of geranyl acetate. One SBO sample containing 0.02% Δ^7 avenasterol and one containing 0.3ppm MS also were tested. The CD data and the percentage retention of 18:1, 18:2, and 18:3 are shown in Fig. 8 and 9, respectively. After 70 hr of heating, LA-C at 0.04% and MS were still protecting the oil, whereas the LA-LP, Δ^7 avenasterol and 0.02% LA-C had lost their protective effect. The retention of the fatty acids showed similar effects. Only about 20% of the 18:3 in the control remained at the end of the heating period. The LA-LP showed some protective effect up to 35 hr of heating, whereas Δ^7 avenasterol was still exerting some effect on 18:3 at 70 hr of heating. The samples containing MS and 0.04% LA-C retained the most unsaturated fatty acids over the heating period. A level of 0.02% LA-C was slightly effective up to about 42 hr.

The LA-C did exhibit antioxidant activity in SBO at deep-fat frying temperature. However, LA-C at 0.02% was less effective than Δ^7 avenasterol at the same concentration. The molar concentrations of Δ^7 avenasterol and LA in the SBO were about 3.0×10^{-5} and 5.5×10^{-5} M, respectively. Although a higher molar concentration of LA was present, its MW of 196 and b.p. 220 C could have caused it to be volatile at

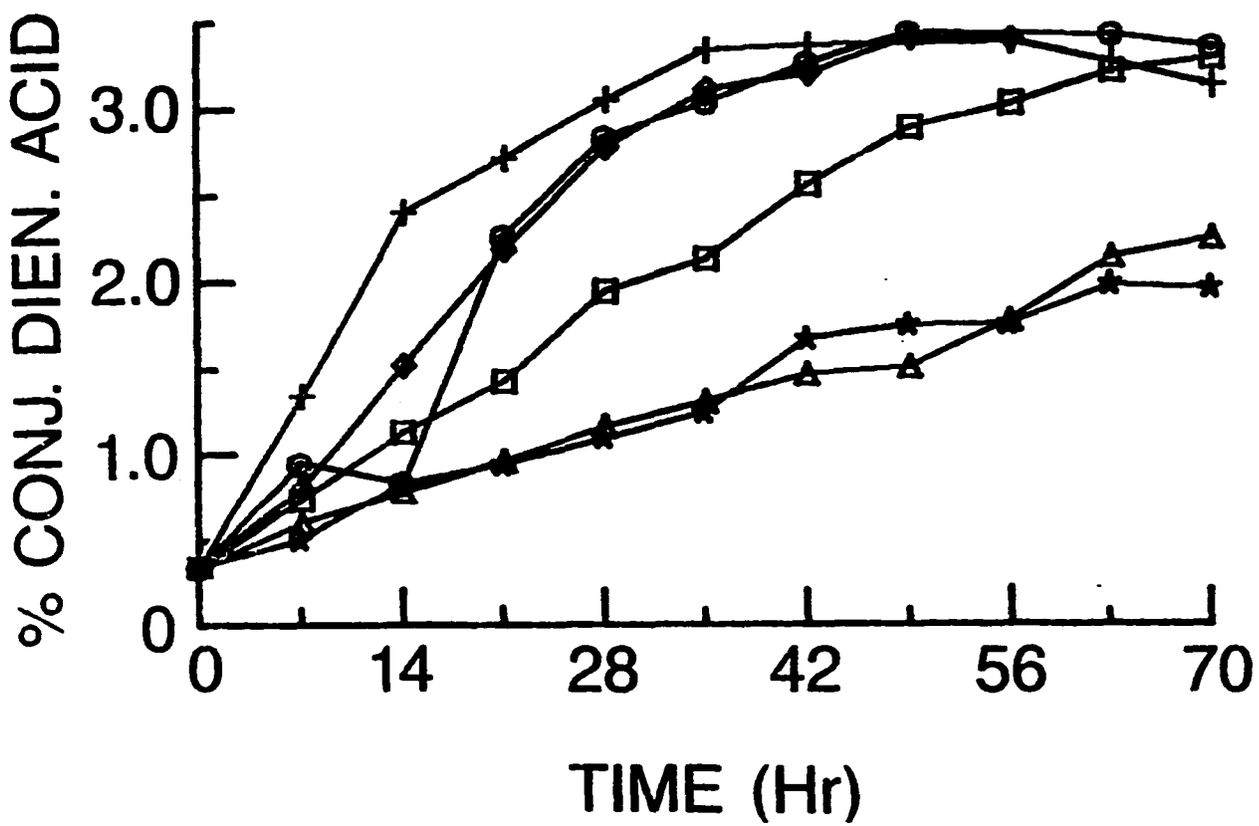


Figure 8. Percentage of conjugated dienoic acid in SBO protected with 0.02% Δ^7 avenasterol (□), 0.02% and 0.04% of LA-C (◇ and △), 0.02% of the material from the LA-LP lower band (⊙), 0.3ppm MS (✱) and a control (+), (Test III).

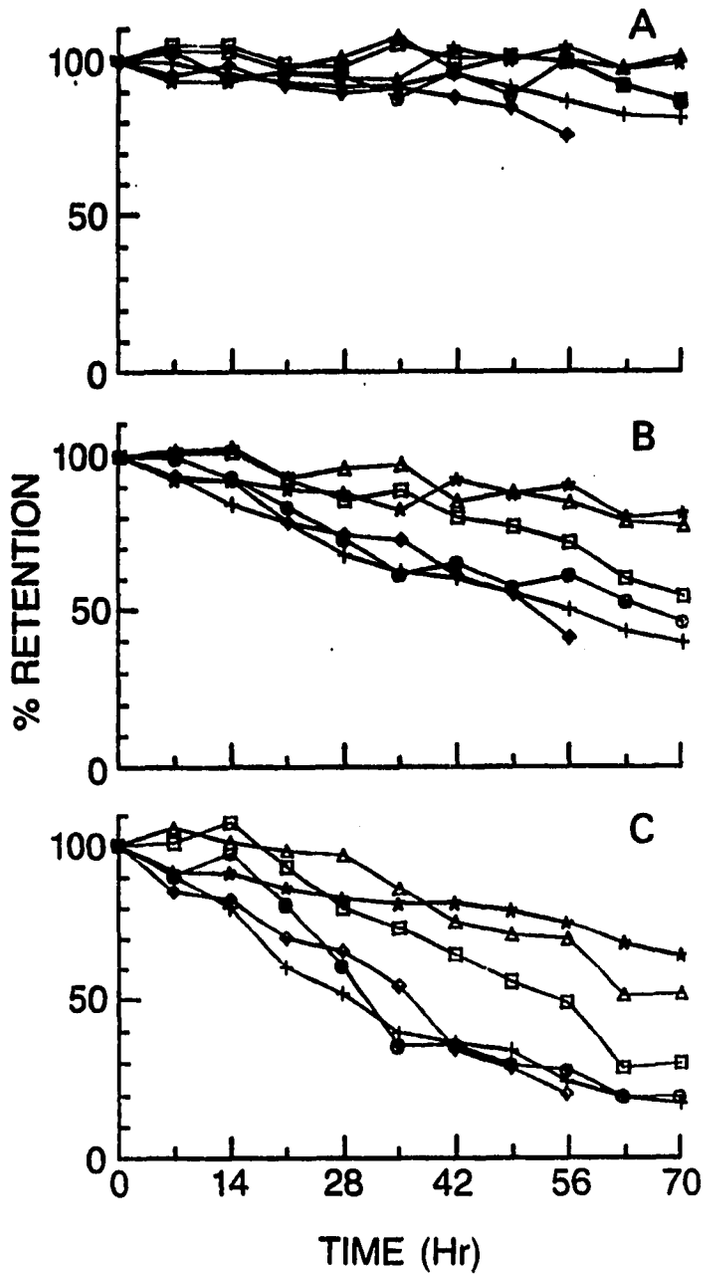


Figure 9. Percentage retention of 18:1 (A), 18:2 (B), and 18:3 (C) in SBO protected with 0.02± Δ^7 avenasterol (□), 0.02% and 0.04% of LA-C (◇ and △), 0.02% of the material from the LA-LP lower band (⊙), 0.3ppm MS (✱) and a control (+), (Test III).

frying temperature, whereas Δ^7 avenasterol (MW 412) might have remained in the SBO longer.

It is likely that the ethylidene group that is found in certain plant sterols and in LA is at least partly responsible for the high-temperature antioxidant activity of these compounds. Boskou and Morton (1976) also suggested that the double bonds within the rings of effective plant sterols contribute towards their total antioxidant activity. The lack of a ring structure with a double bond in LA could help account for the difference in activity between Δ^7 avenasterol and LA.

Registry No. Linalool 78-70-6; linalyl acetate 115-95-7; geranyl acetate 105-87-3; naphthalene 91-20-3; Δ^7 avenasterol 23290-26-8; polydimethylsiloxane 9016-00-6.

Acknowledgment

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SECTION 2. CHOLESTEROL OXIDATION IN HEATED LARD ENRICHED WITH TWO LEVELS OF CHOLESTEROL

Abstract

Cholesterol oxidation in lard containing two levels of added cholesterol was monitored by using a capillary gas-chromatographic with slight modifications. Loss of cholesterol and formation of cholesterol oxidation products (COPs) were measured. Lard samples with 10X (Test I) and 2X (Test II) the amount of cholesterol originally found in each batch of lard were heated at 180 C for 10 hr a day for 240 and 160 hr, respectively. Cholesterol steadily decreased throughout the heating period in Test I. In Test II, cholesterol loss followed a first order reaction rate with rate constant (k) of 0.6×10^{-6} sec^{-1}. There was accumulation of the COPs during both heating tests. However, the total amount of COP formation did not add up to the amount of cholesterol lost. It seems likely that during the heating period, thermal degradation of cholesterol occurred, and those products were not detected. During cooling, hydroperoxides formed, which further oxidized into the COPs that were detected. The 7-ketocholesterol was the predominant COP formed in this study and its formation followed a zero-order reacion rate with a k of 1.6×10^{-5} sec^{-1}. The isomeric 7α- and 7β-hydroxycholesterols also accumulated in the heating tests. Their ratio stayed quite constant in Test I but fluctuated in Test II. The 5α, 6α-epoxycholesterol and the 3β, 5α, 6β-cholestantriol were found at very low levels and the 25-hydroxycholesterol was not detected at all.

Introduction

Cholesterol readily undergoes oxidation to produce a variety of reaction products. Ingestion of some of the cholesterol oxidation products (COPs) by laboratory animals has been shown to be cytotoxic, atherogenic, mutagenic and carcinogenic. Earlier experimental results were summarized by Smith (1981). More recently, Addis (1986) and Sevanian and Peterson (1986) reported the health implications of these COPs. In fact, the impact of cholesterol oxide consumption is recognized by the Food and Drug Administration (FDA) in the U.S. as a potential public health issue (Sheppard and Shen, 1980). The development of new and improved gas chromatographic (GLC), high-performance liquid chromatographic (HPLC) and GC-mass spectrometric (GC-MS) methods has greatly enhanced the research in this area in the past few years (Maerker, 1987).

In a recent publication, Addis (1986) reviewed many of these new GLC, HPLC and GC-MS methods. Among the methods described, some just measured one or two oxides as evidence of cholesterol oxidation, (Tsai and Hudson, 1981); other methods separated and quantified a host of major COPs. These methods included those developed by Missler et al. (1985), Park and Addis (1985), Maerker and Unruh (1986) and Nourooz-Zadeh and Appelqvist (1987). The last two methods were not reviewed by Addis (1986). Briefly, Maerker and Unruh (1986) made use of semi-preparative HPLC to separate cholesterol, triglycerides and other lipids from COPs, thereby enriching the COP fraction. That fraction was later analyzed and quantified by direct on-column capillary GLC with or without trimethylsilyl ether (TMS) derivatization. Nourooz-Zadeh and

Sep-Pak silica cartridge, a Lipidex-5000 column and an anion exchange column. Fractions containing different COPs were collected, TMS derivatized and analyzed by nonpolar capillary GLC.

The presence of COPs in processed foods has been reported and the findings reviewed by Maerker (1987). Many of the COPs have been identified in dried or processed foods (Herian and Lee, 1985; Missler et al., 1985; Tsai and Hudson, 1985; Morgan and Armstrong, 1987; Nourooz-Zadeh and Appelqvist, 1987, 1988, 1989).

The generation of COPs during deep-fat frying was first suggested by Ryan et al. (1981). Since then, several papers have tried to identify and quantify the COPs produced under these conditions in tallow (Park and Addis, 1986a, b; Bascoul et al., 1986), bacon rind fried at 200 C (Nourooz-Zadeh and Appelqvist, 1989) and butter (Csiky, 1982). Since destruction and oxidation proceed at a much higher rate at frying temperatures, researchers detected noticeable losses of cholesterol. Ryan et al. (1981) discovered a marked decrease in the size of the cholesterol spot on TLC plates concomitant with the formation of COPs. Csiky (1982) reported a 4% loss of cholesterol after heating butter for 5 minutes at 180 C. Park and Addis (1986a) noticed that about 40-45% of cholesterol was lost after 200 hr of heating at 190 C or 300 hr at 155 C. Bascoul et al. (1986) detected a loss of 25% after 60 hr of commercial frying. As for the formation of COPs, Park and Addis (1986b) noticed that at 135 C, nearly equal amounts of 7-ketocholesterol and 5α, 6α-epoxide were found, whereas at 165 C, 7-ketocholesterol predominated. In their study, only small peaks were observed at the retention site of COPs when tallow was heated at 190 C.

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Experimental Procedures

Materials

Lard. Distilled lard was purchased from the Meat Laboratory (Iowa State University, Ames, IA) and a distilled and deodorized lard was obtained from Hormel (Austin, MN), for use as the heating media. The original cholesterol content of each lard type was determined by using the colorimetric method of Searcy and Bergquist (1960) as modified by Reitmeier and Prusa (1987). The lard from the Meat Laboratory, which was used in Test I, was found to have 95mg cholesterol/100g lard whereas that from Hormel (used in Test II) had 70mg/100g lard.

Chromatographic standards. Cholesterol (99%+) was purchased from Sigma Chemical Company, St. Louis, MO. Duplicate gas-chromatographic determinations were carried out to ensure its purity. The cholesterol was used as a standard in GLC analyses and also was added to the lard to enhance the accuracy of following cholesterol oxidation. Cholesterol oxide standards, 5, 6α-epoxy-5α-cholestane-3β-OL (α-epoxide), 3β-hydroxycholest-5-en-7-one (7-keto), and 5α-cholestane-3β, 5, 6β-triol (triol) were purchased from Sigma Chemical Company. Cholest-5-ene-3β, 7α-diol (7α-OH), cholest-5-ene-3β, 7β-diol (7β-OH) and cholest-5-ene-3β, 25-diol (25-OH) were purchased from Steraloids, Inc. (Wilton, NH). To quantify and account for the losses during sample preparation and chromatographic analysis, 5α-cholestane (Sigma Chemical Co.) was added prior to saponification as an internal standard.

Heating studies

Test 1. Lard samples (600g) spiked with ten times (i.e., 950mg cholesterol/100g lard) the amount of cholesterol originally present in the lard were heated in Fry Baby Containers at 180±5 C for 10 hr a day for 24 days. The temperature of each individual fryer was controlled by a rheostat. Sample aliquots were taken at time zero and then at the end of each day. They were stored under N_2 in telfon-capped tubes at -10 C until analyzed.

Test 2. Deodorized lard samples (500g) spiked with two times (i.e., 140mg cholesterol/100g lard) the amount of cholesterol originally present in the lard were heated at 180±5 C for 10 hr a day for 16 days.

Sample preparations for cholesterol oxidation analysis

Cold saponification and extraction. The method of Park and Addis (1985) was followed with some modifications. Because of the high initial cholesterol level in our lard samples, a sample size of 0.1g was used instead of the 0.2g sample suggested in the original method. A 30-μg aliquot of 5α-cholestane was added to each sample as an internal standard. The samples were layered with N_2 and cold saponified in 1.0N methanolic KOH for 20 hr. The saponified samples were mixed with deionized water and extracted three times with diethyl ether. The extracts were pooled and back-washed with 0.5N methanolic KOH. Park and Addis (1985, 1986a) recommended washing the extracts containing the unsaponifiables two times with deionized water. We found it difficult to remove the residual methanol after only two washings. But, by increasing the washing step to three or more times, the residual solvent was successfully removed at 35 C. The endpoint of the washing step was

added at the next step are sensitive to any residual alcohols and moisture.

Quantification of sterols by capillary column GLC. For derivatization of the sterols into their corresponding trimethylsilyl (TMS) ethers, the dried ether extracts were redissolved directly into Sylon BTZ (Supelco Inc., Bellefonte, PA) instead of into pyridine, to avoid further dilution of the COPs. This was necessary because the high initial cholesterol levels in the current study required 150 μl of the silylating reagents instead of the 50 μl used in the original method of Park and Addis (1985).

A Varian Aerograph series 3300 Gas Chromatograph equipped with a flame ionization detector was used with a DB-1 capillary column (15m X 0.25mm, 0.1m film thickness; J&W Scientific, Inc., Rancho Cordova, CA) to quantify the TMS ether sterols. Peak areas of eight of these sterols were measured by using the internal standard procedure of a Hewlett Packard (HP) 3390A reporting integrator. The assignments of the peaks were checked visually afterwards due to the close proximities of some unknown peaks to the COP peaks. The cholesterol and COP peaks, in order of increasing retention time (R_T), were: 5α-cholestane, cholesterol, 7α-OH, α-epoxide, 7β-OH, triol, 7-keto and 25-OH. Because helium rather than hydrogen was used as the carrier gas, the total elution time for the products was slightly longer than that of Park and Addis. The same temperature programming was used (3 C/minute at 80-250 C) but a holding period at 250 C was added to accommodate the longer elution time. To confirm the identities of the eight sterols, the same GLC column was installed in a Finnigan 4500 GC-MS. The fragmentation patterns of the

eight peaks, partially identified by their R_T, were compared with those found in the sterol standard solution.

Results and Discussion

For many years, the detection and quantification of COPs were hampered by technical difficulties (Sheppard and Shen, 1980; Addis, 1986). There was a great need for the development of more sensitive and more reliable methods. Recently, there has been a sudden increase in method development for COP analysis, but few interlaboratory evaluations of those methods have occurred. Such evaluations are essential to providing additional confidence that the methods are useful. In the current study, the method followed was the capillary GLC method by Park and Addis (1985), which they adapted for analyzing COPs in heated tallow (1986a, b). Some slight modifications of the procedure were necessary for the current study. The method was easy to follow, but, because of differences in the chromatographic set-up, the gas chromatographic conditions were readjusted to successfully resolve cholesterol and the six COPs. The recovery of COPs in the presence of lard through the cold saponification and extraction procedures was checked by using the recovery procedure described by Park and Addis (1986a). When an internal standard was added prior to sample preparation, the recoveries of five of the COPs were 90% and above with the recovery of triol at about 70%. Park and Addis (1986a) reported recovery in tallow close to 100% except for triol which was about 85%.

In the present study, lard was heated intermittently at 180 C for up to 240 hour with two different levels of added cholesterol. Park and Addis (1986a, b) heated tallow at 135, 150, 165, 180 and 190 C for up to

376 continuous hours. The outcomes from both studies were compared to take advantage of the differences in the research designs and heating media.

Loss of cholesterol in heated lard

In both Tests I and II, the cholesterol content decreased steadily over the heating time. In Test I, where the lard contained 10X the original amount of cholesterol, the cholesterol content dropped during 240 hr of heating, from an average initial level of about 8000 ppm to a final level of about 6500 ppm. See Figure 10A. In Test II, where the lard contained two times the original amount of cholesterol, the cholesterol content dropped during 160 hr of heating from an average of 1900 ppm to a final level of 430 ppm. These data are represented in Figure 10A for Tests I and II and again in Figure 10B as percentage retention of cholesterol in Test II. In Test I, rate calculations on cholesterol loss did not provide good enough r^2 values to show the reaction order it followed. It is possible that the amount of cholesterol (10X) in those samples lay outside of the linearity range of the level of IS added and, thus, was not quantified accurately.

The curve in Figure 10B shows more clearly the slow down of cholesterol loss when about 70% of the initial substrate was consumed. A first order reaction rate was suspected. The natural logarithm of the amount of cholesterol was plotted against hours of heating and the resulting coefficient of variance (r^2) was -0.98. The rate constant (k) of cholesterol loss in Test II is 0.6×10^{-6} sec^{-1}. Park and Addis (1986a) noted that cholesterol loss ceased in two heated tallow samples when 40 to 45% of the initial cholesterol was gone. It took 300 hr of

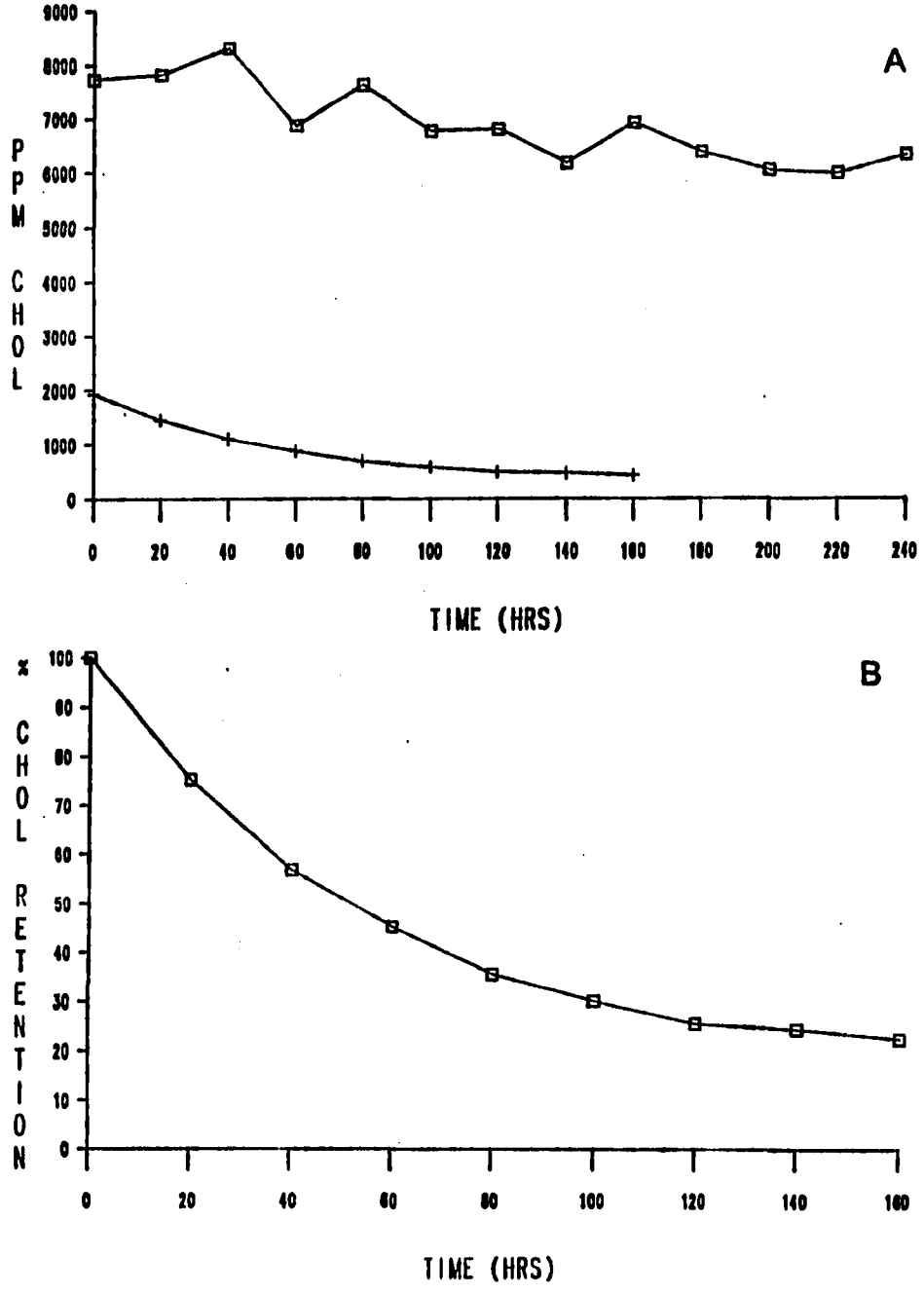

Figure 10. Changes in lard: (A) The amount of cholesterol loss in lard with 10X added cholesterol (□) and 2X added cholesterol (+); (B) Percentage cholesterol retention in lard with 2X added cholesterol in Test II (□).....

heating at 155 C or 200 hr of heating at 190 C to arrive at that point. In the current study, it is likely that the intermittent, rather than continuous, heating caused cholesterol loss to slow down when 70% rather than 40-45% of the cholesterol had been lost, and caused a quicker destruction than in the tallow heated by Park and Addis (1986a, b). The intermittent heating and cooling allows more oxygen to be introduced into the frying medium due to the increased solubility of oxygen at lower temperatures and results in greater oxidation of its components. Kimura et al. (1979) and Bergstrom and Wintersteiner (1941) reported that an aqueous cholesterol mixture arrived at an apparent final state where consumption of cholesterol and formation of COPs were suspended after more than 70% of the initial substrate was consumed. They suggested that the accumulation of COPs in the reaction media may have changed the micellar structure, resulting in the ceasation of cholesterol oxidation. None of the above researchers documented the rate of cholesterol loss observed in their experiments.

Formation of cholesterol oxidation products in heated lard

Figure 11 represents a typical chromatogram of heated lard with the addition of two times the original amount of cholesterol (Test II). Of the eight compounds studied, only six were detected in that sample (5α-cholestane, cholesterol, 7β-OH, β-epoxide, 7β-OH and 7-keto) and the corresponding peaks were labelled. Although baseline separations were achieved among all of the six compounds observed, the initial cholesterol peak covered up the 7α-OH peak that followed it when cholesterol was added at ten times the original level (Test I). However, as the cholesterol content decreased with heating, the 7α-OH

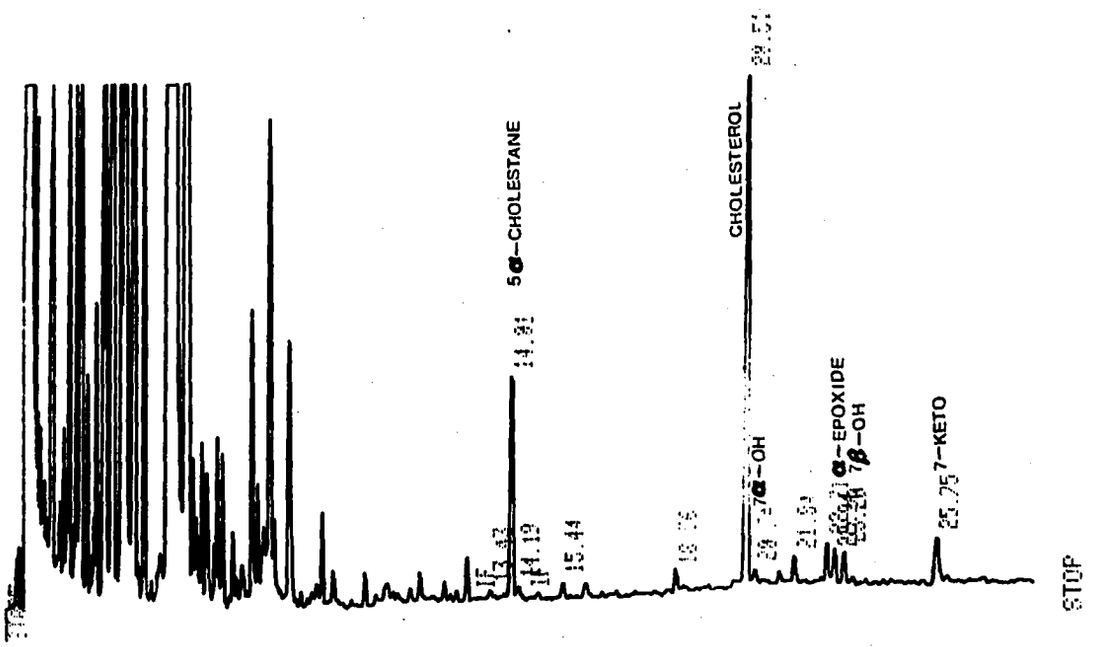

Figure 11. A typical capillary GLC chromatogram of a heated lard sample.

peak gained resolution from the cholesterol peak. This condition occurred after about 80 hr of heating. In Test II, the resolution of 7α-OH was apparent at time zero.

Figures 12A and B show the amount of 7α-OH, α-epoxide, 7β-OH and 7-keto formed in Test I and and the amounts of the above four COPs plus triol formed in Test II. In general, Test I yielded more COPs than did Test II, likely because of the higher level of cholesterol present in the former. However, the higher amount of COPs in Test I did not represent the almost 5-fold difference in initial cholesterol contents between Test I and II. Also, other than α-epoxide which seemed to achieve a steady state after 120 hr of heating, the levels of the other three COPs (7α-OH, 7β-OH and 7-keto) were still increasing at the termination of the heating. Such was not the case in Test II. The COPs other than 7-keto were experiencing either a steady state (triol and 7α-OH) or had achieved a maximum and were decreasing (α-epoxide and 7β-OH) at the end of the heating.

Smith (1981) and later Maerker (1987) summarized the current knowledge on the formation of the commonly found COPs. It was indicated that the autoxidation of cholesterol proceeded via an initial formation of the epimeric 7α- and 7β-hydroperoxides (7-OOHs) which later reduced to their corresponding alcohols with both epimers dehydrating to 7-keto. However, in addition to the homolysis of the peroxide bonds described above, reactions such as isomerization of the axial α-OOHs and α-OHs to the corresponding equatorial β-OOHs and β-OHs (both 5- and 7-isomers) and the disproportionation of 7-OOHs to equal molar concentrations of 7α-OH, 7β-OH and 7-keto complicate the final proportion of each COP in

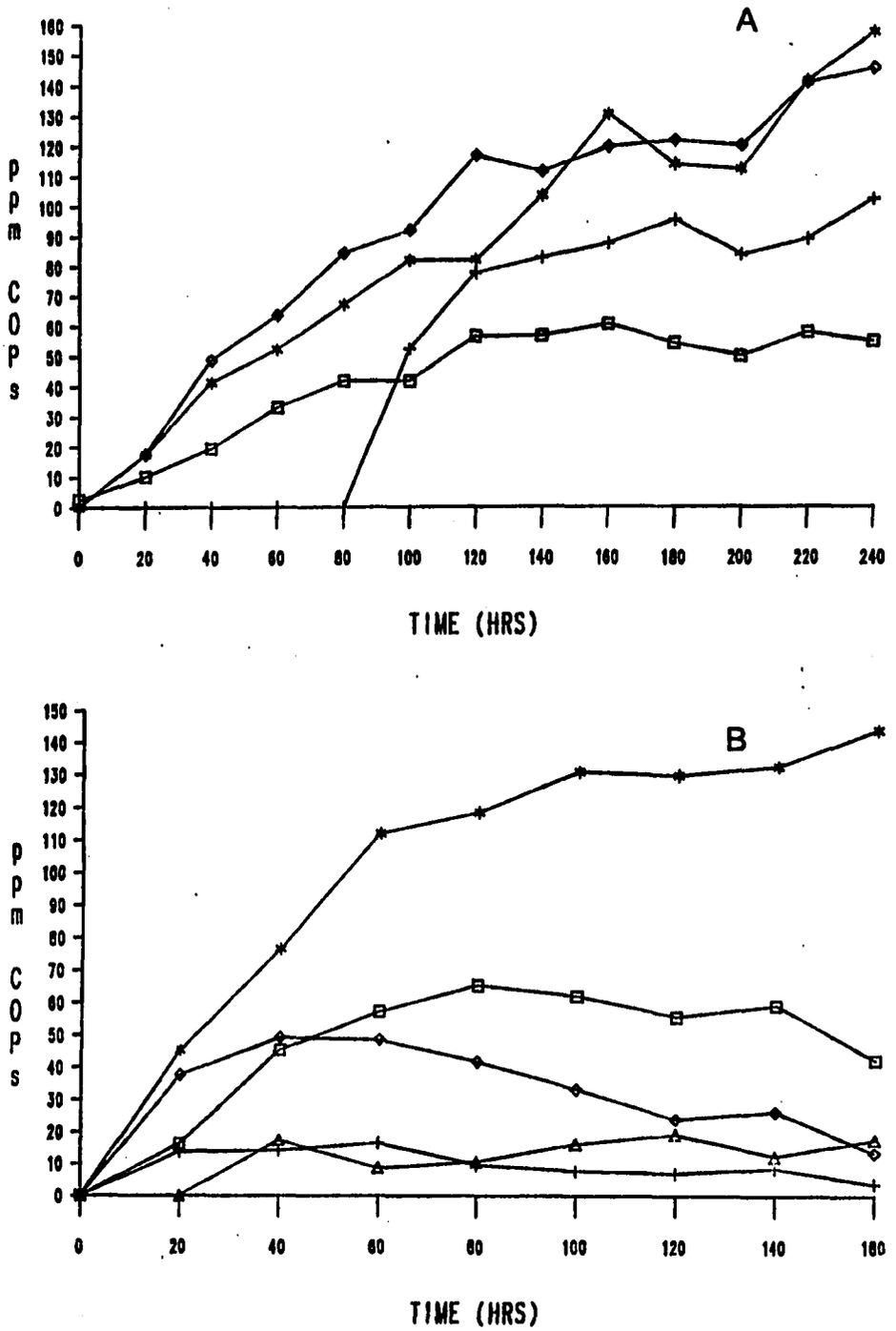

Figure 12. COP formation in lard with (A) 10X added cholesterol (B) 2X added cholesterol. The COPs being analyzed for: (□) α-epoxide, (+) 7α-OH, (◇) 7β-OH, (*) 7-keto, and (△) triol.

an oxidized mixture. Furthermore, heating a frying medium results in the formation of free fatty acids which reduces the pH and causes the hydrolysis of α- and β-epoxides to triol.

In our tests, the amount of total COP formed (480 and 320 ppm in Test I and II respectively) was not equal to the amount of cholesterol loss (1550 and 1470 ppm in Test I and II, respectively). There may be many other degradation products formed for which analyses were not done or that were lost through volatilization. Park and Addis (1985, 1986a) also proposed that COP degradation was the cause for the low levels of COP accumulation in tallow heated at 180 and 190 C.

Reaction rates for formation of COPs. The formation of most commonly found COPs in heated lard samples cannot be explained by simple reaction rates because of the many possible routes of formation within this complex system. One exception may be 7-keto. The formation of 7-keto in Test I exhibited a straight line relationship with heating time. When a regression line was fitted through the data points, it had an r^2 of 0.98. Thus, the formation of 7-keto in lard sample with 10X added cholesterol appeared to follow a zero-order reaction rate and the rate constant was 1.6×10^{-5} sec^{-1}. In Test II, the formation of 7-keto followed a straight line relationship with heating time up to 60 hrs. When a regression line was fitted through those data points, an r^2 of 1.0 and a zero-order rate constant of 5.1×10^{-5} sec^{-1} was observed. Beyond 60 hr of heating, a regression line with an r^2 of 0.92 and a zero-order rate constant of 7.3×10^{-6} sec^{-1} was calculated. The initial rate constant for the formation of 7-keto in Test I was greater than that for Test II. No reason can be offered at this point to

explain such observation. In Test II, the rate constant for 7-keto formation decreased with increased heating, possibly due to the diminished cholesterol content in the heated lard. The observed zero-order rate constants may be purely coincidental. The systems used in this study were complex ones and not model systems that are generally used to calculate rate constants.

Another important parameter observed in this study as well as by other researchers was that the β-isomer of 7-OHs accumulated to a greater extent than the α-isomer, likely because of the presence of less steric hindrance in the formation of the former isomer. In Test I, the α/β ratio was 1 : 1.3-1.8. In Test II, it was 1 : 2.7-4.3. A wide range of α/β ratios is reported in the literature ranging from 1 : 8-11 (Smith and Kulig, 1975) in the autoxidation mixture of cholesterol to 1 : 1.0-22.7 in commercial spray-dried egg products (Tsai and Hudson, 1984). Nourooz-Zadeh and Appelqvist (1987) suggested that differences in the relative proportions of the epimeric 7-OHs and the isomeric epoxides in several skim milk samples may have reflected differences in the storage conditions. In a model system carried out by Maerker and Bunick (1986), they found that at alkaline pH's, the ratio of α/β epoxides remained constant even though the level of accumulation increased with heating at 80 C. In acidic pH's, this trend did not hold, possibly because β-epoxides were preferrentially hydrolyzed to triol. The α/β ratios of epoxides were not monitored in this study. The α/β ratios of the 7-OHs remained fairly constant throughout the heating in Test I (for those samples where the 7α-OH peak was sufficiently separated from the cholesterol peak) but increased

initially followed by a decrease indicating possible preferrential degradation of 7β-OH in Test II as indicated by Smith (1981) and Maerker (1987).

The accumulation of COPs in a high-temperature, cholesterol-rich food system may be a dynamic one, depending upon the amount of cholesterol present, the treatment of the oil (intermittent or continuous heating) and the severity of the heat treatment. Further studies where food is actually fried in the fat would contribute to practical information about the accumulation of COPs in such a system. On the other hand, adding pure COPs individually to heating media such as pure triolein would allow one to monitor the rate of COP formation in a model system. Information from both types of study will provide further understanding of the occurrence of COP in our food system.

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SECTION 3. THE EFFECT OF LINALYL ACETATE AND POLYDIMETHYL SILOXANE ON CHOLESTEROL AND TRIGLYCERIDE OXIDATION IN HEATED LARD

Abstract

In a previous study, linalyl acetate (LA) was shown to be effective in reducing triglyceride oxidation in soybean oil heated at 180 C for 7 hr a day for up to 70 hr. In the current study, the effectiveness of LA at 0.02 and 0.04% was monitored in lard with two levels of added cholesterol (2X and 10X). Lard samples with and without added LA and polydimethyl siloxane (MS, 0.2 and 1.0ppm) were heated at 180 C for 10 hr a day for up to 240 hr. The percentage cholesterol retention, the formation of cholesterol oxidation products, and the percentage fatty acid retention were measured over the heating periods. The addition of MS was slightly effective in reducing both fatty acid, cholesterol oxidation, and 7-ketocholesterol formation when added at 1.0ppm in lard with 2X added cholesterol. The MS at 0.2ppm was not effective in lard with 10X added cholesterol. The addition of LA at both levels had little effect at reducing changes in cholesterol and triglyceride in either lard sample. Data from another study suggested the need for adding LA at a higher concentration or including other antioxidants such as MS to cause synergism to occur.

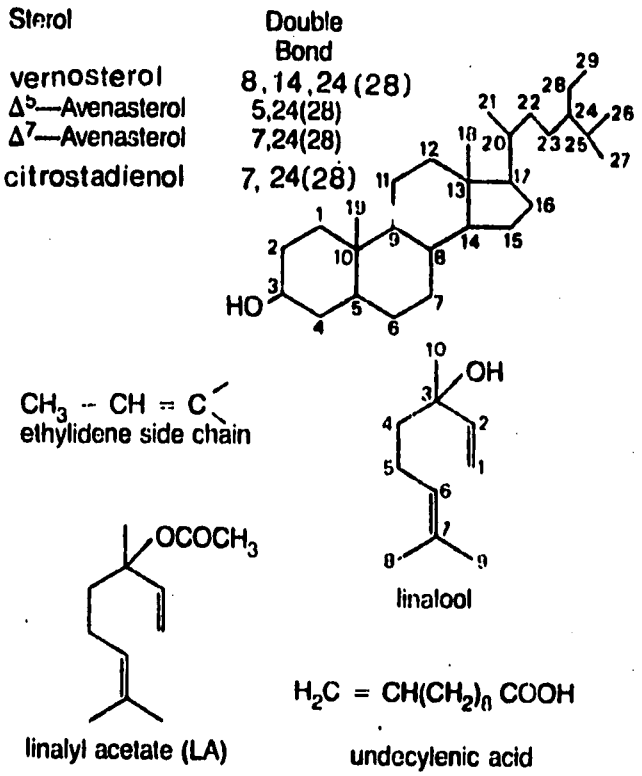
Introduction

The mechanism and the use of antioxidants in the food industry have been reviewed extensively (Sherwin, 1976, 1978; Porter, 1980; Burton and Ingold, 1984; Simic and Hunter, 1985; Houlihan and Ho, 1985; Dziezak, 1986). Because of consumer demands, the food industry is using fewer

saturated animal fats and more polyunsaturated vegetable oils. These changes prompted the introduction and the approval for food uses of monotertiarybutyl hydroquinone (TBHQ) in 1972. A recent survey (Haumann, 1987) reported that the fast food industry has taken steps to switch from animal to vegetable fats in its frying operation. However, major food chains such as McDonald's and Burger King Corporation continue to fry french fries in shortenings containing some tallow to produce a beefy flavor. Thus, the reduction in thermally-induced changes in fats and oils from both vegetable and animal sources is desirable.

Most of the phenolic antioxidants undergo steam-distillation or destruction at deep-frying conditions (Peled et al., 1975). To date, polydimethyl siloxane (MS) remains the only antioxidant used by the industry to protect frying media. Because of its synthetic nature, processors avoid adding it to bottled salad oil in order to label the oil "all natural".

Antioxidants isolated from plant sources may provide alternatives to the current choices of effective high temperature antioxidants. A number of plant sterols including Δ^5- and Δ^7-avenasterol, vernosterol and citrostadienol were found to be effective at frying temperatures (Sims et al., 1972; Boskou and Morton, 1975; Gordon and Magos, 1983; White and Armstrong, 1986). Gordon and Magos (1983) theorized that the ethylidene side chain (Figure 13) reacts rapidly with lipid free radicals to form stable allylic tertiary free radicals that interrupt the oxidation chain. The ethylidene side chain forms free radicals rapidly due to the presence of unhindered hydrogen atoms on an allylic



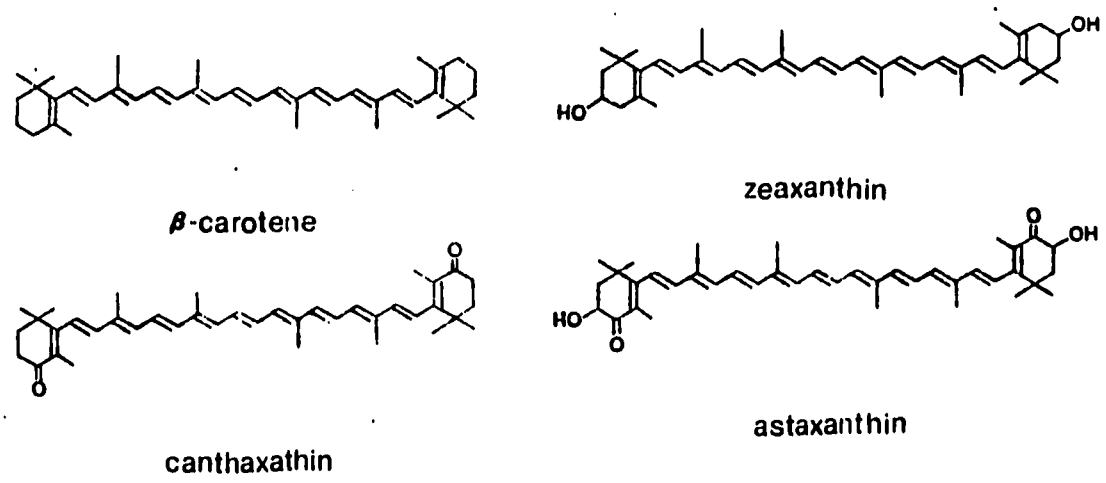


Figure 13. Chemical structures of compounds containing an ethylidene group and carotenoids.

carbon atom. This theory was further tested by examining the antioxidative effect of compounds containing an ethylidene-like group but without the sterol moiety (Yan and White, 1987). Compounds fitting these requirements include a group of monoterpene alcohols. The high temperature antioxidant effects of linalyl acetate and undecylenic acid (not a terpene) on soybean oil heated at 180 C were reported in a previous study (Yan and White, 1987, 1989). The rate of triglyceride oxidation was reduced in the protected oils. Linalyl acetate at 0.04% was found to be as effective as MS at 0.3ppm but linalyl acetate at 0.02% was only slightly effective.

Attempts have been made to arrest cholesterol oxidation by incorporating known antioxidants. Park and Addis (1985) studied the effects of adding 500ppm ascorbyl palmitate and 100ppm dl-α-tocopherol to tallow heated at 135 C. They reported that no COPs were detected for up to 70 hr of heating in tallow protected with the antioxidants. However, after 70 hr, the inhibitory effect of antioxidants was no longer observed. Morgan and Armstrong (1987) added butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and propyl gallates (PG) alone or in combination to liquid egg products prior to spray-drying. The antioxidants did not significantly affect the levels of COP found in the final products. Tsai and Hudson (1985) evaluated scrambled egg mixes that contained a substantial amount of soybean oil or corn oil that was protected with BHA, BHT, PG and citric acid (CA). Those that had been stored for 5 years had substantially more epoxides than those stored for 3 years.

Nourooz-Zadeh and Appelqvist (1987) studied Swedish dehydrated egg products. One type of dehydrated egg mix indicated that freshly dried products and those stored for only 2 months contained traces of COPs. Yet one egg material, Petit-Choux mix, was free from oxidation products at 2 and 6 months of storage. Presumably the antioxidants added to the vegetable oil present in that formulation were exerting an effect. When Maerker and Unruh (1987) carried out hot alkaline saponification in the presence of 0.3% BHT, there was no substantial reduction of COP formation.

In the current study, the antioxidant effect of linalyl acetate at 0.02 and 0.04% was tested in lard heated to 180 C with two levels of added cholesterol. The triglycerode oxidation of lard with two times the original amount of added cholesterol was followed to further examine the effects of linalyl acetate.

Experimental Procedures

Materials

Lard. Distilled lard was purchased from the Meat laboratory (Iowa State University, Ames, IA) and a distilled and deodorized lard was obtained from Hormel (Austin, MN), for use as the heating media. The original cholesterol content of each lard type was determined by using the colorimetric method of Searcy and Bergquist (1960) as modified by Reitmeier and Prusa (1987). The lard from the Meat Laboratory, which was used in Test I, was found to have 95mg cholesterol/100g lard whereas that from Hormel (used in Test II) had 70mg/100g lard.

Antioxidants. Linalyl acetate (LA) was purchased from Aldrich Chemical Company (Milwaukee, WI). Polydimethyl siloxane (MS) was purchased from Sigma Chemical Company (St. Louis, MO).

Chromatographic standards. Cholesterol (99%+) was purchased from Sigma Chemical Company. Duplicate gas-chromatographic determinations verified its purity. The cholesterol was used as a standard in GLC analyses and also was added to the lard to enhance the accuracy of following cholesterol oxidation. The following cholesterol oxide standards were purchased from Sigma Chemical Company (St. Louis, MO): 5α, 6α-epoxy-5α-cholestan-3β-OL (α-epoxide), 3β-hydroxycholest-5-en-7-one (7-keto), and 5α-cholestane-3β, 5, 6β-triol (triol). Additional standards were purchased from steraloids, Inc. (Wilton, NH): cholest-5-ene-3β, 7α-diol (7α-OH), cholest-5-ene-3β, 7β-diol (7β-OH) and cholest-5-ene-3β, 25-diol (25-OH). To quantify and account for the losses due to sample preparation and chromatographic analysis, 5α-cholestane (Sigma Chemical Company) was added prior to saponification as an internal standard.

Heating studies

Test 1. Lard samples (600g) spiked with ten times (i.e., 950mg cholesterol/100g lard) the amount of cholesterol originally present in the lard were heated in Fry Baby Containers at 180±5 C for 10 hr a day for 24 days. The temperature of each individual fryer was controlled by a rheostat. Four treatments were tested, including a control, lard with 0.2ppm MS and lard with 0.02 and 0.04% LA. The appropiate amount of antioxidant was dissolved in hexane and aliquots equal to the desired amounts were added to the lard. An equal amount of hexane was added to

the control. Sample aliquots were taken at time zero and then at the end of each day. They were stored under N_2 in telfon-capped tubes at -10 C until analyzed.

Test 2. Deodorized lard samples (500g) spiked with two times (i.e., 140mg/100g lard) the amount of cholesterol originally present in the lard were heated at 180±5 C for 10 hr a day for 16 days. The level of antioxidants tested were: MS at 1.0 ppm, LA at 0.02 and 0.04% and a control sample.

Sample preparations for cholesterol oxidation analysis

The method of Park and Addis (1985) was followed with modifications as described (Yan and White, 1989). Briefly, 0.1g of lard and a 30μg aliquot of 5α-cholestane was added to each sample as an internal standard. The samples were layered with N_2 and cold saponified in 10 ml 1.0N methanolic KOH for 18 to 20 hr. The saponified samples were mixed with deionized water and extracted three times with diethyl ether. The extracts were pooled and back-washed with 0.5N methanolic KOH. The unsaponifiables were washed with deionized water until the wash water achieved neutrality. Samples were dried with anhydrous sodium sulfate and the solvent was removed under vacuum. A 150μl of Sylon BTZ was added to each sample, mixed and injected onto a capillary GLC column after derivatization for 30 minutes at room temperature.

Triglyceride oxidation in heated lard

A Varian Aerograph series 3700 GLC equipped with a flame ionization detector was used. The method of Metcalfe et al. (1966) was followed for the preparation of fatty acid methyl esters (FAMES). The GLC contained a stainless steel packed column (100/120 Gas Chrom Q II with

10% Silar 10C coating; Alltech Associates, Deerfield, IL) of 6.0ft. X 0.085in.. Peak areas were measured by using the internal standard procedure of an HP3390A reporting integrator. Triheptadecanoin was added to all the samples as an internal standard. This method of measurement was suggested by Waltking and Zmachinski (1970) to be the preferred method in determining total polyunsaturated fatty acids. The fatty acid data reported in this study list the percentage retention for each fatty acid over the heating time, based on the amount at time zero. All test results are the average of duplicate samples.

Results and Discussion

Levels of added polydimethyl siloxane

Freeman et al. (1973) tested MS in sunflower seed oil heated at 180 C. The MS added at 0.02 to 1.00ppm significantly protected the oil in comparison with the control and the effects were similar throughout the range tested. They concluded that MS gave its full protective effect at 0.1ppm. The level of MS added in Test I (0.2ppm) was close to their suggested effective range. For Test II, a higher level of MS (1.0ppm) was tested because levels higher than 0.2ppm were used by other researchers. Sims et al. (1972) tested MS at 0.5ppm in heated safflower oil. Ishikawa et al. (1984) reported that processors regularly add up to 2.5ppm MS to corn oil. Drew (1961) patented the use of 1 through 25ppm MS in hydrogenated vegetable oils to increase the smoke point.

Loss of cholesterol during heating of lard

In both heating tests, the cholesterol content decreased steadily over the heating time. In Test I, where the lard contained ten times the original amount of cholesterol, no apparent protective effect of LA

at 0.02 and 0.04% or MS at 0.2ppm was seen when compared with the control. See Figure 14A. All lard samples lost about 1500ppm over the 240 hr of heating. In Test II, where the lard contained two times the original amount of cholesterol, the cholesterol content dropped from an average of 1900ppm to a final level of 430ppm during 160 hr of heating. These data are represented in Figure 14B as percentage retention of cholesterol to illustrate the slowing down of cholesterol loss. In this test, LA was tested at 0.02 and 0.04% while MS was added at 1.0ppm. Throughout the heating period, the lard sample containing MS had a slightly greater retention (about 10%) of cholesterol than did all other samples. When first order rates were calculated using the cholesterol data from Test II, r^2 ranging from -0.975 to -0.979 were obtained. Lard with no additives and lard with 0.02% LA had first order rate constants of 2.6×10^{-6} sec^{-1}. Lard protected with 0.04% LA and 1.0ppm MS had rates of 2.4 and 2.3×10^{-6} sec^{-1} respectively. This shows that MS at 1.0ppm (and may be LA at 0.04%) helped to retain slightly more cholesterol than the control and 0.02% LA in the heated lard. In Test I, rate calculations on the cholesterol loss did not provide good enough r^2 values to report the reaction orders of the samples. It is possible that the amounts of cholesterol in those treatments lay outside of the linearity range of the level of IS added and thus were not quantified accurately.

Formation of COPs during heating of lard

Figures 15A and B illustrate the levels of 7α-OH accumulation in Tests I and II. In Test I, the large excess of cholesterol (10X the original amount) covered up the 7α-OH peak that elutes very close to it.

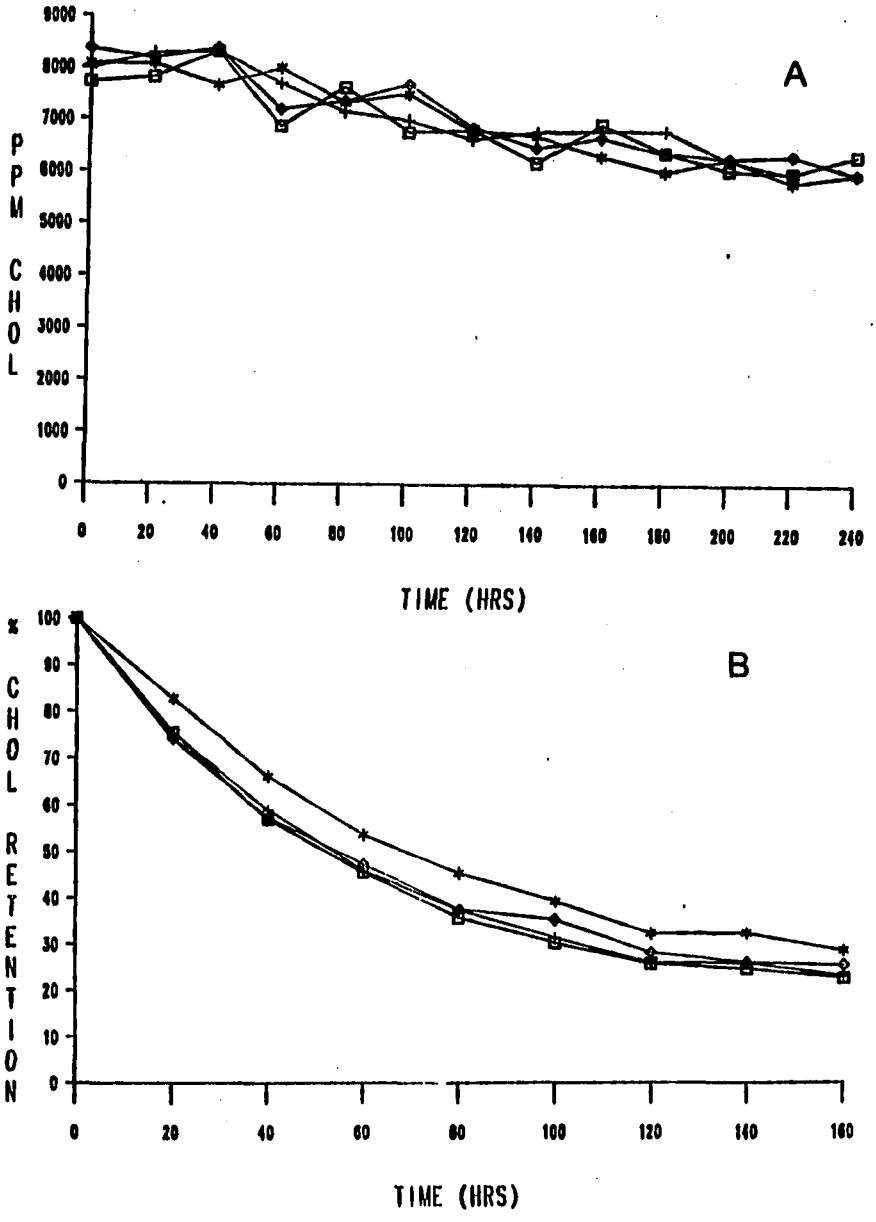


Figure 14. The amount of cholesterol loss in lard with 10X added cholesterol (A) and the percentage cholesterol retention in lard with 2X added cholesterol (B) protected with: (□) control, (+) LA at 0.02%, (◇) LA at 0.04%, (✱) MS at 0.2ppm in A and 1.0ppm in B.

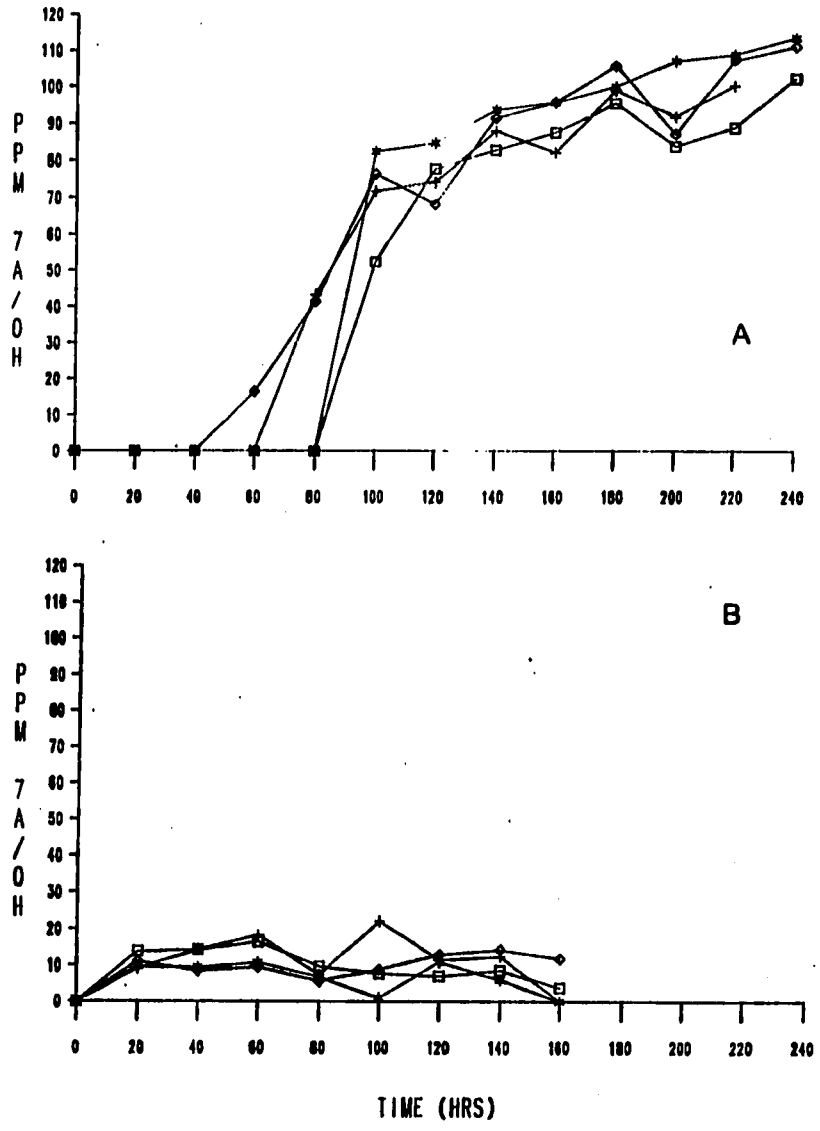


Figure 15. The formation of 7α-OH in lard with (A) 10X added cholesterol and (B) 2X added cholesterol protected with: (□) control, (+) LA at 0.02%, (◇) LA at 0.04%, (*) MS at 0.2ppm in A and 1.0ppm in B.

As a result, an apparant delay in 7α-OH formation was seen in Figure 15A but not in Figure 15B. About five-folds more 7α-OH were observed in Test I compared with Test II. This is the only COP that reflected the five-fold difference in added cholesterol between Tests I and II. Rate calculations were done and none of the COPs in lard heated with or without additives fit a first order reaction rate. This was likely because the formation of most COPs follows more than one pathway including transformation of one COP into another. Mechanisms that describe these pathways were discussed by Smith (1981) and later summarized by Maerker (1987). The various routes from which a COP can be formed and/or degraded precludes that its rate of formation be described by any one simple reaction rate. The difficulty of assigning reaction rates is especially true in a non-model system condition such as that used in this study. Regardless of the final accumulation, no antioxidant effects on 7α-OH formation was evident in either test.

The levels of α-epoxide accumulation are shown in Figures 16A and B. The α-epoxide concentrations were quite similar in both Tests I and II. No obvious antioxidant effects on α-epoxide formation was observed.

The formation of 7β-OH was more prevalent than 7α-OH (See Figures 15A and B and 17A and B). The preferential formation of 7β-OH over 7α-OH was observed by other researchers (Nourooz-Zadeh and Appelqvist, 1987; Maerker and Unruh, 1987) and were attributed to the presence of less steric hindrance in the formation of the β-epimer. The plot in Figure 17B showed a gradual decline of 7β-OH after a peak was reached while no such trend was observed in Figure 17A (Test I). Once again,

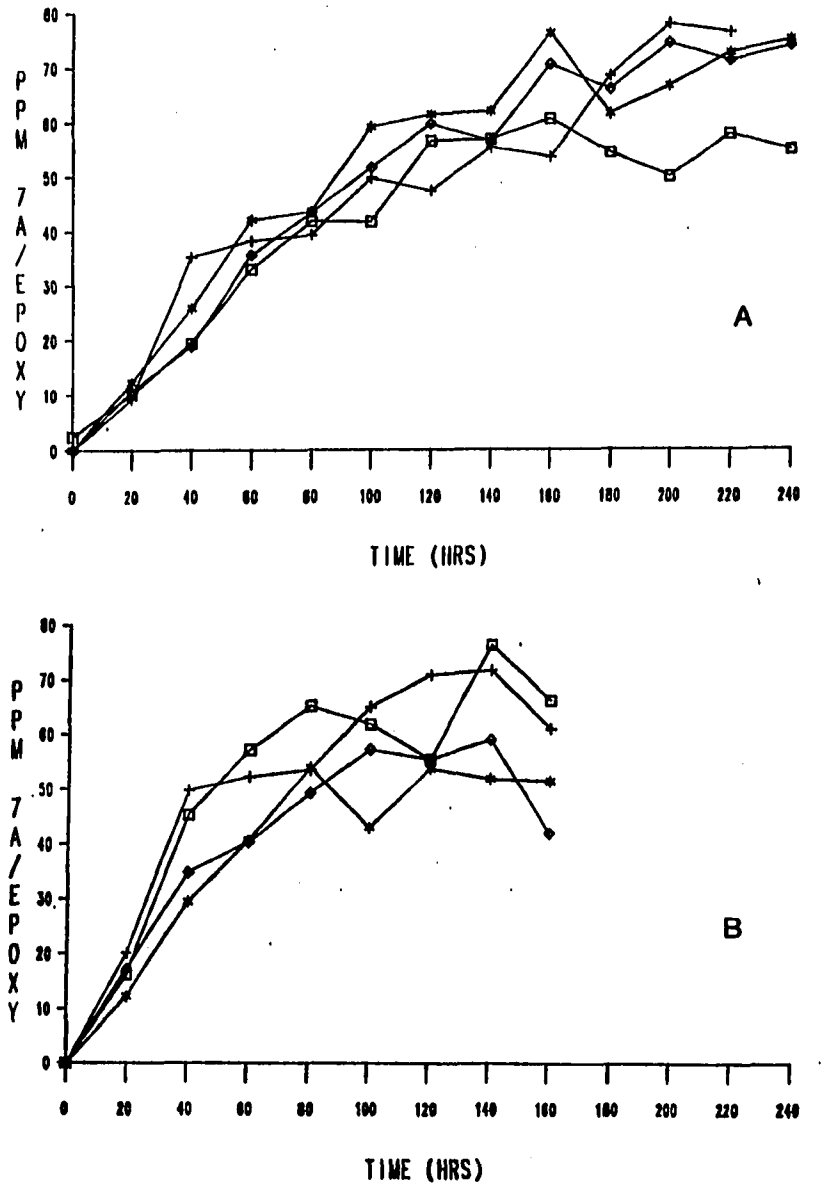


Figure 16. The formation of α-epoxide in lard with (A) 10X added cholesterol and (B) 2X added cholesterol protected with: (□) control, (△) LA at 0.02%, (◇) LA at 0.04%, (✱) MS at 0.2ppm in A and 1.0ppm in B.

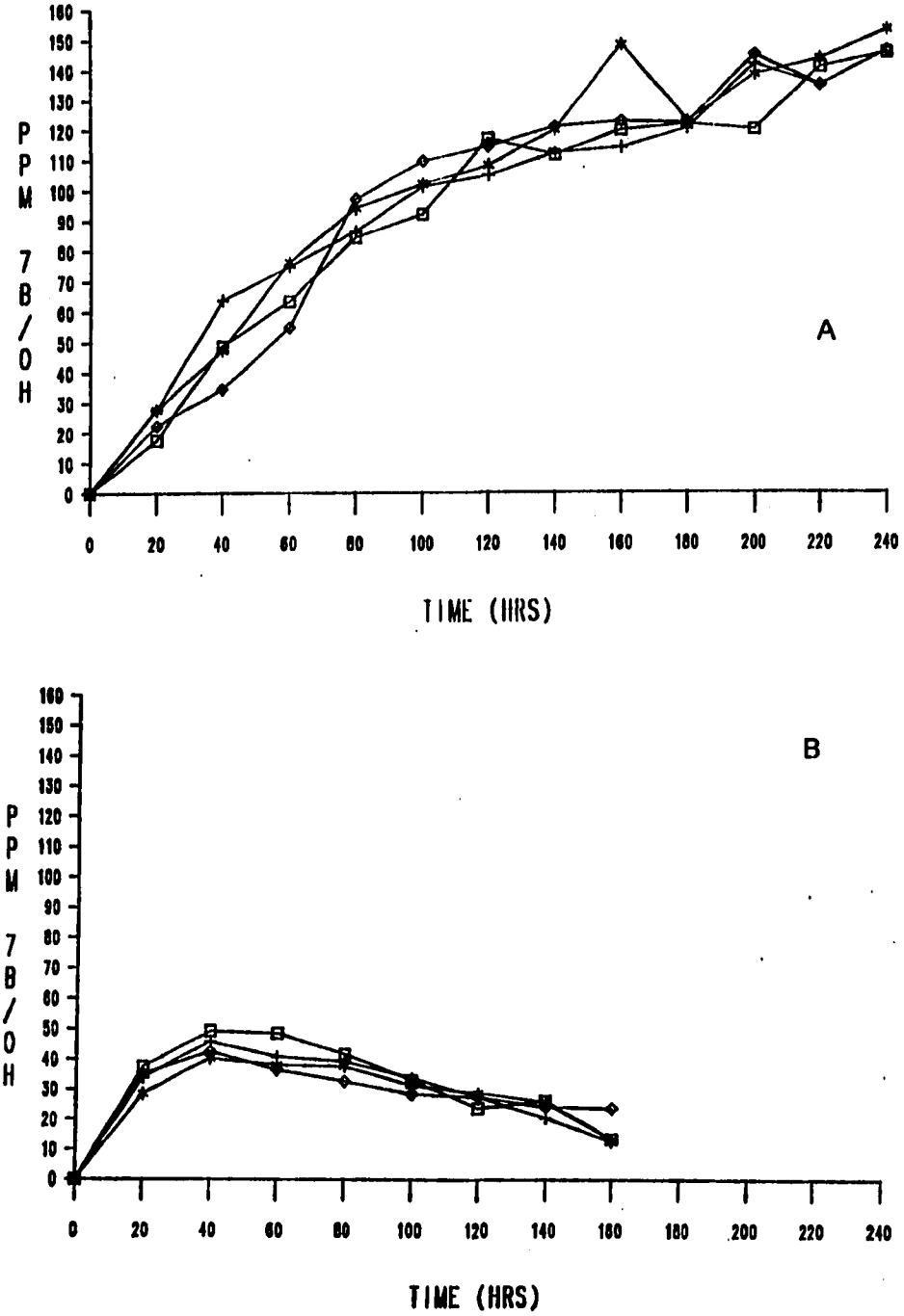


Figure 17. The formation of 7β-OH in lard with (A) 10X added cholesterol and (B) 2X added cholesterol protected with: (□) control, (△) LA at 0.02%, (◇) LA at 0.04%, (*) MS at 0.2ppm in A and 1.0ppm in B..

the addition of any level of MS or LA in Tests I and II had no protective effect.

Figure 18 represents the level of triol measured during Test II. Formation of triol in Test I was so erratic that we chose not to include those data. Triol results from the hydrolysis of the epoxy ring of the epimeric epoxides. It is usually included in the total epoxide formation (Zulak and Maerker, 1989). No evidence of antioxidant protection was seen in the formation of the triol.

Figures 19A and B present the levels of 7-keto accumulation in the heated lards. Nearly straight line relationships between 7-keto accumulation and the heating time were seen throughout Test I (Figure 19A) and were found to follow a zero order reaction rate. The rate constants for all treatments of lard (with and without additives) were similar in Test I at 1.6×10^{-5} sec^{-1}. In Test II (Figure 19B), the rates of 7-keto formation were linear with heating up to 60 hr. All samples in Test II with 60 hr of heating had r^2's of 0.99. The rate constants were 5.1×10^{-5} sec^{-1} for the control, 4.4×10^{-5} sec^{-1} for lard containing LA at 0.02%, 4.6×10^{-5} sec^{-1} for lard containing LA at 0.04% and 3.7×10^{-5} sec^{-1} for lard containing MS at 1.0ppm. These rate constants suggest that MS added to lard at 1.0ppm reduced 7-keto formation in lard with 2X added cholesterol when compared with other treatments. The LA appeared to have a small effect by these calculations.

The 7-keto was the predominant COP formed in this study and in other studies (Park and Addis, 1986a, b) possibly due to the many pathways by which it can be formed (e.g., disproportionation of 7-OOHs

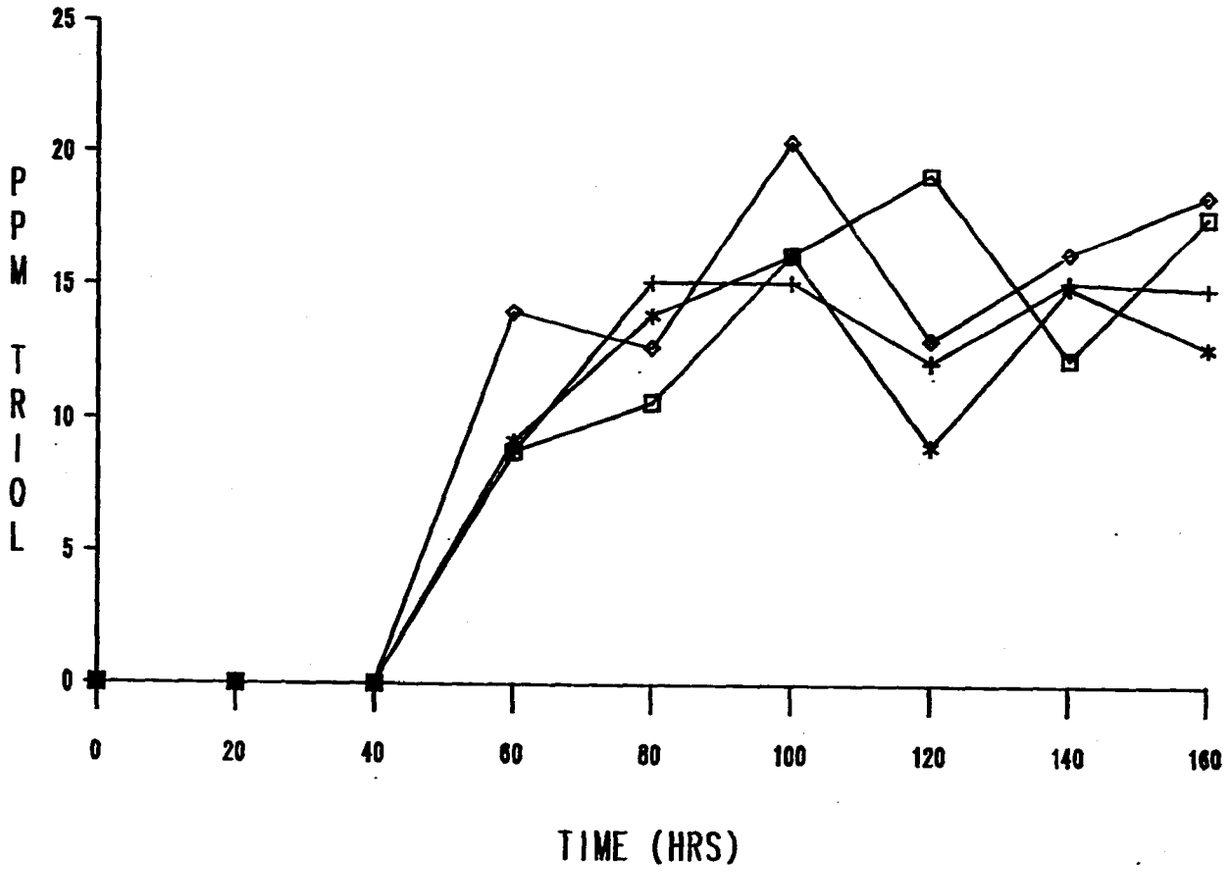


Figure 18. The formation of triol in lard with 2X added cholesterol protected with: (□) control, (+) LA at 0.02%, (◇) LA at 0.04%, (*)MS at 1.0ppm.

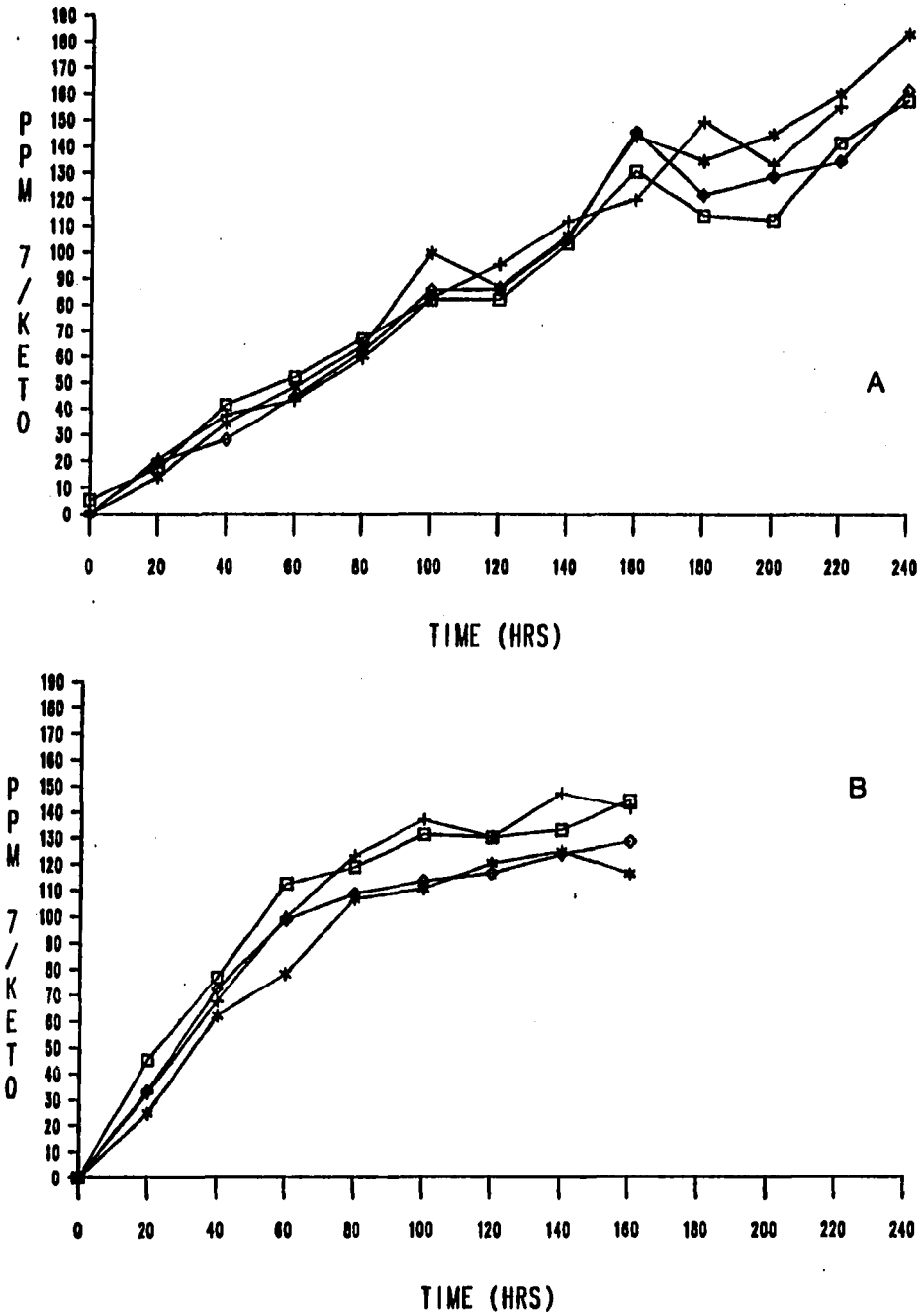


Figure 19. The formation of 7-keto in lard with (A) 10X added cholesterol and (B) 2X added cholesterol protected with: (□) control, (+) LA at 0.02%, (◇) LA at 0.04%, (✱) MS at 0.2ppm in A and 1.0ppm in B.

and dehydration of 7-OHs). Also, 7-keto is quite stable in acidic media and does not degrade into other commonly found COPs. The large amount of 7-keto formed compared with the other COPs, made the small antioxidative effects of MS and LA more evident when measuring this COP.

Triglyceride oxidation during heating of lard

In previous studies, LA and MS protected the fatty acids in triglycerides of soybean oil (Yan and White, 1987, 1989). Thus, fatty acid analyses were carried out on the lard samples from Test II where MS, and possibly LA, slowed the disappearance of cholesterol (Figure 14B) during heating. The amounts of palmitic and stearic acid were not affected by treatment, therefore, the data are not shown. The percentages of retention of oleate (18:1) and linoleate (18:2) from all treatments in Test II are plotted in Figures 20A and B, respectively. The oleate disappearance rate did not show a treatment effect although all samples followed a zero order reaction rate with an r^2 of -0.98 and a k of -8.5×10^{-6} sec^{-1}. Lard with added MS clearly showed a higher retention of 18:2 compared with the other treatments (Figure 20B). The disappearance of 18:2 in the sample containing MS followed a zero order reaction rate (r^2 = -0.97, k = -2.1×10^{-5} sec^{-1}). The disappearance of 18:2 in the control sample also followed a zero order reaction rate with an r^2 of -0.99 and a k of -2.7×10^{-5} sec^{-1}.

Data from the oxidation of cholesterol, formation of 7-keto and retention of fatty acids confirmed that there was some antioxidant effect of MS on lipid oxidation in lard with 2X added cholesterol. Little or no antioxidant effect was seen with the addition of LA, when examining fatty acid retention, COPs formation and cholesterol

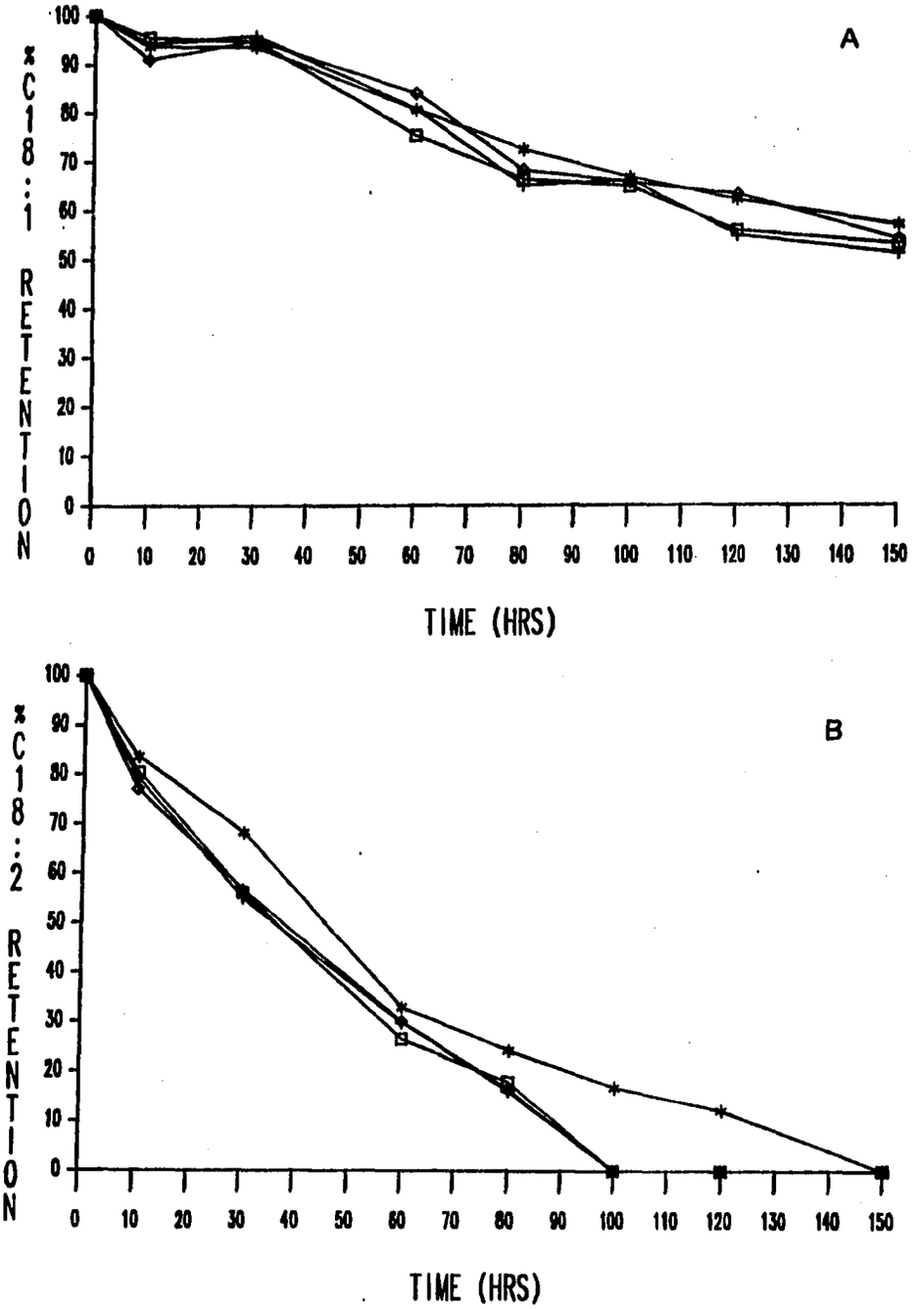


Figure 20. Percentage retention of 18:1 (A) and 18:2 (B) in lard with 2X added cholesterol protected with: (□) control, (+) LA at 0.02%, (◇) LA at 0.04%, (*) MS at 1.0ppm.

oxidation. In the current study, the large excess of cholesterol (10X the original amount) in Test I may have overwhelmed the effects of all the antioxidants. In Test II, when MS was added at 1.0ppm and cholesterol was added at 2X the original amount, the antioxidant effect of MS was manifested in reduced cholesterol disappearance, decreased 7-keto formation and increased 18:2 retention. The MS did not appear to reduce other COP formation, however, even a 10% reduction in the COP formation (the level observed in cholesterol retention in Test II) may have been hard to detect because of high variability and low accumulation of COPs other than 7-keto.

In 1989, Willemse received a patent on the use of silicon (at 0.02 to 50ppm) and linalool and/or linalyl acetate or their mixtures. The patent shows that the mixture were effective in lowering the development of unpleasant flavors in the presence of a small amount of MS when fat and oil products are subjected to prolonged heating above 120 C. Essential oils with weight ratios of linalool: LA between 1-2 : 1-10 at a total of 4-40ppm were effective antioxidants.

The high temperature antioxidant effect of essential oils seen in the patent of Willemse could be due to the synergism between LA or linalool and MS. Synergism between primary antioxidants and MS was also observed by Frankel et al. (1985). In their study, after 19 hr of heating and intermittent frying of bread cubes at 190 C, hydrogenated SBO with the addition of TBHQ, citric acid (CA) and MS had the lowest room odor score among all other treatments. When the above compounds were used singly, they were less effective antioxidants. This suggested a synergistic effect. In the current study, the lard samples were free

of additives such as MS and CA, therefore, the presence of LA at a higher concentration than was used might be needed to show a similar protective effect.

Among compounds containing an ethylidene group, Gordon and Magos (1983) observed that the antioxidative activity of vernosterol was higher than Δ7-avenasterol and fucosterol, possibly due to the presence on the former of one or more endocyclic double bonds (in addition to the ethylidene group). The endocyclic bonds create other sites for free radical formation and delocalization. Recently, Burton and Ingold (1984) demonstrated the effectiveness of β-carotene as an antioxidant at very low oxygen pressures (e.g. 15 torr). They theorized that the β-carotene antioxidant effect was based on the stability of a carbon-centered radical. This very stable radical subdues further oxidative chain reactions by trapping the peroxyl radicals on the conjugated double bond system of β-carotene and other effective carotenoids (Terao, 1989). There are similarities between the theories for antioxidant effectiveness of β-carotene and of compounds containing an ethylidene group, although the compounds work at different temperatures.

Although LA was not particularly useful at reducing oxidation of cholesterol or even of triglycerides in heated lard under the present conditions, it has been shown to be effective in other systems (Yan and White, 1987; Willemse, 1989). It may be helpful to study other structure-reactivity (e.g. polarity of an antioxidant and its effectiveness in different food matrices) relationships that would enhance the effectiveness of LA in different loci of autoxidation, such

as autoxidation of bulk fats and oils and the autoxidation of membranes as suggested by Simic and Hunter (1985) and Porter (1980).

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SUMMARY

Several studies have shown that sterols that contain an ethylidene side chain can act as high-comparative antioxidants (Sims et al., 1972; Boskou and Morton, 1975; Gordon and Magos, 1983; White and Armstrong, 1986). It was suggested by Gorden and Magos (1983) that the ethylidene side chain reacts rapidly with lipid free radicals to form stable allylic tertiary free radicals that interrupt the oxidation chain. This theory was further tested and strengthened in the current study. A compound with the ethylidene structure but without the sterol moiety (linalyl acetate) and another compound with an ethylidene-like structure (undecylenic acid) were shown to delay triglyceride oxidation in soybean oil heated at 180 C for 7 hr a day for 56 to 70 hr when compared to a control sample. Fatty acid changes and conjugated diene formation were monitored. Purchased and laboratory-prepared LA were effective in delaying oleate, linoleate and linolenate oxidations. Undecylenic acid provided some protective effect but less than that of LA.

The use of these antioxidants to reduce oxidation of cholesterol in lard was studied. Establishment of an appropriate method for measuring cholesterol oxidation products was needed so the method of Park and Addis (1985, 1986a) for quantification of cholesterol oxidation products (COPs) was evaluated. Slight modifications of the procedure were necessary to adapt it to the samples and laboratory set-up in the current study. Good resolutions between the internal standard, cholesterol and the six cholesterol oxidation products (COPs) were achieved in most cases. Recoveries of the COPs ranged from 70 to 95%.

Lard with two different levels of added cholesterol were heated at 180 C for up to 240 hr. Loss of cholesterol and formation of COPs were monitored. Cholesterol steadily decreased throughout the heating period in Test I but followed a first order reaction rate in Test II. Cholesterol oxidation products accumulated in both Tests I and II. However, the total amount of COP formation did not add up to the amount of cholesterol lost. It seems likely that cholesterol undergoes thermal degradation at frying temperatures. But when the frying media were coolded down nightly, it resulted in enough hydroperoxides that further oxidized into COPs such as 7-ketocholesterol (7-keto) and the epimeric 7-hydroxycholesterols (7-OHs). In this study, 7-keto was the predominant COP while the very atherogenic 3b, 5a, 6b-cholesterol and 25-hydroxycholesterol were not detected or detected in very low concentration.

Finally, the antioxidants that had been previously shown to be effective at reducing triglyceride oxidation in soybean oils were tested in lard. Two tests were designed in which cholesterol was added to enhance the ability to measure COP formation. In lard samples with 10X added cholesterol, LA was tested at 0.02 and 0.04% and polydimethyl siloxane (MS) was tested at 0.2 ppm. In lard samples with 2X added cholesterol LA was tested at levels same as above and MS was tested at 1.0ppm. MS was effective in delaying triglyceride and cholesterol oxidation and 7-keto formation in lard with 2X added cholesterol in Test II where it was present at a level of 1.0ppm. The LA at 0.02 and 0.04% showed little effects. It is possible that linalyl acetate was not present at a high enough concentration to show an effect.

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