2015

Analysis of co-crystallized free phytosterols with triacylglycerols as a functional food ingredient

Danielle Franchetti
Iowa State University

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Analysis of co-crystallized free phytosterols with triacylglycerols as a functional food ingredient

by

Danielle Franchetti

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Food Science and Technology

Program of Study Committee:
Nuria Acevedo, Major Professor
Lester Wilson
Suzanne Hendrich

Iowa State University
Ames, Iowa
2015
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>FHSO</td>
<td>Fully Hydrogenated Soybean Oil</td>
</tr>
<tr>
<td>SO</td>
<td>Soybean Oil</td>
</tr>
<tr>
<td>FPS</td>
<td>Free Phytosterol</td>
</tr>
<tr>
<td>β-Sit.</td>
<td>β-Sitosterol</td>
</tr>
<tr>
<td>Stig.</td>
<td>Stigmasterol</td>
</tr>
<tr>
<td>DSC</td>
<td>Differential Scanning Calorimetry</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>WXRD</td>
<td>Wide Angle X-Ray Diffraction</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential Interference Contrast</td>
</tr>
<tr>
<td>OL</td>
<td>Oil Loss</td>
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CHAPTER 1
GENERAL INTRODUCTION

Phytosterols have been studied for many years for their health benefits. In previous studies, an intake of 1-3 g/day of plant sterols has been shown to produce a 10-15% reduction in LDL cholesterol (Ling & Jones, 1995; Kritchevsky & Chen, 2005; Kuhlmann et al., 2005). However, the typical western diet contains only 150-400 mg/day and therefore does not reach desired levels to obtain the aforementioned health benefits (Ostlund, 2002). Consequently, supplemental doses are required.

Previous strategies of phytosterol utilization include ultrafine powders, chemically modified esterified phytosterols, emulsified phytosterols, and the most susceptible to oxidation are phytosterols in water-oil microparticulate suspensions (Perlman, Hayes, & Pronczuk, 2006). These strategies, along with being susceptible to oxidation, cause inconvenience during processing and added costs.

Thus, the objective of this research project included the health benefits of phytosterol supplementation, specifically chemically unmodified free phytosterols to reduce production cost in industry, as well as increase convenience in processing. Furthermore, the use of local soybean oil and low amounts of fully hydrogenated soybean oil to create a semisolid product as a substitute for partially hydrogenated fats containing unhealthy trans fatty acids. Puff pastry shortening typically contains high amounts of trans fats due its vast quantity of partially hydrogenated fats. Therefore, this study sought to mimic desired properties of this high-trans shortening by comparatively analyzing phytosterol enriched plastic fats against a commercial puff pastry shortening.
CHAPTER 2
LITERATURE REVIEW

1. Phytosterols/stanols chemical composition

The general term ‘phytosterols’ describes plant-derived sterols and stanols with a chemical structure related to cholesterol, with a different side chain configuration (Cantrill, 2008; Spitzer & Maggini, 2013) (Figure 2.1). The differentiating feature in the chemical structure of stanols, as compared to sterols, is the absence of a double bond at the Δ5 in the steroid skeleton B-ring (Spitzer & Maggini, 2013). Studies have shown that this minor structural difference does not have an influence in regards to clinical effectiveness (Hallikainen & Sarkkinen, 2000). However, stanols comprise only about 10% of total dietary phytosterols, and are therefore considered less significant than sterols (Spitzer & Maggini, 2013). Sitosterol is the most common phytosterol found in various foods, followed by campesterol and stigmasterol (Ostlund, 2002).

While in humans, most of the total body cholesterol is free, plants have a much greater amount of total sterols in long-chain fatty acid ester form (Figure 2.2, top) (Kochhar, 1983). While, free phytosterols are present, they are in much smaller concentrations (Ostlund, Racette, Okeke, & Stenson, 2002). Besides long-chain fatty acid esters, phytosterols are also found in ferrulate esters in foods (Figure 2.2, middle) (Ostlund, Racette, Okeke, & Stenson, 2002). Glycosylated phytosterols are also a minor component in most foods, except in the case of potatoes where about 82% of total phytosterols are glycosylated (Figure 2.2, bottom) (Ostlund, 2002).
Figure 2.1 Common phytosterols and cholesterol structure
Figure 2.2 Forms of phytosterols present in plants with modifications of the 3-β-hydroxyl group. R represents an acyl group found in some phytosterol glycosides.

However, the alkaline conditions used to quantify sterols by hydrolyzing the sterol esters cannot cleave glycosidic linkages (Ostlund, 2002). Glycosidic linkages require acidic hydrolysis and therefore glycosylated phytosterols are not measured by common procedures for quantification of phytosterols (Ostlund, 2002).
It should also be noted that pancreatic enzymes could not cleave glycosylated phytosterols during in vitro studies, therefore their bioactivity has been doubted (Moreau & Hicks, 2004).

2. Functional Use: phytosterols

Phytosterols are found in all plant foods, with the highest concentrations occurring in vegetable oils (Ostlund, 2002). Other common sources of phytosterols and stanols include wood pulp, leaves, nuts, seeds, and cereals (Noakes, Sullivan, Nestel, & Kritharides, 2009). Commercially available phytosterols are obtained from the deodorized distillate from soybean, palm, or pine oil (Noakes et al., 2009). In general, the refining process of vegetable oils gradually reduces the sterol content of the oil (Kochhar, 1983). It has been reported that the degree of reduction differs according to the quantity of sodium hydroxide, type of bleaching earth, conditions of hydrogenation and steam deodorization time and temperature or physical deacidification (Kochhar, 1983). Overall, due to processing and the type of oil, a 10-70% decrease in sterols has been notable.

For commercial use, phytosterol- and stanol powders are esterified with fatty acids in vegetable oils (Cantrill, 2008). The process of esterification manipulates the physical properties of the high melting powders that have low solubility in oils (Cantrill, 2008). The characteristics of the esters are similar to edible fats and oils, classified as liquid or semi-liquid (Cantrill, 2008). The phytosterol fatty acid esters, as well as free phytosterols, are incorporated into processed foods such as spreads, juices, oils, and other foods. Table 2.1 lists several food products from the United States of America and the amount of phytostanol esters present per serving (Cantrill, 2008).
Table 2.1 USA food products containing phytostanol esters; modified from (Cantrill, 2008)

<table>
<thead>
<tr>
<th>Type of Food</th>
<th>Phytostanol ester content/daily portion (equals 2g phytostanols)</th>
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<tbody>
<tr>
<td>Spread</td>
<td>3.4g/56g</td>
</tr>
<tr>
<td>Dressing</td>
<td>3.4g/30ml</td>
</tr>
<tr>
<td>Snack Bars</td>
<td>3.4g/62g</td>
</tr>
<tr>
<td>Candy Chews</td>
<td>3.4g/2 candies</td>
</tr>
<tr>
<td>Capsules</td>
<td>3.4g/6 capsules</td>
</tr>
</tbody>
</table>

3. Non-esterified phytosterols

Until recently, the majority of studies involving phytosterols or phytostanols have been strictly on the use of esterified phytosterols or phytostanols. There have been few studies addressing the use of free (non-esterified) phytosterols and phytostanols in commercial foods due to their limited solubility and disputes on bioavailability as compared to their esterified counterparts (Hayes, Pronczuk, & Perlman, 2004).

However, researchers have proven when free phytosterols are effectively heated and then recrystallized in fat upon cooling, they are made bioavailable and therefore effective in reducing cholesterol absorption. In a study utilizing free phytosterols recrystallized in frying oil to create phytosterol enriched tortilla chips, researchers proved that even though non-esterified phytosterols have low solubility (1.5% by weight) in dietary fat, free sterols can be heated and recrystallized in fat during cooling to obtain a triglyceride-recrystallized non-esterified phytosterol complex (TRPs) (Hayes et al., 2004; Perlman et al., 2006).
The researchers of this study created a patent on the use of these TRPs. It was found that when TRPs are ingested, regardless of the crystalline size, they were successful at lowering mammalian plasma cholesterol levels (Perlman et al., 2006). The utilization of non-esterified phytosterols in fat, by recrystallization, lowers the cost of processing to make previously insoluble non-esterified phytosterols bioavailable and soluble in dietary fats. Previous strategies of phytosterol utilization include ultrafine powders, chemically modified esterified phytosterols, emulsified phytosterols, and the most susceptible to oxidation are phytosterols in water-oil microparticulate suspensions (Perlman et al., 2006).

There has been much debate on the degree of cholesterol lowering by free phytosterols as compared to esterified phytosterols. In a study where free versus esterified phytosterols were used to compare cholesterol lowering abilities, a similar (60%) decrease was found in cholesterol absorption for both free and esterified phytosterols (Richelle et al., 2004). The free phytosterols also presented a lower level of α-tocopherol and β-carotene reduction as compared to their esterified counterparts (See side effects of phytosterols section) (Richelle et al., 2004). Furthermore, in another study comparing free phytosterols versus free phytostanols, it was found that both constituents when supplemented in their free form result in equal LDL and total cholesterol concentrations (Vanstone, Raeini-Sarjaz, Parsons, & Jones, 2002).

Data was plotted from a review combining the results of 60 clinical studies with an average phytosterol dose of 2.8g/day and an average LDL cholesterol reduction of 10.9% (Normen, Frohlich, & Trautwein, 2004).
Despite previous controversy addressing free phytosterol bioavailability, the results from the plotted studies indicate free phytosterols/stanols had a slightly higher level of cholesterol lowering ability than phytosterol/stanol esters (Normen et al., 2004).

4. Stability of phytosterol, phytostanols, and their fatty acid esters

Typically, phytosterols and their fatty acid esters are stable compounds, but in the presence of oxygen under conditions such as high temperatures (>100°C), oxidation can occur (Cantrill, 2008). These conditions can occur during shallow frying of foods in phytosterol oils (Cantrill, 2008). In shallow frying, temperatures range from 160-200°C for 5-10 minutes (Cantrill, 2008). However, even at these harsh conditions phytosterol esters in liquid margarine remained below 1.3% oxidation levels (Cantrill, 2008). Most phytosterols and ester oil products on the market are typically low fat spreads with less than 40% total fat (Cantrill, 2008). These products are made for spreading, not with the intention for use in shallow frying (Cantrill, 2008). Phytosterol enriched spreads with higher levels of fat, greater than 60% total fat are intended for use in shallow frying, but still not for the more severe conditions of deep fat frying (Cantrill, 2008).

Concerning free versus phytosterol esters, the esters were found to be more susceptible to oxidation at higher temperature conditions than free phytosterols (Cantrill, 2008). Phytostanols and phytostanol esters are also relatively heat stable and show low oxidation (Cantrill, 2008). Other food processing and long-term storage conditions for phytosterols that can influence the autoxidation free-radical process include light, water or metal exposure (Soupas, Huikko, Lampi, & Piironen, 2006).
These same conditions along with quality of packaging and packaging materials could lead to sterol oxidation in long-term storage (Soupas et al., 2006).

In regards to microbiological stability, phytosterols and phytostanols are inactive as shown by no effect on yogurt processing in the fermentation steps (Cantrill, 2008). Not only do phytosterol and phytostanol esters show stability in products with large quantities of active bacteria like yogurt and yogurt-based beverages, they also have shown stability in milk and fermented milk (Cantrill, 2008). In long term storage, up to one year, phytostanol and phytosterol ester products show superior stability at various pH values (Cantrill, 2008).

5. Regulations on phytosterols

Official health claims and safety guidelines have been established addressing the use of phytosterols and phytosterol containing foods that are low in saturated fat and cholesterol as an option in lowering cholesterol levels (Spitzer & Maggini, 2013). The use of phytosterols as supplement to lower cholesterol has been well studied. More than 80 studies have been conducted with the use of phytosterols, and no major adverse effects have been reported (Spitzer & Maggini, 2013). The U.S. Food and Drug Administration (FDA) issued an official health claim regarding phytosterols in the year 2000 and has also recognized phytosterol esters as safe ingredients as long as a ‘self-GRAS’ (Generally Recognized As Safe) procedure has been followed (Spitzer & Maggini, 2013).
The approval of phytosterols in functional foods and supplements has been achieved outside of the United States as well, such as in the European Union (EU), Australia, Switzerland, Norway, Iceland, Brazil, South Africa, Japan, Turkey, and Israel (Spitzer & Maggini, 2013).

6. Side effects from phytosterol supplementation

A rare genetic disorder (approximately one in 6 million) associated with phytosterol absorption termed sitosterolemia or phytosterolemia is caused by mutations in the genes encoding the ABCG5 and ABCG8 cholesterol transport proteins (Noakes et al., 2009; Spitzer & Maggini, 2013). This mutational genetic disorder is characterized by promoting an increased absorption of phytosterols and cholesterol, resulting in an elevated risk for CHD for patients with this disorder (Patel & Thompson, 2006; Noakes et al., 2009). However, studies with the more common heterozygous carriers of the mutation showed that no abnormally elevated serum phytosterols could be observed after consumption of 3 g/day of phytosterols for 4 weeks and 2.2 g/day of phytosterols for 6–12 weeks (Spitzer & Maggini, 2013). The genetic disorder is rare and involves defective cholesterol as well as phytosterol transport, so it may not be directly relevant to phytosterol intake (Kwiterovich et al., 2003).

In regards to maximum dosages on effectiveness for cholesterol lowering, phytosterol intake which exceeded 3 g/day did not show any additional benefits (SFC, 2003). Long term studies have proven phytosterols to be well tolerated when consumed every day over the span of several months (Spitzer & Maggini, 2013). A few minor side effects have been reported in rare cases such as slight gastrointestinal discomfort including gas, diarrhea, and constipation (St-Onge & Jones, 2003).
Intestinal bacteria and male and female hormone levels are not affected by phytosterols (Hendriks, Brink, Meijer, Princen, & Ntanios, 2003; Spitzer & Maggini, 2013). However, there have not been any studies addressing the consumption of phytosterols during pregnancy in regards to safety and toxicity for the fetus (Spitzer & Maggini, 2013). Also, phytosterols have been proven to reduce β-carotene levels, which is of concern for populations with greater vitamin A needs, such as pregnant and lactating women (Richelle et al., 2004). Therefore, phytosterols are not yet recommended for pregnant or breastfeeding women (Spitzer & Maggini, 2013).

There are conflicting results on the effects of consumption of phytosterols on the resulting concentrations of fat-soluble vitamins. Fat-soluble vitamins and fat-soluble micronutrients known as carotenoids are transported by lipoproteins; lipoproteins are lowered by the consumption of phytosterols (Spitzer & Maggini, 2013). In a study involving phytosterol esters ingested in the form of a spread up to 1 year at 1.6g/day, plasma vitamin A concentrations did not drop (Hendriks et al., 2003). However, in the same study a slight but statistically significant decrease (7%) in plasma 25 hydroxyvitamin D3 concentrations could be detected (Hendriks et al., 2003). The study also proved Vitamin E levels diminished slightly, however those decreases are not relevant when plasma alpha-tocopherol concentrations are standardized to the concentration of LDL in cholesterol (Hendriks et al., 2003). In regards to vitamin K, studies have shown an absence of information providing a significant decrease in concentrations when consuming phytosterols (Raehini-Sarjaz, Ntanios, Vanstone, & Jones, 2002).

Even after standardizing carotenoid levels to LDL cholesterol concentrations, some studies have indicated consumption of phytosterols may lead to a 10–20% reduction in
plasma carotenoids (Ntanios & Duchateau, 2002). Further studies are needed to prove if this decrease will cause any health risks. However, researchers have noted that the reduced levels of carotenoids can be prevented by increasing the dietary intake of fruits and vegetables (Ntanios & Duchateau, 2002). It should also be noted that in a study comparing free versus esterified phytosterols on the reduction of α-tocopherol and β-carotene, free phytosterols had significantly lower levels of reduction (Richelle et al., 2004).

A possible explanation for the esters having a greater effect on fat-soluble vitamins and micronutrients, is that in the intestine, esters disperse into the oil phase whereas free sterols move into the micellar phase (Nissinen, 2002).

7. Cardiovascular Disease

The number one cause of death worldwide is cardiovascular disease (CVD) (WHO, 2011). In 2008, an estimated 17.3 million people died from CVD and by the year 2030 it is predicted that this number will grow to 23.3 million (WHO, 2011). Additionally, CVD comprises 17% of overall national health expenditures (Heidenreich et al., 2011). Total direct medical costs of CVD from 2010 to 2030 are expected to triple, from $273 billion to $818 billion (Heidenreich et al., 2011). Therefore, it is crucial to reduce the risk of CVD, ultimately reducing overall health care expenses.

There is a plethora of research proving dietary saturated and trans fats lead to an elevated LDL to HDL ratio and total cholesterol in the blood (Noakes et al., 2009). Hypercholesterolemia, a precursor for atherosclerosis, is a significant contributor in the development of cardiovascular disease (Noakes et al., 2009).
Plaque buildup on artery walls, from a diet high in cholesterol, restricts normal levels of blood flow in the artery, causing the heart to work harder to pump and circulate blood throughout the body.

8. Trans fats

*Trans* fatty acids ascend from either industrial hydrogenation, or from the natural process of biohydrogenation in ruminant animals (Brouwer, Wanders, & Katan, 2010). In industrial hydrogenation, partial hydrogenation of vegetable or fish oils with the use of hydrogen in the presence of a metal catalyst is used to produce trans fatty acids (Brouwer et al., 2010). *Trans* fatty acids naturally present in the rumens of cows and sheep, which are present in the fat in milk, butter, cheese, and beef, are produced from partial hydrogenation and/or isomerization of cis-unsaturated fatty acids from the feed (Brouwer et al., 2010). Hydrogen and a catalyst are still necessary for the formation of *trans* fatty acids in nature. However, the hydrogen in this process is produced during oxidation of substrates and bacterial enzymes are used as catalysts (Brouwer et al., 2010).

In industrial production of *trans* fats, the process of hydrogenation with a catalyst converts previously liquid vegetable and fish oils into semisolid fats. These semisolid fats are then used in margarines, commercial cooking, and manufacturing processes in the food industry (Mozaffarian, Katan, Ascherio, Stampfer, & Willett, 2006). For use in food applications, partially hydrogenated oils are attractive because of their lengthy shelf life, stability under harsh conditions such as deep-frying, and their semisolidity, which can be utilized to heighten the palatability of baked goods and sweets (Mozaffarian et al., 2006).
Studies have shown that the consumption of industrial produced *trans* fatty acids increases the LDL to HDL ratio of total cholesterol in the blood, and therefore raising the risk of coronary heart disease (Mozaffarian et al., 2006). In fact as of January 2006, the FDA changed the Nutrition Facts panel regulations stating that products containing *trans* fat must be labeled to include the amount of trans fat content (FDA, 2006). This modification was done to assist consumers in choosing heart-healthy foods (FDA, 2006). Common sources of *trans* fats include deep-fried fast foods, bakery products, processed snack foods, margarines, and crackers (Mozaffarian et al., 2006).

9. Cholesterol reduction medications

Statins are the most common type of drugs prescribed to control high cholesterol levels in patients. The interference with the production of endogenous cholesterol by the liver is responsible for the cholesterol lowering mechanism of statins (Normen, Holmes, & Frohlich, 2005; Spitzer & Maggini, 2013) The main enzyme responsible for endogenous cholesterol synthesis, hydroxy-methylglutaryl-coenzyme A reductase, is blocked by statins (Stancu & Sima, 2001; Normen et al., 2005). The process by which phytosterols and statins lower cholesterol is different; therefore when both compounds are taken collectively cholesterol reduction can be elevated (Spitzer & Maggini, 2013). Clinical studies using statins and phytosterols in combination have validated that the addition of phytosterols to a statin regimen can reduce LDL levels by an additional 4.5% per gram of ingested phytosterol (Normen et al., 2005). This overall level of phytosterol and statin combined reduction is analogous to the level achieved by doubling the prescribed statin dose (Normen et al., 2005).
This knowledge is beneficial due to statins having unwanted side effects caused by their interference with the rate-limiting step in endogenous cholesterol production (Normen et al., 2005). Statins work by interfering with HMG-CoA reductase, which is synthesized from mevalonate. Mevalonate also has a key role in the synthesis of several compounds such as coenzyme Q10 (CoQ10) (Ghirlanda & Caputo, 1993). The reduction of CoQ10 caused by statins can lead to possible cellular damage due to its pertinent role in the electron transport chain and role as an effective lipid-soluble antioxidant (Ghirlanda & Caputo, 1993).

10. Phytosterols and cholesterol reduction

Even though the human diet contains almost equal proportions of cholesterol and phytosterols, it seems greater than 95% of dietary phytosterols undergo straight fecal excretion while about 50% of dietary cholesterol is absorbed (Spitzer & Maggini, 2013). These findings suggest that the human body is capable of differentiating between cholesterol and non-cholesterol phytosterols (Spitzer & Maggini, 2013). Greater intake of phytosterols is necessary to compete for absorption against cholesterol. The competitive mechanism between phytosterols and cholesterol are still being further studied. Some researchers have proposed possible effects at the absorption site, physico-chemical effects, and controlling intracellular trafficking of sterols (Spitzer & Maggini, 2013).

Dietary cholesterol, when consumed, is incorporated into mixed micelles formed in the small intestine. These mixed micelles are responsible for transporting cholesterol across the intestinal lumen comprised of enterocytes. If phytosterols and cholesterol are collectively consumed, competition at the absorption site occurs due to the similar
chemical structures (Spitzer & Maggini, 2013). As phytosterols are incorporated into mixed micelles over cholesterol, any free cholesterol gets excreted in the feces because cholesterol in its free form cannot be absorbed. After undergoing micelle formation, the esterified phytosterols are hydrolyzed to free phytosterols presumably by enzymes cholesterol esterase and pancreatic lipase (Normén et al., 2006). Another proposed mechanism, of less significance, is a co-crystallization process with the formation of an insoluble mixed phytosterol-cholesterol crystal that can no longer pass through the intestine lumen (Mel’nikov, Seijen ten Hoorn, & Bertrand, 2004).

A cyclic mechanism has been proposed regarding a transporter protein involved in the absorption of phytosterols and cholesterol. The Niemann-Pick C1L1 transporter assists in the absorption of cholesterol and phytosterols at the enterocyte (Spitzer & Maggini, 2013). Once inside the enterocyte, adenosine triphosphate (ATP)-binding cassette proteins are induced through the presence of phytosterols (Spitzer & Maggini, 2013). The ABC transporters use energy produced from ATP hydrolysis to assist cholesterol and phytosterols across the membrane (Spitzer & Maggini, 2013). The increase in ABC transporters from a high intake of phytosterols has been studied as a main constituent in the cholesterol-lowering effect. The ABC transporters in the enterocytes secrete phytosterols and free cholesterol back into the intestinal lumen where the cycle continues for the sterols to get incorporated into the mixed micelles, continuing to get taken back up by the enterocytes again (Calpe-Berdiel, Escolà-Gil, & Blanco-Vaca, 2009). Phytosterols are secreted back into the intestinal lumen at a much higher rate than cholesterol, therefore supplementation is needed for an increased absorption (Spitzer & Maggini, 2013).
After phytosterols and cholesterol are taken up by the enterocytes, they must be incorporated into chylomicrons for further transport throughout the body. However, phytosterols must undergo esterification for the further integration into chylomicrons. Phytosterols are not esterified as efficiently as cholesterol, this rate limiting step in the competition between phytosterols and cholesterol leads to smaller concentrations of phytosterols reaching the tissues (Spitzer & Maggini, 2013). The liver takes up the phytosterols that do end up getting transported by chylomicrons where they are quickly excreted into bile by hepatic ABCG5/G8 transporters. Cholesterol also undergoes this same process. However, the secretion rate of phytosterols into the bile is much higher than cholesterol (Sudhop & von Bergmann, 2002). Cholesterol excreted into the bile is either reabsorbed or further excreted into the feces. The increased consumption of phytosterols with the intention of displacing cholesterol reduces reabsorption of cholesterol through the bile. When there is a decrease in levels of cholesterol, LDL-receptor expression is increased. This results in an increase of LDL clearance in circulation (Ostlund, 2007).

11. Phytosterols and cancer prevention

In addition to their cholesterol-lowering benefits, numerous studies on phytosterols indicate an anti-cancer effect as well (Choi et al., 2007). Research has revealed protective effects against cancer of the lung (Mendilaharsu, De Stefani, Deneo-Pellegrini, Carzoglio, & Ronco, 1998), stomach (De Stefani et al., 2000), ovary (McCann, Freudenheim, Marshall, & Graham, 2003), as well as estrogen-dependent human breast cancer (Ju, Clausen, Allred, Almada, & Helferich, 2004).
Researchers have proposed various anti-cancer effects from phytosterols such as the inhibition of carcinogen production, cancer-cell growth, invasion and metastasis, and the promotion of cancer cell apoptosis (Méric et al., 2006).

Carcinogenesis, from damaged DNA, occurs from reactive oxygen species produced by oxidatively stressed cells (Woyengo, Ramprasath, & Jones, 2009). Phytosterols work to protect against reactive oxygen species by increasing the activity of antioxidant enzymes such as superoxide dismutase and glutathione peroxidase (Vivancos & Moreno, 2005). In regards to cancer cell growth, a study done on the treatment of leukemia cells, breast cancer cells, and prostate cancer with β-sitosterol all showed a significant decrease in cell growth (Park et al., 2007). Another study involving β-sitosterol determined the mechanism by which phytosterols promote apoptosis of cancer cells. It seems the treatment of human leukemia cells with β-sitosterol resulted in an increased activity of an essential protein involved in cell death, caspase-3 (Park et al., 2007). In the invasion of cancer cells, angiogenesis, while important in wound healing, also has a major role in increased cancer cell growth and multiplication (Prescott, 2000). Metastasis, or the spreading of cancer throughout the body’s organs, is the main cause of death following cancer diagnosis (Awad, Fink, Williams, & Kim, 2001). Phytosterols have been proven to have an effect on both of these cancer-promoting events, and the mechanisms by which the phytosterols interfere are still under investigation.

12. Soybean oil and fully hydrogenated soybean oil

The use of fully hydrogenated oil eliminates all trans fatty acids. Full hydrogenation and likewise saturation of the fatty acid does not allow a shift from the cis to trans
configuration, unlike in partially hydrogenated oils. Alternatives to achieve the semisolid consistency of partially hydrogenated oils includes utilizing blends of fully hydrogenated oils with liquid oils to create a semisolid product with zero trans fat.

Due to current recommendations to decrease trans and saturated fats, there is a demand to find appropriate solid fat substitutes that do not increase the risk for CVD (Hunter, Zhang, & Kris-Etherton, 2010). One saturated fat in particular, fully hydrogenated soybean oil, has a fatty acid profile high in stearic acid (18:0) at ~84% (Acevedo, Block, & Marangoni, 2012). Stearic acid has been under investigation for its use in saturated fats.

In a study comparing effects on serum lipoproteins between stearic acid and trans fatty acid, 80 healthy subjects consumed a dairy fat-based diet as a baseline, then an experimental diet high in either stearic acid (Aro, Jauhiainen, Partanen, Salminen, & Mutanen, 1997). When compared with the dairy fat diet, similarly both stearic and trans fatty acids decreased total serum cholesterol concentrations by 13% and 12% (Aro et al., 1997). However, the trans fatty acid diet decreased HDL cholesterol by 17% while stearic acid only decreased HDL cholesterol by 11% (Aro et al., 1997). In regards to lowering harmful LDL cholesterol, only the stearic acid diet decreased LDL concentrations significantly (Aro et al., 1997). In fact, the trans fatty acid diet increased the harmful LDL to HDL ratio by 19% as compared to the dairy fat baseline diet (Aro et al., 1997). Altogether, the outcomes from the stearic acid diet emerged to be less harmful than those of the trans fatty acid diet.

Furthermore, the utilization of locally produced oils such as soybean oil can reduce costs and boost local economy instead of importing foreign oils. In fact, the United States is the largest producer and exporter of soybeans in the world (USDA, 2012).
13. Research justification

The justification of this research project includes the health benefits of phytosterol supplementation, specifically chemically unmodified free phytosterols to reduce production cost in industry, as well as increase convenience in processing. Furthermore, the use of local soybean oil and low amounts of fully hydrogenated soybean oil to create a semisolid product as a substitute for partially hydrogenated fats containing unhealthy trans fatty acids.
REFERENCES


Ntanios, F. Y., & Duchateau, G. S. M. J. E. (2002). A healthy diet rich in carotenoids is effective in maintaining normal blood carotenoid levels during the daily use of plant sterol-enriched spreads. *International journal for vitamin and nutrition research, 72*(1), 32-39. doi: 10.1024/0300-9831.72.1.32


Free phytosterols (FPS) have been utilized in recent research compared to their esterified or soluble counterparts in order to reduce costs and inconvenience associated with esterified phytosterols during processing. Furthermore, bans on trans fats have led to the need for substitutes that can mimic their desirable properties. This research focuses on the analysis of FPS co-crystallized with fully hydrogenated soybean oil (FHSO) and soybean oil (SO) as a zero-trans substitute for puff pastry shortening and various other types of shortenings. Differential interference contrast microscopy and wide angle x-ray diffraction demonstrated co-crystallization of FPS with FHSO and SO. Polymorphic forms were characterized as β’ and β for all samples. The addition of FPS decreased oil loss (OL) and melting profiles of FPS samples were extended to higher temperatures compared to commercial puff pastry shortening. Rheological properties suggested FPS blends may be acceptable for bakery applications. With further research, FPS co-crystallized with FHSO and SO may be used as a suitable trans-fat free substitute for several types of shortening, including puff pastry shortening.
Highlights

- Co-crystallized free phytosterol triacylglycerol blends as a substitute for trans fat containing puff pastry shortenings
- Free phytosterol triacylglycerol blends are in desirable polymorphic forms
- Free phytosterols decrease oil loss in triacylglycerol blends
- Rheological properties of free phytosterol triacylglycerol blends are comparable to puff pastry shortening

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>FHSO</td>
<td>Fully Hydrogenated Soybean Oil</td>
</tr>
<tr>
<td>SO</td>
<td>Soybean Oil</td>
</tr>
<tr>
<td>FPS</td>
<td>Free Phytosterol</td>
</tr>
<tr>
<td>β-Sit.</td>
<td>β-Sitosterol</td>
</tr>
<tr>
<td>Stig.</td>
<td>Stigmasterol</td>
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<tr>
<td>DSC</td>
<td>Differential Scanning Calorimetry</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear Magnetic Resonance</td>
</tr>
<tr>
<td>WXRD</td>
<td>Wide Angle X-Ray Diffraction</td>
</tr>
<tr>
<td>DIC</td>
<td>Differential Interference Contrast</td>
</tr>
<tr>
<td>OL</td>
<td>Oil Loss</td>
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</table>
1. Introduction

Phytosterols are found in all plant foods, with the highest concentrations occurring in vegetable oils (Ostlund, 2002). The general term 'phytosterols' describes plant-derived sterols and stanols with a chemical structure related to cholesterol and having a different side chain configuration (Cantrill, 2008; Spitzer & Maggini, 2013). Even though the human diet contains almost equal proportions of cholesterol and phytosterols, it seems greater than 95% of dietary phytosterols undergo straight fecal excretion while about 50% of dietary cholesterol is absorbed (Spitzer & Maggini, 2013). These findings suggest that the human body is capable of differentiating between cholesterol and non-cholesterol phytosterols (Spitzer & Maggini, 2013). In previous studies, an intake of 1-3 g/day of plant sterols has been shown to produce a 10-15% reduction in LDL cholesterol (Ling & Jones, 1995; Kritchevsky & Chen, 2005; Kuhlmann et al., 2005). However, the typical Western diet today contains only 150-400 mg/day (Ostlund, 2002). Therefore, greater intake of phytosterols is necessary to compete for absorption against cholesterol and supplemental doses may be required.

For commercial use, phytosterol- and stanol powders are esterified with fatty acids in vegetable oils (Cantrill, 2008). The process of esterification manipulates the physical properties of the high melting powders that have low solubility in oils (Cantrill, 2008). The characteristics of the esters are similar to edible fats and oils, classified as liquid or semi-liquid (Cantrill, 2008). The phytosterol fatty acid esters are incorporated into processed foods such as spreads, juices, oils, and other foods.

Until recently, the majority of studies involving phytosterols or phytostanols have been strictly on the use of esterified phytosterols or phytostanols. There have been few
studies addressing the use of free (non-esterified) phytosterols and phytostanols in commercial foods due to their limited solubility and disputes on bioavailability as compared to their esterified counterparts (Hayes et al., 2004). However, researchers have proven when free phytosterols are effectively heated and then recrystallized in fat upon cooling, they are made bioavailable and therefore effective in reducing cholesterol absorption (Hayes et al., 2004; Perlman et al., 2006). The utilization of non-esterified phytosterols in fat, by recrystallization, lowers the cost and increases convenience of processing to make previously insoluble non-esterified phytosterols bioavailable and soluble in dietary fats (Hayes et al., 2004). Previous strategies of phytosterol utilization include ultrafine powders, chemically modified esterified phytosterols, emulsified phytosterols, and the most susceptible to oxidation are phytosterols in water-oil microparticulate suspensions (Perlman et al., 2006).

The justification of this research includes the health benefits of phytosterol supplementation. Furthermore, the use of local soybean oil and low amounts of fully hydrogenated soybean oil to create a semisolid product as a substitute for various bakery shortenings. Puff pastry shortening was specifically selected due to the large amount of partially hydrogenated fats present in the product. Partially hydrogenated fats are a major source of unhealthy trans fatty acids. Due to current recommendations to decrease trans fats, there is a demand to find appropriate solid fat substitutes that do not increase the risk for CVD (Hunter et al., 2010).
2. Materials and Methods

2.1. Materials

Fully hydrogenated soybean oil (FHSO) and liquid soybean oil (SO) were generously donated from ADM oils (Decatur, IL). Beta-sitosterol powder (purity ≥70 %) was obtained from Sigma-Aldrich (St. Louis, MO). Stigmasterol powder (purity > 90%) was obtained from TCI America (Portland, OR). Super Bowl® puff pastry shortening consisting of partially hydrogenated soybean oil and cottonseed oils with artificial flavor and artificial colors was generously provided by Stratas Foods (Memphis, TN).

2.2 Blend preparation

Samples consisted of 48% solids and 52% liquid oil. For free phytosterol (FPS) fat blends, the solid material consisted of either 20% or 25% FPS and 28% or 23% FHSO (totaling 48%); the 52% liquid portion consisted of soybean oil. The control sample contained 0% FPS, 48% FHSO, and 52% soybean oil. Blends were prepared by heating the mixture up to ~180°C and agitating with a Caframo Real Torque Digital overhead stirrer (Ontario, Canada) at 200 RPM. Once the mixtures were clear, they were held at 180°C for additional 20 minutes to ensure the crystal memory had been erased. Subsequently, all samples were cooled at room temperature until ~20°C was reached and once crystallization was complete they were stored at 4°C until use in analysis.

2.3. Wide Angle X-ray Diffraction (WAXRD)

Wide angle x-ray diffraction (WAXRD) patterns of crystallized FPS, FPS fat blends, and control fat blends were measured using a Rigaku Ultima (Rigaku, Japan) IV X-ray
diffractometer. The operating conditions during experiments were 44 mA and 40kV. The
angular range using a 10 mm slit was from 1 to 30° (2θ) with steps of 1°, and the measuring
time was 1 minute/step. XRD patterns were analyzed with MDI Jade 9.0 software (Rigaku,
Japan). In this study, three replicates of each sample were performed.

2.4. Differential Scanning Calorimetry (DSC)

The thermal properties of phytosterol and control samples were measured by
differential scanning calorimetry (DSC) using a Perkin Elmer Diamond DSC (Shelton, CT,
USA). Heat flow calibration was made by reference to the known melting enthalpy of
indium (purity >90 %). Temperature calibration was made with zinc (purity >90 %).
Approximately 10mg of sample was placed in aluminum pans and sealed hermetically, an
empty pan served as a reference. All measurements were performed at a heating rate of
10°C/min. Thermograms were analyzed using Pyris Series Diamond DSC 9.0 software
(Shelton, CT, USA). The peak melting temperature (T\text{m}) and the enthalpy of melting (ΔH\text{m})
were determined. The reported data corresponds to the average of three replicates of each
sample.

2.5. Proton-Nuclear Magnetic Resonance (\textsuperscript{1}H-NMR)

Solid fat content (SFC) was analyzed by proton nuclear magnetic resonance (\textsuperscript{1}H-
NMR) by using a Bruker MiniSpec Bench Top NMR (Billerica, MA, USA). Co-crystallized
samples stored at 4°C were introduced into NMR glass tubes, then incubated for 30
minutes at 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100°C to allow for a homogenous
distribution of temperature at the time of measurement. The reported data correspond to the average of three replicates of each sample.

2.6. Oil Loss Determination (OL)

Once crystallized, oil loss (OL) experiments were performed according to previously described techniques (Dibildox-Alvarado, Rodrigues, Gioielli, Toro-Vazquez, & Marangoni, 2004; Acevedo et al., 2012). Blends were molded into PVC molds of 35 mm diameter and 3.2 mm thickness to form disks that were then transferred to filter papers (Whatman #1, 125 mm diameter). The amount of oil lost over time was determined by the difference in weight of the filter papers before and after placing the disc on the paper for the designated time at 20°C. A “blank” filter paper was included in all experiments to account for differences in possible humidity of the storage environment. Filter papers were large enough to avoid paper saturation with oil during the experiment. The reported data correspond to the average and standard deviation of five replicates, each separate disk on an individual filter paper. Oil loss (%) was calculated according to previously described technique and OL rate (g/h) was calculated from the average slope of OL replicates of each sample:

\[
\text{OL(\%)} = \frac{\text{wt.paper(X h)} - \text{wt.paper (0 h)}}{\text{wt.paper (0 h)}} \times 100
\]

2.7. Differential Interference Contrast Microscopy (DIC)

Differential Interference Contrast (DIC) Microscopy was used to observe the FPS fat blends microstructure. Approximately 0.01 g of the melted sample was placed on a heated
glass slide and a heated glass cover was carefully laid over the sample to remove air and homogenously spread the sample. After cooling at room temperature 20°C, slides were stored at 4°C until analysis. Samples were imaged using an Olympus BX53 System Microscope (Tokyo, Japan) with polarized light (X-Cite 120 LED) and equipped with an Olympus Q Imaging camera. All images were obtained using a 40X objective lens. Autoexposure of the camera was adjusted according to the sample. CellSens Dimension 1.9 software (Tokyo, Japan) was used to acquire focused images. At least twenty-five images in total were captured from each of the 9 replicates.

2.8. Small Deformation Rheology

Crystallized samples were spread into the wells of polyvinylchloride (PVC) molds of 3.2 mm thick and 35 mm in diameter. Rheological measurements were obtained using a HAAKE RS 150 RheoStress rheometer (Paramus, NJ, USA). A 35 mm diameter serrated stainless steel plate (PP35Ti) was used in the analysis. Each sample was kept at a constant temperature 20°C controlled by the base of the TC 81 Peltier temperature control system through a HAAKE F6 (Paramus, NJ, USA) water bath.

Oscillatory stress sweeps were performed within the range of 50 to 2000 Pa (with a frequency of 1 Hz) inside the linear viscoelastic region (LVR). Normal force was set at 3N for all samples. Sandpaper (grade 60) was attached to the base of the rheometer to prevent slippage of samples during analysis. The yield stress ($\sigma^*$) values (Pa), elastic ($G'$) and storage ($G''$) modulus values (MPa) were determined from the stress sweep curves. The reported data are the average of eight individual replicates from each sample.
2.9. Statistical Analysis

GraphPad Prism 5 software (GraphPad Software, Inc., San Diego, CA) was used to process data. Reported values correspond to means and standard deviations of the measurements. Statistical analysis was carried out by one-way ANOVA (P < 0.005) with Tukey's multiple comparisons as a post-test (P < 0.05).

3. Results and Discussion

3.1. Analysis of thermal properties

DSC is a well-established method conducted to assess the thermal properties of fats and oils during heating and cooling. Thermograms showed the melting point $T_m$ range between 63.02-65.36°C for the 48:52 FHSO:SO (control sample) and all FPS fat blend samples, i.e. 20:28:52 β-Sit:FHSO:SO, 25:23:52 β-Sit:FHSO:SO, 20:28:52 Stig:FHSO:SO and 10:10:28:52 β-Sit:Stig:FHSO:SO (Table 3.1); which is in agreement with previous studies on FHSO:SO blends (Acevedo et al., 2012). Thermograms also showed $T_m$ for β-sitosterol powder of 137.78°C in agreement with (Vaikousi, Lazaridou, Biliaderis, & Zawistowski, 2007) and stigmasterol powder of 169.99°C. In FPS fat blends, no peaks were observed at temperatures corresponding to the ($T_m$) of FPS powders suggesting co-crystallization of the species (Figure 3.1). The type of FPS appears to have affected the enthalpy of melting ($\Delta H$) (Table 3.1). The addition of 20% β-sitosterol or stigmasterol reduced $\Delta H$ by 32.71% and 29.73% respectively when compared to the control. However, when both β-sitosterol and stigmasterol were added to the FHSO:SO fat blends (10:10:28:52 β-Sit:Stig:FHSO:SO), $\Delta H$ was not significantly different from the control. These results may be indicating interference by FPS in the thermodynamic system.
Figure 3.1 DSC thermograms of the fat blends with and without FPS as well as pure FPS

Under thermal conditions such as in DSC, the heat given off by the system is proportional to the internal energy occurring in the reaction (Durland, 2015). The addition of both FPS in FHSO:SO blends could have generated greater internal energy in the system, resulting in a higher enthalpy of melting. However, when only one type of FPS was added to the fat blends, the internal energy of the system was lower, thus a lower enthalpy of melting was found. Thus, the sample containing both types of FPS had a higher enthalpy of melting than the sample containing only one type of FPS.
Table 3.1. Melting temperature (T_m) and enthalpy of melting (ΔH) of the control sample, pure phytosterols and their blends

<table>
<thead>
<tr>
<th>Sample</th>
<th>T_m (°C) ± SD</th>
<th>ΔH (J/g) ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>48:52 FHSO:SO</td>
<td>65.30 ± 2.50^a</td>
<td>71.88 ± 6.90^a</td>
</tr>
<tr>
<td>20:28:52 β-Sit:FHSO:SO</td>
<td>63.02 ± 2.00^a</td>
<td>48.37 ± 6.43^b</td>
</tr>
<tr>
<td>25:23:52 β-Sit:FHSO:SO</td>
<td>63.05 ± 0.17^a</td>
<td>41.18 ± 2.74^b</td>
</tr>
<tr>
<td>20:28:52 Stig:FHSO:SO</td>
<td>65.36 ± 0.72^a</td>
<td>50.51 ± 7.37^b</td>
</tr>
<tr>
<td>10:10:28:52 β-Sit:Stig:FHSO:SO</td>
<td>63.07 ± 0.37^a</td>
<td>86.64 ± 8.14^a</td>
</tr>
<tr>
<td>β-Sitosterol</td>
<td>137.78 ± 0.79^b</td>
<td>43.27 ± 4.34^b</td>
</tr>
<tr>
<td>Stigmasterol</td>
<td>169.99 ± 1.54^c</td>
<td>72.80 ± 4.64^a</td>
</tr>
</tbody>
</table>

Note: Different letters within columns indicate statistical difference at 5% probability level.

3.2. Polymorphic form characterization

Polymorphic form characterization is important to assess the stability of fats and oils. WAXRD patterns of the control consisting of 48:52 FHSO:SO and all FPS fat blend samples showed a presence of both polymorphs β' and β (Figure 3.2a, b and c) (Sato, 2001). However, XRD patterns from the control showed greater characteristics of β polymorphism, which is in agreement with previous studies on FHSO:SO blends (Acevedo et al., 2012). Furthermore, β-sitosterol d-values were in agreement with previous research (Christiansen, Rantanen, von Bonsdorff, Karjalainen, & Yliuruusi, 2002; von Bonsdorff-Nikander, Karjalainen, Rantanen, Christiansen, & Yliuruusi, 2003). However, there is a lack of previous studies performed to assess the nanostructure of stigmasterol using WAXRD.
Figure 3.2 Wide angle x-ray diffraction (WAXRD) patterns of control (a), β-sitosterol and its mixture with FHSO:SO (b), and stigmasterol and its mixture with FHSO:SO (c). d-values of possible new structures formed in free phytosterol fat blends as compared to free phytosterols are indicated with arrows (b and c)
Polymorphic form is predominately determined by the rate of nucleation, being governed by thermodynamic and kinetic influences (Sato, 2001). When nucleation is induced under sizeable kinetic factors, e.g., supercooling or supersaturation the metastable form nucleates first prior to the most stable form (Sato, 2001). However, when some other external influences (pressure, temperature fluctuation, ultrasonic stimulation, template, seeding, etc.) are applied the nucleation of the more stable forms is hindered (Sato, 2001; Rousseau, Hodge, Nickerson, & Paulson, 2005). It appears the addition of FPS as an external influence into the FHSO:SO system is impeding the polymorphism transition from the metastable form β' to most stable β. The addition of FPS may be kinetically entrapping the crystals in the metastable form due to the higher viscosity of the system, preventing the polymorphic transformation toward the more stable form.

There is a lack of literature on this topic to elucidate the nanostructure of FPS crystallized with FHSO:SO blends. However, the XRD patterns of FPS fat blends presented new d-values absent in the control or FPS powder XRD patterns (Figure 3.2b and c). These new d-values, indicated with arrows, could be attributed to the presence of new structures formed by co-crystallization of FPS with triacylglycerols, also suggested by DSC thermograms (Figure 3.1). Further analysis needs to be carried out to elucidate the nanostructure of these matrices.

3.3. Microstructure analysis by DIC

Differential interference contrast (DIC) microscopy was used for microstructural analysis due to the polarized light microscope’s ability to exploit the high contrast between the solid and liquid fraction in fats and oils (Murphy, 1998). Fig. 3.3 shows digitalized
images of samples thermostatically crystallized at 20 °C. The microstructure of the control sample consisted of aggregated spherulites, similar to previous research where similar fat blends were analyzed (Acevedo et al., 2012; Bouzidi, Omonov, Garti, & Narine, 2013). The morphology of the β-sitosterol and stigmasterol powder presented shiny needle-like crystals, similar to previous research on phytosterols (AlHasawi & Rogers, 2013). The addition of stigmasterol into the FHSO:SO matrix caused morphological differences in the aggregated spherulite crystalline structure; long needle-like crystals arose as indicated in the powder stigmasterol sample. An apparent concentration difference between the higher concentration of β-sitosterol in the 25:23:52 β-Sit:FHSO:SO versus the 20:28:52 β-Sit:FHSO:SO sample presented shiny flower-like β-sitosterol crystals as displayed in the pure β-Sitosterol. The addition of β-sitosterol did not seem to drastically modify the aggregated spherulite structure of the FHSO:SO matrix, but rather exhibited a trivial presence within the network.

These results correspond to previous research in that the addition of phytosterol esters (PE) into milk fat (MF) did not seem to drastically modify the structure of MF crystals, nonetheless the PE still showed a small presence within the MF crystal network (Rodrigues, Torres, Mancini-Filho, & Gioielli, 2007). The inhabitance of both FPS, β-sitosterol and stigmasterol, in the 10:10:28:52 β-Sit:Stig:FHSO:SO sample displayed a branch-like crystal structure differing from both the β-sitosterol and stigmasterol structures. Non-uniform structures were observed in all DIC FPS fat blend images. Previous research stated non-uniform crystal structures may be elucidated by FPS in the matrix impeding nucleation of the triacylglycerol crystals (Bouzidi et al., 2013). The addition of FPS into the matrix by impeding further nucleation and growth may be hindering
polymorphic transformations due to the viscosity of the matrix, as seen in XRD results (Figure 3.2) (Sato, 2001; Rousseau et al., 2005; Bouzidi et al., 2013).

Figure 3.3 DIC microstructure of FPS powders, FPS fat blends and control

3.4. Oil loss analysis

Fat crystal networks possess physicochemical properties that bind and entrap oil (Acevedo et al., 2012). Oil loss (OL) in this study and previous studies has been determined through the measurement of the maximum oil loss over time, which is the mass of oil lost from the fat sample under controlled conditions (Dibildox-Alvarado et al., 2004; Acevedo et al., 2012; Bouzidi et al., 2013). Figure 3.4 reports OL values as a function of time and Figure 3.5 reports OL rates in g/h. The 48:52 FHSO:SO control sample presented 41.52 ± 12.56% OL after reaching the plateau at about 1,000 h with an OL rate of 0.18 ± 0.09 g/h. While the FPS fat blends had OL values ranging from 28.07-46.00% and OL rates of 0.09-0.16 g/h. The addition of β-sitosterol into the FHSO:SO matrix at 20% decreased the amount of total OL compared to the control by 13.45% respectively. The decrease in OL % with lesser
FHSO conflicts with previous studies stating decreasing the FHSO leads to more oil loss over time (Acevedo et al., 2012).

However, there is a lack of literature discussing the effects of FPS powders on OL % in FHSO:SO blends. According to previous research, two important parameters which successfully and predictively measure the relative tendency of a solid network to lose/hold oil were defined: (1) the rate of oil loss, which is a quantified representation of the kinetics of oil loss and (2) the initial amount of oil receptive to be lost, *i.e.*, the propensity for oil loss (POL), which is a representation of the thermodynamics of oil binding (Bouzidi et al., 2013). The initial amount of oil receptive to be lost is dependent upon how much oil is experimentally added to the system.

Researchers suggested that the mechanism of oil binding is complex, depending on the structurant’s crystalline form locked within the oil network (Bouzidi et al., 2013). Furthermore, the POL and oil loss rate values do not always trend in the same fashion, which coincides with the results from the addition of FPS into a FHSO:SO matrix. The control and all FPS samples had equal amounts of initial oil receptive to be lost (POL) at 52% total weight of SO, yet the final OL values differed. These results suggest the addition of FPS have a thermodynamic effect on the system’s ability to lock oil within the network.

OL % and OL rate analysis was conducted on commercial puff pastry shortening to ascertain the differences between FPS:FHSO:SO blends. Similar OL % resulted between 20:28:52 β-Sito:FHSO:SO (28.07 ± 3.71%) and puff pastry shortening (29.80 ± 1.60%) over time. However, all FPS fat blends presented significantly lower OL rates (0.09-0.16 g/h) than puff pastry shortening (0.52 ± 0.09 g/h), suggesting the FPS fat blends may release oil at a slower rate, which would be desirable in shelf life (Figure 3.5).
Figure 3.4. Oil loss of control (a), FPS fat blends (b) and puff pastry shortening (c) as a function of time.
3.5. Solid fat content (SFC) analysis by pulsed nuclear magnetic resonance

Solid fat content (SFC) analysis was conducted to determine the SFC profiles of all samples compared to commercial puff pastry shortening and SFC at room temperature (Figure 3.6a and b). SFC profile results from puff pastry shortening were in agreement with previous research ~24% SFC at 20°C (Ghotra, Dyal, & Narine, 2002) (Figure 3.6b). The control sample reached 0% SFC at 65-70°C, however FPS blends did not achieve 0% SFC even at temperatures higher than 65-70°C (Figure 3.6a).
This phenomenon could be due to a threshold in the co-crystallization of FPS with FHSO and SO; beyond a certain ratio of FPS with FHSO and SO not all of the FPS present may be co-crystallizing. There may be a phase separation of the components where some FPS did not integrate into the FSHO:SO matrix. XRD patterns of new d-values suggested co-crystallization occurred. However, the more sensitive method of NMR indicated that 100% of FPS in the matrix are not co-crystallized.

Previous research has been conducted on the addition of phytosterols into a matrix on SFC. However the work encompassed phytosterol esters (PE) blended with milk fat (MF), instead of FPS (Rodrigues et al., 2007). The MF PE blends presented a decrease in SFC when a higher proportion of PE was present, even though PE independently presented higher SFC than MF at all temperatures (Rodrigues et al., 2007). A phenomenon was hypothesized regarding the interaction between MF and PE in that the PE interfered with
the formation of a MF crystalline structure, resulting in lower SFC values (Rodrigues et al., 2007). FPS may still have this effect on FHSO:SO crystalline structure, but at a lower percentage than 20-25% total weight of FPS. Results may be suggesting a threshold in the capability for the matrix to co-crystallize. At 20°C no differences were observed in SFC for the FPS blends, even though different types of FPS and different percentages were utilized (Figure 3.6b). The 20% versus 25% FPS and the β-sitosterol versus the stigmasterol were not significantly different. Therefore, it was proposed that the type or percentage of FPS did not affect the SFC at room temperature but the presence of FPS versus the FHSO did affect the SFC at room temperature as shown by significant differences between all FPS containing samples versus the control. Again, this may be due to a threshold in co-crystallization where the percentage of FPS incorporated was beyond the threshold.

3.6. Analysis of macro structural properties by small deformation oscillatory rheology

Small deformation rheology with an oscillatory stress sweep was conducted in order to maintain a linear viscoelastic region (LVR) where measurements of $G'$ and $G''$ were obtained. No significant differences were observed in the $G'$ values of the blends except for commercial puff pastry shortening and 25:23:52 β-Sit:FHSO:SO (Figure 3.7a). These results indicate the sample with a higher percentage of phytosterols (25% vs. 20%) and lower percentage of FHSO (23% vs. 28%) had similar properties, in regards to $G'$, as compared to commercial puff pastry shortening. $G'$ is the storage modulus which represents the elastic behavior of the sample (Marangoni & Wesdorp, 2013). $G'$ has been related to the strength or hardness and has also been a strong indicator of the SFC of the fat crystal network (Acevedo et al., 2012; Marangoni & Wesdorp, 2013).
Figure 3.7 $G'$ (a), $G''$ (b) and yield stress ($\sigma^*$) (c) at 20°C
Therefore, it is not surprising that the sample containing a lower proportion of FHSO at 23% (25:23:52 β-Sit:FHSO:SO) resulted in a lower G’ value as compared to the rest of the samples containing 28% FHSO.

G” represents the viscosity of the fat crystal network also known as the loss modulus (Marangoni & Wesdorp, 2013). Similar to the aforementioned G’ results, there were no significant differences in G” between the 25:23:52 β-Sit:FHSO:SO sample and the commercial puff pastry shortening suggesting parallel viscoelastic properties (Figure 3.7b). Again, this may be related to the decrease in FHSO in the sample containing a higher proportion of phytosterols.

The measurement of yield stress is one of the most imperative macroscopic properties in regards to assessing fats; it is strongly correlated to the sensory perception, material stability and spreadability (Marangoni & Wesdorp, 2013). Previous research states that if the yield stress for shortenings falls within the 200-800 Pa range, the product may be acceptable for bakery applications (Haighton, 1959). Not only did the puff pastry commercial shortening fall within this range, but so did all of the FPS:FHSO:SO samples (Figure 3.7c). Additionally, the 20:28:52 β-Sit:FHSO:SO sample and the commercial puff pastry shortening were not significantly different from each other in respect to yield stress values, suggesting analogous sensory, stability and spreadability characteristics. In puff pastry shortening applications, the shortening is layered between sheets of dough (Ghotra et al., 2002). In order to create separate flaky layers during the baking process it is imperative that replacements for puff pastry shortenings have similar spreadability (Ghotra et al., 2002).
The sample containing higher percentages of FPS at 25% had lower mechanical properties compared to samples containing lower percentages of FPS at 20%. This could be explained by incompatibilities between components of the FHSO:SO versus FPS systems (Marangoni & Wesdorp, 2013). Incompatibility within fat systems has previously been studied in chocolate; it has been correlated to the cause of undesirable chocolate blooming (Marangoni & Wesdorp, 2013). Perhaps the explanation for the lower mechanical properties is that, similar to the incompatibilities between cocoa butter and milk fat used in chocolate, the FPS are not compatible with the FHSO:SO matrix.

4. Conclusions

The absence of peaks at higher temperatures of the FPS $T_m$ suggested co-crystallization by DSC. XRD patterns revealed the presence of new d-values not present in the control sample or in the FPS powders XRD patterns. These new d-values insinuated new structures present from the co-crystallization of FPS with triacylglycerols. Fat crystal networks were characterized as either spherulites for β-sitosterol or long needle-like structures for stigmasterol. Overall, the addition of FPS decreased the OL % of the matrix, which was comparable to commercial puff pastry shortening. In regards to SFC, the type of FPS (β-sitosterol or stigmasterol) and the percentage (20% or 25%) did not affect the SFC of the FPS blends at room temperature. However, the addition of FPS into the FHSO:SO matrix extended the melting profile compared to the control and commercial puff pastry shortening. The extended melting profile could affect how the FPS shortenings perform during baking. Furthermore, NMR may have detected a threshold in co-crystallization, the FPS shortenings did not achieve 0% SFC at the $T_m$ of ~65°C reported in DSC thermograms.
Despite differences in SFC melting profiles, the rheological properties of the FPS enriched shortenings were comparable to those of commercial puff pastry shortening, notably all FPS shortenings fell within the 200-800 Pa range. With further research in regards to SFC melting profile and determining the threshold of co-crystallization, the FPS shortenings may be suitable for bakery applications and utilized as a desirable replacement for puff pastry shortenings containing \textit{trans} fats.
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CHAPTER 4

GENERAL CONCLUSION

The overall objective of this research was to prepare a trans-fat free substitute for commercial shortenings typically consisting of partially hydrogenated fats, specifically puff pastry shortening. The prepared substitute was also enriched with free phytosterols adding previously studied health benefits such as lowering cholesterol and anti-cancer effects. Free phytosterols were utilized in order to reduce processing costs and inconvenience in the food industry. Phytosterol percentages were incorporated in high amounts of 20-25% as compared to other studies. WAXRD patterns and DSC suggested co-crystallization at these percentages, but NMR suggested the entire matrix was not co-crystallized. Oil loss and rheological values were comparable to commercial puff pastry shortening. Overall, this research demonstrated FPS FHSO:SO blends may be suitable for bakery applications due to comparative results amongst analyses with the blends and commercial puff pastry shortening.
Future Work

Future research should focus on identifying the threshold at which the matrix can be 100% co-crystallized. This may allow for greater absorption of FPS during consumption if all the FPS were effectively entrapped and protected in the lipid system. A nutritional study would be beneficial to prove the absorption of FPS in this system. A range of percentages of FPS from low to high could be analyzed to compare differences in XRD patterns and NMR melting profiles. Furthermore, small angle x-ray diffraction should be conducted to further analyze the new structures suggested by WAXRD patterns. Cryo-TEM analyses could also be utilized to determine how the FPS and triacylglycerols are stacking within the lamella during the crystallization process. If necessary, emulsifiers could be added to the matrix to improve the equal dispersion of FPS with triacylglycerols. Sensory testing would also be appropriate in comparing puff pastries constructed with commercial puff pastry shortening versus puff pastries utilizing FPS enriched shortening.