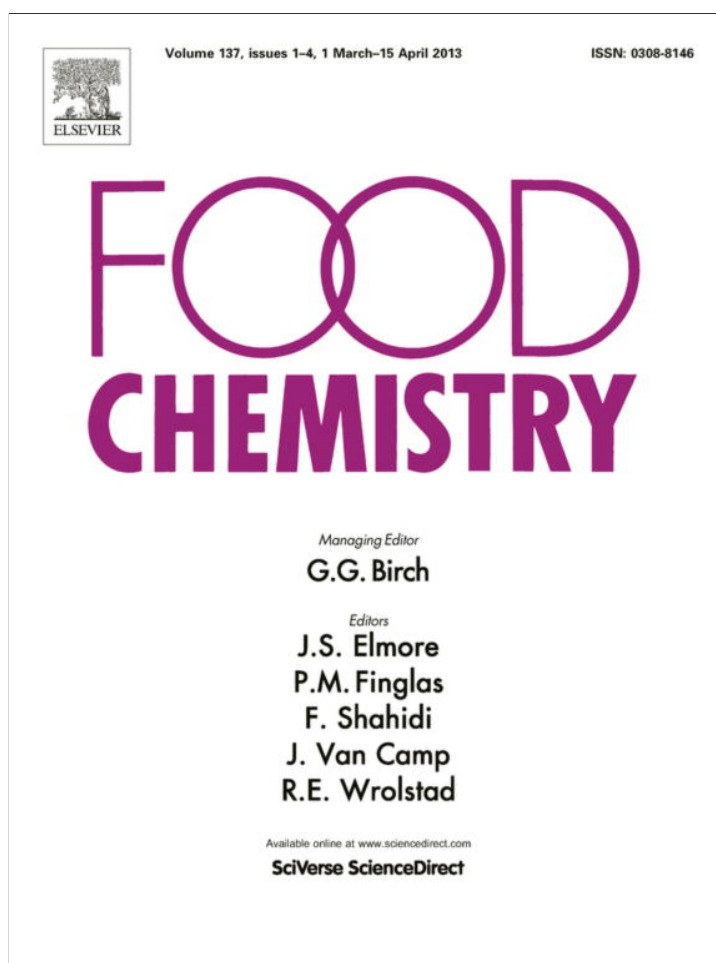


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Sitosterol as an antioxidant in frying oils

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ABSTRACT

The antioxidative effect of sitosterol at 1, 2 and 5% levels, in triolein, refined canola, high oleic sunflower and flaxseed oils, continuously heated for a period of up to 72 h at frying temperature of 180 °C, was studied. High Pressure Size Exclusion Chromatography (HPSEC) was used to monitor changes in peak areas of triacylglycerol (TG) polymer, monomer and ester hydrolysis products. The presence of enhanced levels of sitosterol was found to significantly decrease TG polymer formation in triolein and the vegetable oil samples after heating at 180 °C for a period of 72 h. A corresponding increase in the level of intact TG monomer and the extent of TG ester hydrolysis was observed in all samples with enhanced levels of sitosterol. Conversion of sterol to steradiene, by the 1, 2 elimination of water, may be responsible for the antioxidative effect of sitosterol at frying temperatures.

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1. Introduction

Phytosterols are cholesterol-like molecules, found in all plants with the highest concentration occurring in vegetable oils (Ostlund, 2002). They make up a major portion of the unsaponifiable content of vegetable oils either as free sterols or esterified to fatty acids. The major plant sterols present in vegetable oil include sitosterol, campesterol, stigmasterol, brassicasterol, and avenasterol (Moreau, Whitaker, & Hicks, 2002). Despite, their widespread occurrence, there are relatively few published reports on the effect of plant sterols on vegetable oil stability at frying temperature, as described below.

Gertz, Klostermann, and Kochhar (2000) have suggested that the stability of vegetable oils at frying temperature may be a function of more than just the fatty acid composition and have reported a co-relationship between the unsaponifiable matter content and oxidative stability at elevated temperatures (OSET), which is the inverse percentage of polymerized triacylglycerols (TG) that form when oil is heated at 170 °C in the presence of water-conditioned acid silica gel for 2 h. The addition of phytosterols in refined rapeseed and sunflower oils at levels of 0.25% (w/w) was found to significantly increase the OSET value, implying a decrease in the formation of TG polymers.

Gordon and Magos (1983) hypothesized that sterols with an ethylidene containing side chain may function as antioxidants by enabling lipid free radicals to react with the unhindered allylic

protons on the side chain, with the resulting radical rearranging to a relatively more stable tertiary free radical. They found antioxidant activity for Δ -5 avenasterol and fucosterol, each of which possess an ethylidene side chain, in a triglyceride mixture similar in composition to olive oil, heated at 180 °C for 72 h. Sims, Fioriti, and Kanuk (1972) have also reported antioxidant activity for vernosterol, Δ -7 avenasterol and fucosterol, in safflower oil heated to 180 °C but found sterols with no ethylidene side chain, ergosterol, lanosterol, β -sitosterol, stigmasterol and cholesterol, ineffective as antioxidants.

Recent reports on the impact of phytosterols on the thermal and oxidative stability of frying oils have been mixed. A phytosterol blend, which included, 3.8% brassicasterol, 26.9% campesterol, 0.6% campestanol, 17.2% stigmasterol, 48.2% β -sitosterol, 1.1% sitostanol, 1.3% Δ 5-avenasterol, and 0.8% Δ 7-stigmastanol, was found to significantly decrease polymerization in stripped soybean oil heated over a period of 8 h, at a concentration of 1.0% and 2.5%. However, the same phytosterol blend at a concentration of 2.5% was found to significantly increase polymerization of stripped high-oleic sunflower oil over a 12 h heating period (Winkler & Warner, 2008).

Studies assessing the anti-polymerization activity of oat sterols found significantly lower amounts of polar compounds in soybean and cottonseed oil samples heated at 180 °C for 10 days in the presence of oat extract, in comparison to oils containing tertiary butyl hydroquinone (TBHQ), dimethyl polysiloxane (DMS) and oils with no additives (Tian & White, 1994). White and Armstrong (1986) found soybean oil samples heated at 180 °C to deteriorate more slowly in the presence of oat sterol fractions containing

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$\Delta 5$ -avenasterol when compared to samples containing pure β -sitosterol, which altered at a rate similar to that of the controls. Another study evaluating the effect of soy sterols consisting of β -sitosterol (45.7%), campesterol (27.3%), stigmasterol (15.3%), and brassicasterol (4.4%) on soybean oil and distilled soybean oil methyl esters found no improvement in the oxidative stability index (OSI) of the oil substrates (Wang, Hicks, & Moreau, 2002).

Commercially used antioxidants, such as butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT) and tertiary butyl hydroquinone (TBHQ), have been reported to be heat-sensitive and volatile, hence, lack antioxidant activity at frying temperatures. Buck (1981) analyzed the volatility of BHA, BHT and TBHQ in soybean oil and reported a 70% reduction in concentration of all additives after 4 h of heating at 180 °C. Dimethyl polysiloxane (DMS) has been known to be effective in extending frying life of oils by functioning as an antifoaming agent and providing a protective inert surface that inhibits oxidation (Fritsch, Egberg, & Magnuson, 1979). However, synthetic additives in edible oil are not desirable and so it is useful to study the anti-polymerization effect of natural vegetable oil sterols at frying temperature.

Refining of vegetable oils is known to result in the removal of considerable amounts of unsaponifiables, including sterols and endogenous antioxidants, thus lowering their natural oxidative stability at frying temperatures in comparison to the non-refined vegetable oils (Andrikopoulos, Tzamtzis, Giannopoulos, Kalantzopoulos, & Demopoulos, 1989). Hence, a study was designed to highlight the need to minimize sterol loss during refining and gain more insight into the effect of vegetable oil sterols on TG polymer formation in refined vegetable oils and triolein heated to a frying temperature of 180 °C for up to 72 h. A sterol blend, comprising of β -sitosterol, β -sitostanol and campesterol, which together account for around 75–80% of sterols commonly found in vegetable oils (Przybylski, Mag, Eskin, & McDonald, 2005), was used at different levels. A mechanistic explanation based on the structural features of β -sitosterol and campesterol is proposed, which is indicative of their potential importance as antioxidants at frying temperatures.

2. Materials and methods

2.1. General

Refined canola and flaxseed oil samples were obtained from Richardson Oilseed Company, Canada and high oleic sunflower oil was obtained from Adams Company, USA. Triolein (99% pure) was obtained from Nucheck Inc., USA. Test samples with 1%, 2% and 5% additional sterol were prepared by adding 20 mg, 40 mg and 100 mg of β -sitosterol (assay ~60%, residual campesterol and β -sitostanol) obtained from Fluka Analytical, to 2 g of each oil, canola, high oleic sunflower, flaxseed and triolein contained in 7 ml open mouth glass vials, followed by gentle mixing by vortex. The samples and controls with no additional sterol were then placed in a Stabil-Therm gravity oven (Illinois, USA) set at 180 °C for durations that ranged from 12 h to 72 h. Small aliquots, drawn in duplicate from each vial at time intervals of 12, 24, 36, 48, 60, and 72 h, was dissolved in THF to a concentration of 5 mg/ml and analyzed twice by High Pressure Size Exclusion Chromatography (HPSEC). The final result was an average of the four measurements.

The HPSEC system included an Agilent 1200 series binary pump (G1312 A) and a degasser (G1379 B), Agilent 1200 series (G13229 A) auto sampler and Agilent 1200 series evaporative light scattering detector (ELSD). Two size exclusion columns were used in series, a Waters styragel HR 100 Å (4.6 × 300 mm) and a Phenomenex phenogel 500 Å (4.6 × 300 mm). Tetrahydrofuran (HPLC-grade;

Fischer scientific) was used as the mobile phase at a flow rate of 0.3 ml/min.

The chromatogram of triolein before heating with a single TG monomer peak at retention time ~17.7 min is shown in Fig. 1a. A typical chromatogram of triolein heated at 180 °C for 72 h (Fig. 1b) included peaks corresponding to the TG polymer at retention time ~12.8 min, TG monomer at retention time ~17.7 min and TG ester hydrolysis products at a retention time of ~18.4 min. Percentage peak area, obtained by drawing vertical lines connecting the starting and ending retention points of a peak to the base line using the Chemstation software, served as the basis to follow changes in TG polymer formation, level of intact TG monomers and the extent of TG ester hydrolysis.

2.2. Statistical analysis

Treatment effects on TG polymer formation, level of TG monomers and the extent of TG ester hydrolysis in triolein, canola, high oleic sunflower and flaxseed oils were compared by repeated measures analysis of variance using Sigma Plot 11 (Systat Software Inc., San Jose, CA). The mean peak areas for TG polymer, TG monomers and TG ester hydrolysis products for different treatments at each time point were compared using Tukey's multiple comparisons test, where $p \leq 0.05$ was deemed statistically different.

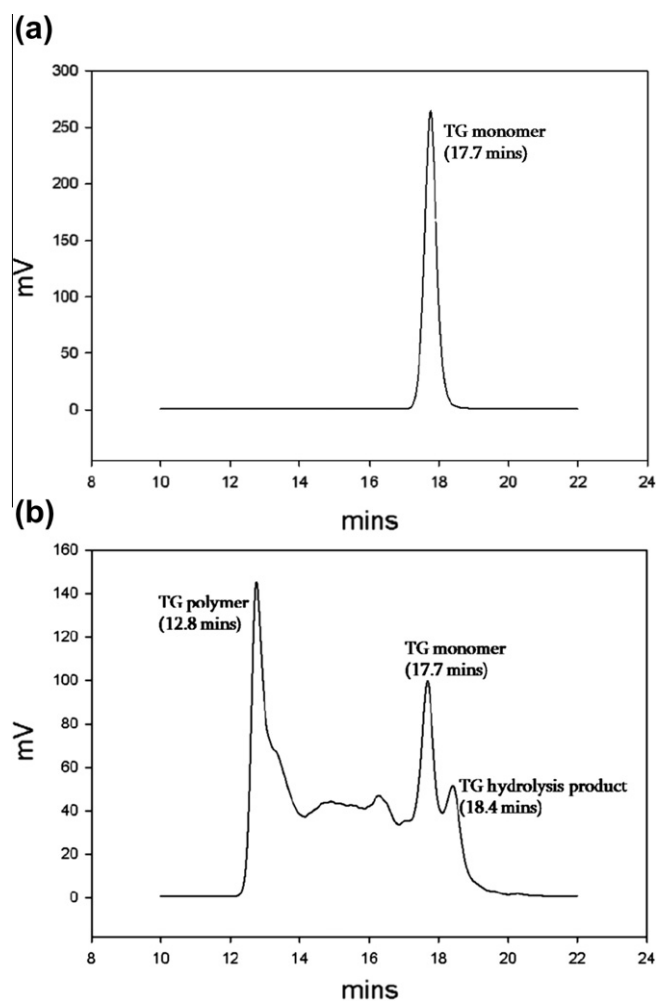


Fig. 1. HPSEC chromatograms for triolein, (a) before heating, (b) after heating at 180 °C for 72 h.

3. Results and discussion

As expected, the level of unsaturation was found to influence the rate of TG polymerization, as shown by the appearance and growth of the TG polymer peak at retention time of ~ 12.8 min in the HPSEC chromatogram for the vegetable oil and triolein samples heated at 180°C for 48 h. (Fig. 2a) In triolein, canola and sunflower oil samples, the TG polymer peak first appeared after 48 h of heating at 180°C . On the other hand, flaxseed oil, with a linolenic content of about 53% required only 24 h of heating for TG polymer formation and with the exception of samples with the highest level of additional sterol (5%) all flaxseed oil samples were solid after 24 h of heating. Fig. 2b represents the appearance and growth of the TG polymer peak in triolein over a 72 h heating period.

The effect of sterol addition on the formation of TG polymer in triolein heated for 72 h at 180°C is shown in Fig. 3a and plotted in Fig. 3b. These two figures are also representative of the data obtained for canola, high oleic sunflower and flaxseed oils. In triolein, about 40% decrease in TG polymer formation was observed in the presence of 5% sterol after 72 h of heating at frying temperature. Similarly for canola, after 72 h of heating in the presence of 5% additional sterol, a 21% reduction in TG polymer formation was seen compared to samples with no additional sterol. In sunflower oil, 5% additional sterol reduced TG polymer formation by about

50% after 72 h of heating. The extent of TG polymer formation in flaxseed oil after 24 h of heating was about 54% less in samples with 5% additional sterol compared to samples without any additional sterol. Overall, the addition of sterols was found to significantly decrease the extent of TG polymer formation in triolein, canola, high oleic sunflower and flaxseed oils heated at frying temperature.

The presence of additional sterol was also seen to impact the amount of intact TG monomer remaining in oils after heating at 180°C for 72 h. This is illustrated by the HPSEC peak areas at retention time 17.7 min for TG monomer in triolein samples with different levels of sterol (Fig. 3a and c). The impact of sterol addition on the relative increase in % peak area for TG monomer remaining after heating at 180°C was found to be substantial during the 48–72 h period, corresponding with the appearance and growth of the TG polymer peak, in triolein samples with 5% additional sterol.

Based on the retention time of 18.4 min for a diolein standard under identical conditions, it was concluded that the peak at retention time 18.4 min in the heated triolein HPSEC chromatogram (Fig. 1b) was indicative of the extent of TG ester hydrolysis that would occur during frying. The addition of sterol appeared to have a significant impact on the extent of TG ester hydrolysis in each of the vegetable oil samples and triolein. The effect of additional sterol on the extent of TG ester hydrolysis in triolein is shown in Fig. 3a and plotted in Fig. 3d. In the case of triolein, addition of 5% sterol resulted in an average increase in TG ester hydrolysis of about 19% during the first 24 h of heating compared to samples without any additional sterol. In contrast, during the 48–72 h heating period, the average increase in TG ester hydrolysis in triolein samples was found to be 74%. In canola, the percentage increase in the extent of TG ester hydrolysis for samples with 5% additional sterol was 55% during the first 24 h and 96% in the times between 48 and 72 h. With sunflower oil, the average increase in TG ester hydrolysis in samples with additional 5% sterol was found to be about 63% in the first 24 h and 94% in the following 48–72 h period. In flaxseed oil, in the 24 h heating period the extent of TG ester hydrolysis was found to increase by almost 33% in the presence of additional 5% sterol.

The increase in TG ester hydrolysis that occurred during the initial 48 h of heating for all samples, both with and without sterol addition, may be due to the interaction of TG esters with hydronium ions resulting from atmospheric moisture at the air–oil interface. Once the TG ester hydrolysis attained equilibrium after ~ 48 h, effects due to higher levels of sterol became more prominent (Fig. 4). This is not unexpected, given the higher proton affinity of TG esters in comparison to the sterol OH group. The greater extent of TG ester hydrolysis during the 48–72 h heating period, seen for all vegetable oil and triolein samples with additional 5% sterol in comparison to samples with no additional sterol, may be attributed to the water released in the system during the conversion of Δ^5 -sterol to $\Delta^3, 5$ -steradiene by the 1, 2 – elimination reaction (Scheme 1). Conversion of sterol to steradiene by the elimination of water has also been reported during the bleaching and deodorization steps of vegetable oil refining (Verleyen, Cortesa, Verhea, Dewettinck, Huyghebaert, & De Greyt, 2002).

Despite the absence of structural features of avenasterol and vernosterol, known to be responsible for their antioxidative effect, (Gordon & Magos, 1983; Sims et al., 1972) the sitosterol blend used in this experiment was found to be effective in limiting TG polymer formation in triolein, canola, high oleic sunflower and flaxseed oils heated at frying temperature.

In the case of avenasterol, it was hypothesized that the presence of an ethylidene side chain, with unhindered allylic hydrogen atoms, promotes release of hydrogen for the termination of lipid oxidation chains with the resulting radical isomerizing into a more

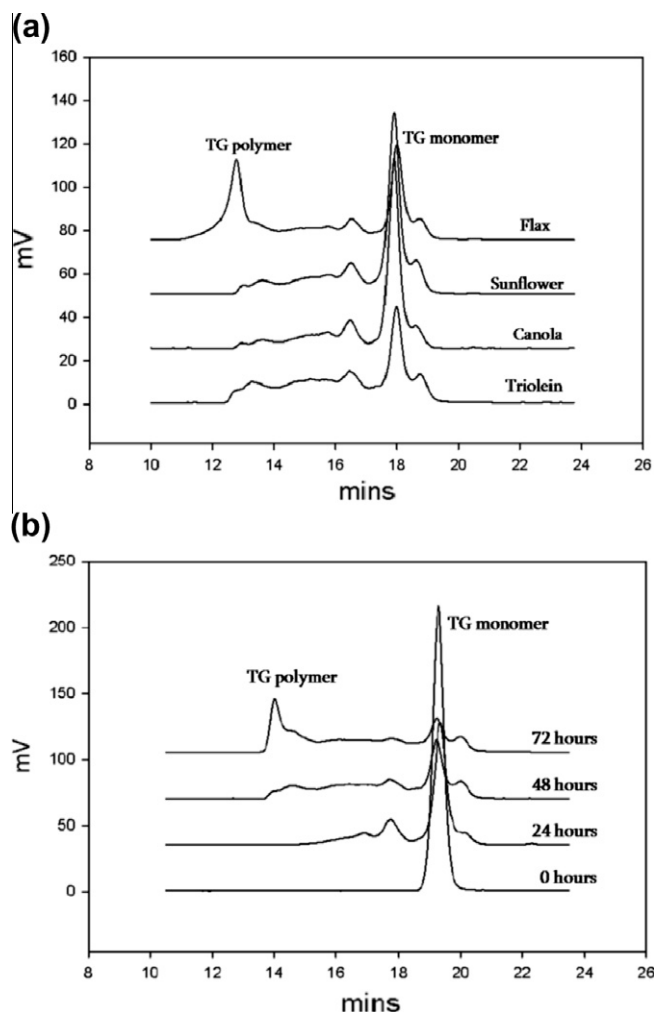


Fig. 2. HPSEC chromatograms for oil samples and triolein heated at 180°C in the presence of 5% sterol, (a) triolein, canola, sunflower and flax heated for a period of 48 h, (b) triolein heated for a period of 24, 48, 60 and 72 h.

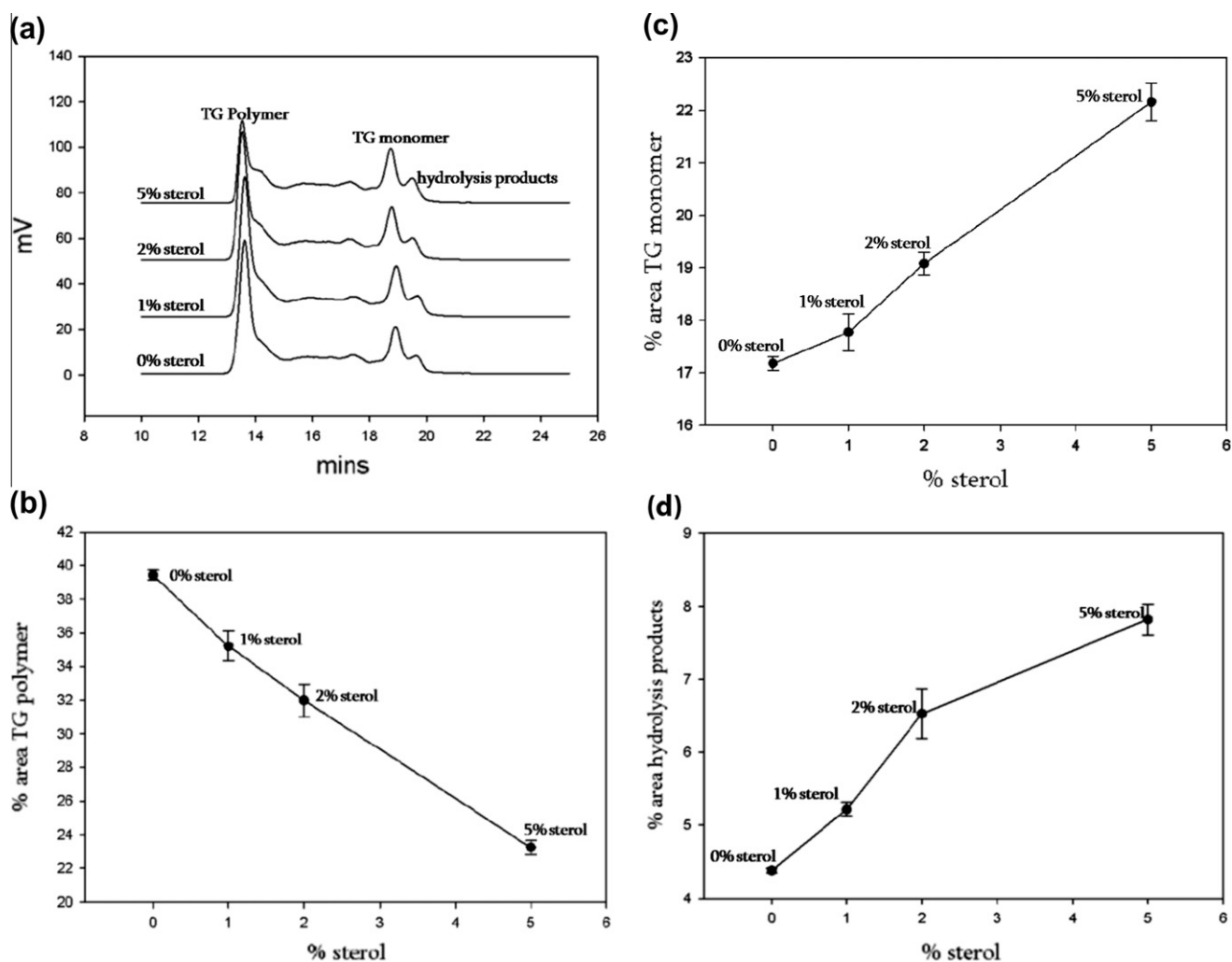


Fig. 3. Triolein heated at 180 °C for 72 h in the presence of different levels of sterols, (a) HPLC chromatograms, (b) effect on TG polymer formation, (c) effect on the level of intact TG monomer, (d) effect on the extent of TG ester hydrolysis.

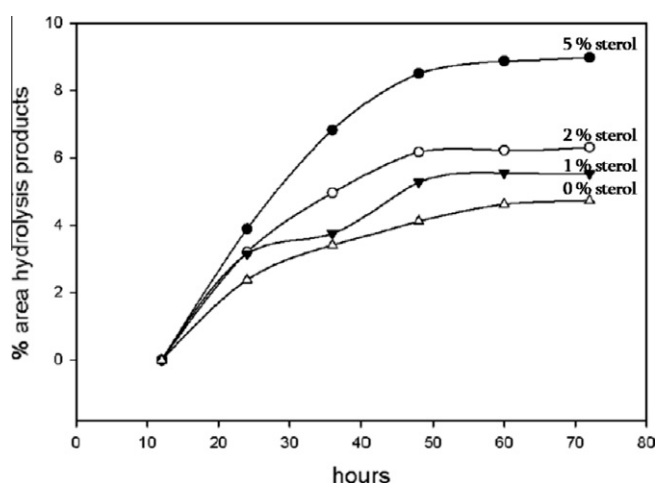


Fig. 4. Progression of the extent of TG ester hydrolysis with time in triolein heated at 180 °C over a 72 h period in the presence of different levels of sterols.

stable tertiary free radical. The higher antioxidative effect in vernosterol has been attributed to the presence of two endocyclic double bonds at the C₈ and C₁₄ positions in addition to the ethylidene side chain. This makes available two further allyl hydrogens

at C₁₁ and C₁₆ to interact with and terminate lipid oxidation chains, with the resulting radicals being stabilized by delocalization over the conjugated diene system.

The structural features in sitosterol and campesterol include an OH group at C₃ position and one endocyclic double bond at the C₅ position. Based on physical organic chemistry principles, (Sykes, 1986) the presence of a double bond at the C₅ position with its ability to stabilize through conjugation a developing double bond at the C₃ position, would strongly favour a 1, 2 - elimination reaction involving the OH group at C₃ and the allylic H at C₄ carbon atoms. The loss of water involving the OH group at C₃ and the allylic H at C₄ would convert sterol to steradiene generating a conjugated diene system comprising of C₃-C₄-C₅-C₆ atoms. This would make the allylic H at C₂ and C₇ more labile and hence available to interact with and terminate lipid oxidation chains, with the resulting radicals being stabilized by delocalization over the conjugated diene system (Scheme 1). Hydronium ions resulting from atmospheric moisture at the air-oil interface may catalyze the elimination reaction resulting in the conversion of sterol to steradiene. Hydronium ions with a known proton affinity of 166.4 kcal/mol would readily protonate the OH group at C₃ position in sterol, which would be comparatively more basic, based on the known proton affinities for CH₃OH (181.0 kcal/mol) and CH₃CH₂OH (186.8 kcal/mol) (Mackay, Tanner, Hopkinson, & Bohme, 1979).

Elimination E1 reactions are normally accompanied by S_N1 substitution as both proceed through a common carbocation

intermediate. However, at higher temperatures, elimination would be favoured over substitution, which may be attributed to the fact that elimination is accompanied with an increase in number of particles in comparison to substitution, resulting in a more positive entropy term (ΔS). This would result in a lower free energy of activation (ΔG^\ddagger), given by the, $\Delta G^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger$ as at higher temperatures the $T\Delta S^\ddagger$ term would increasingly outweigh a less favorable ΔH^\ddagger term. Also, given the large size of the attacking sterol nucleophile, steric effects would play a role in restricting substitution and ensuring a higher proportion of elimination. In addition, sp^2 hybridized carbon atom in the carbocation would remain sp^2 hybridized on elimination, but would have to undergo a change in hybridization to sp^3 on substitution, which would be another factor favoring elimination.

In order to demonstrate the possible conversion of sitosterol to sitostadiene at frying temperature, HPSEC chromatograms were obtained for sitosterol heated at 180 °C for 48 h, using both ELSD and UV detectors. For comparison, cholestadiene, a commercially available steradiene obtained from cholesterol, was used as a standard.

In Fig. 5, trace 1 represents the ELSD chromatogram for sitosterol heated at frying temperature with the single peak at retention time 20.3 min representing sitosterol. Trace 2 represents the UV absorbance of heated sitosterol at wavelength 232 nm, which is the absorbance maximum for cholestadiene. Trace 3 represents the ELSD chromatogram for heated sitosterol and cholestadiene, used as a retention time standard and shown by the peak at 21.1 min. Trace 4 is part of the ELSD chromatogram for heated sitosterol, covering a retention time range of 20.3–22.0 min. The single peak with a retention time of 20.9 min in trace 4 may represent the sitostadiene peak as it closely matches the cholestadiene retention time of 21.1 min. The slight shift (0.2 min) to the left for sitostadiene in comparison with cholestadiene may be attributed to the comparatively bigger size of sitostadiene due to the presence of an additional ethyl group in the side chain at the C-24 position. In addition, appearance of a peak at the retention time of 20.9 min in trace 2, representing the UV absorbance of heated

sitosterol at wavelength 232 nm, may also indicate the likely presence of sitostadiene in sitosterol heated at frying temperature.

Corroboration of the fact that conjugated diene system of steradiene played a role in limiting TG polymer formation in frying oils was obtained by comparing the effects of the presence of sitosterol and sitostanol on TG polymer formation in triolein heated at 180 °C. Sitostanol, due to the absence of a double bond at the C₅ position would not generate a conjugated diene system, that would readily form in sitosterol upon the 1, 2 – elimination of water involving the OH group at C₃ and the allylic H at C₄.

Samples of triolein, triolein + 5% sitostanol and triolein + 5% sitosterol were heated at 180 °C for a period of 72 h. The resulting HPSEC chromatogram for each of the samples is shown in Fig. 6. Trace 1 represents triolein with no sitosterol or sitostanol addition, heated at 180 °C for 72 h, giving a relative TG polymer peak area of

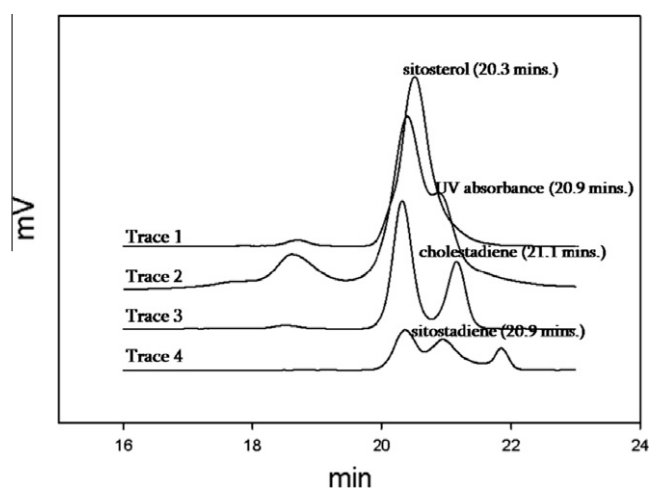
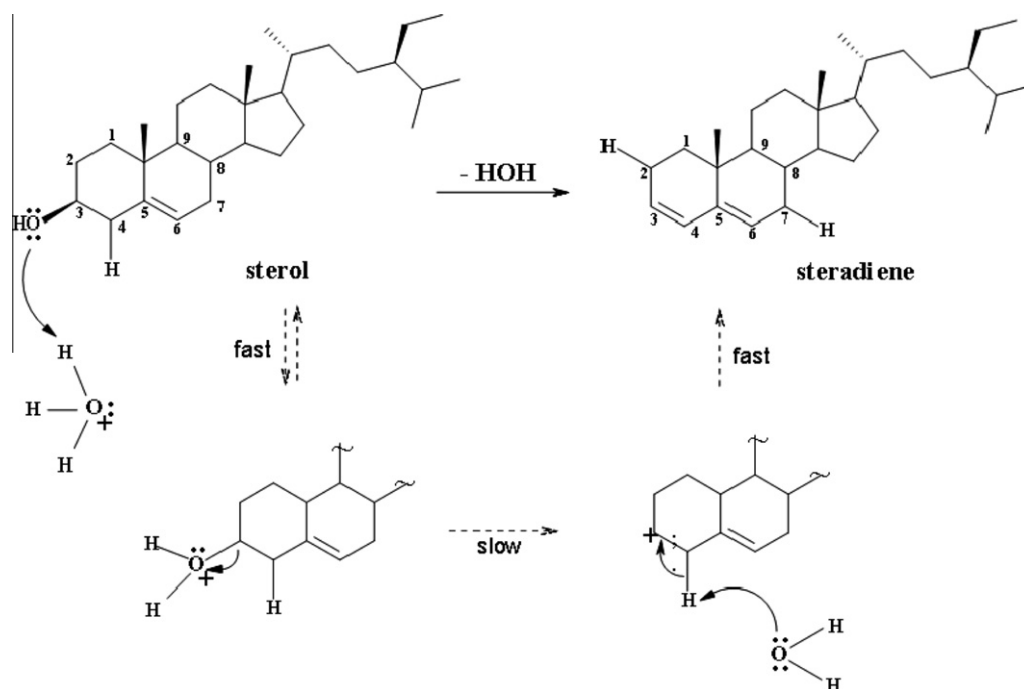


Fig. 5. HPSEC chromatograms with ELSD (trace 1, 3, 4) and UV (trace 2) detection for sitosterol heated at 180 °C for 48 h.



Scheme 1. 1, 2 – Elimination of water from sitosterol to generate sitostadiene.

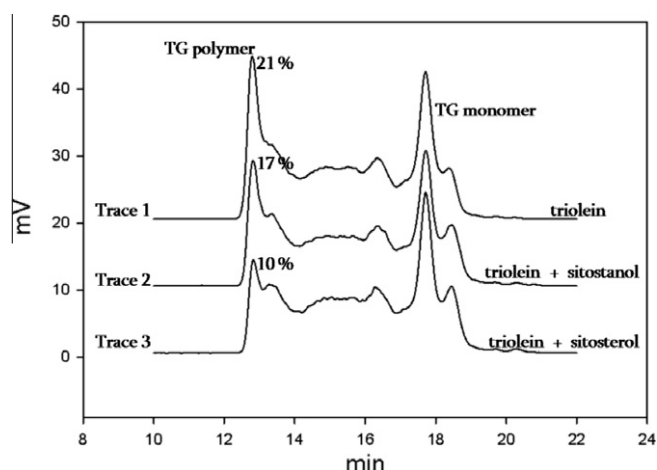


Fig. 6. Effect of the presence of sitosterol and sitostanol on TG polymer formation in triolein heated at 180 °C for 72 h.

21%. Trace 2 represents triolein heated in the presence of 5% sitostanol showing a reduced TG polymer peak area of 17%. Trace 3 indicating a comparatively low TG polymer peak area of 10%, represents a triolein sample heated in the presence of 5% sitosterol. The inability of sitostanol to greatly limit TG polymer formation in triolein samples heated to frying temperatures may be attributed to the fact that unlike sitosterol, sitostanol cannot generate a conjugated diene system. Hence this observation is further evidence that formation of a conjugated diene system in sitosterol plays an important role in limiting the extent of TG polymerization in frying oils.

4. Conclusion

Presence of additional sitosterol was found to limit TG polymer formation in triolein, refined canola, high oleic sunflower and flaxseed oils heated at frying temperature. The anti-polymerization effect of sitosterol has been attributed to the conversion of sterol to steradiene by the 1, 2 - elimination of water. This fact was further corroborated by the significant increase in TG ester hydrolysis during the 48–72 h period, corresponding with the appearance and growth of the TG polymer peak.

Recent findings have established that unrefined oils have higher stability at frying temperature compared to refined oils which tend to lose some of the natural components including sterols at various stages of refining, particularly during neutralization and deodorization. The unsaponifiable fraction in the soap stock generated from the neutralization process has been found to contain on an average 70% sterols (Gutfinger & Letan, 1974) while the concentration of sterols in the deodorizer distillate has been reported to range from 2% to 20% (Verleyen, Verheea, Garcia, Dewettinck, Huyghebaert, & De Greyt, 2001). Overall, sterol losses during the complete refining of vegetable oils, has been reported to range between 10% and 70% (Kochhar, 1983).

Hence, given the effectiveness of sterols, naturally found in vegetable oils, in limiting TG polymer formation at frying temperature, ensuring presence of an optimum level of sterols in refined oils is of critical importance.

Also, triolein and vegetable oil samples with 5% additional sterol were found to have the lowest level of TG polymer formation, in comparison to control and samples with 1% and 2% additional sterol, after heating at 180 °C for 72 h. Hence, vegetable oils enriched

with sterols, at levels higher than those naturally present, may be considered as a means of further enhancing oxidative stability at frying temperatures. This would also ensure higher levels of sterols in fried foods, which may be desirable given their known anti-cholesterolemic effects (Hayes, Pronczuk, & Perlman, 2004). Interest in foods enriched with sterols has soared in recent times with products, including cooking oils, diacylglycerol, spreads, salad dressings, mayonnaise, chocolate, yogurt, bread and breakfast cereal being marketed globally (Moreau, 2003).

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