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OPTIMIZATION OF PHYSICOCHEMICAL AND SENSORY PROPERITES OF REDUCED-CHOLESTEROL MILK & QUESO-FRESCO CHEESE WITH β-SITOSTERYL OLEATE

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Synthesis of β-Sitosteryl Oleate for the Optimization of Physicochemical and Sensory Properties of Reduced-Cholesterol Milk & Cheese

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ABSTRACT

In this study, the incorporation of β-sitosteryl oleate was investigated for improving the textural properties of reduced-cholesterol milk and cheese. This became necessary because cholesterol removal usually leads to textural changes that consumers find undesirable. β-sitosteryl oleate is similar in structure to the cholesteryl fatty acid esters that are removed during the formulation of reduced-cholesterol products, and their melting points are about the same. As such, the incorporation of β-sitosteryl oleate into reduced-cholesterol milk and cheese should significantly improve its consistency. Response surface methodology was used to optimize the synthesis of β-sitosteryl oleate. β-sitosteryl oleate was synthesized by reacting β-sitosterol with oleic acid at 140°C for 9 h (under vacuum). The reaction was catalyzed by sodium bisulfate (2% w/w). Different amounts of β-sitosteryl oleate (BSO) were added to the reduced-cholesterol cream, and the treatment containing 3% BSO resulted in reduced-cholesterol cream with a milk fat melting profile similar to that of regular cream. It was then used to constitute 3.5% milk and was analyzed for its fatty acid profile, fat microstructure, color, sensory properties

and viscosity. The constituted 3.5% milk was comparable to regular milk (control) with respect to its fatty acid profile, fat microstructure, and sensorial and rheological properties. Additionally, the experimental milk was used to prepare queso fresco cheese, which was analyzed for moisture, ash, fat content, pH, titratable acidity, NaCl, total plate count, color and meltability. Our findings showed there were no significant differences with respect to pH, meltability and microbial load.

TABLE OF CONTENTS

List of Tables vi

List of Figures vii

Common Abbreviations viii

Chapter 1: Introduction 1
Objectives 2

Chapter 2: Literature Review 4
2.1. Dairy 4
2.2. Cheese 4
2.3. Impacts of Cholesterol on Human Physiology 6
2.4. β-Cyclodextrin 7
2.5. Phytosterols 10
2.6. Phytosteryl Esters 11

Chapter 3: Materials and Methods 16
3.1. Synthesis of β-Sitosteryl Oleate 16
3.2. Quantification of β-Sitosteryl Oleate 17
3.3. Cholesterol Removal from Cream 19
3.4. Treatment of Reduced-Cholesterol Cream with β-Sitosteryl Oleate 19
3.5. Cholesterol Content Determination 19
3.6. Differential Scanning Calorimetry 20
3.7. Preparation of Reduced-Cholesterol Milk & Queso-Fresco Cheese 21
3.8. Fatty Acid Profile Analysis of Milk 21
3.9. Sensory Evaluation of Milk 22
3.10. Fat Globule Structural Analysis by Scanning Electron Microscopy 22
3.11. Compositional Analysis 22
3.12. Color and Meltability 23
3.13. Statistical Analysis 23

Chapter 4: Results and Discussion 24
4.1. Specific Objective 1 24
4.1.1. Synthesis of β-Sitosteryl Oleate 24
4.1.2. Differential Scanning Calorimetry 25

4.2. Specific Objective 2 27
4.2.1. Fatty Acid Profile Analysis 27
4.2.2. Scanning Electron Microscopy 27
4.2.3. Color & Viscosity 28
4.2.3. Sensory Analysis 28
4.3. Specific Objective 3 30
4.3.1. Compositional, Physicochemical and Microbiological Analysis 30
4.3.2. Color & Meltability 30

Chapter 5: Conclusion and Recommendations 50

References 52

Curriculum Vitae 56

LIST OF TABLES

Table 3.1: Experimental settings for RSM study 18

Table 4.1: Synthesis of β-sitosterol oleate 32

Table 4.2: Regression analysis for the synthesis of β-sitosterol oleate 33

Table 4.3: Analysis of Variance for the Synthesis of β-sitosteryl Oleate 34

Table 4.4: Fatty Acid Profile of Milk 38

Table 4.5: Chi-squared results for aroma 40

Table 4.6: Chi-squared results for color 41

Table 4.7: Chi-squared results for viscosity 42

Table 4.8: Color & viscosity of milk 43

Table 4.9: Compositional, physicochemical and microbiological analyses of cheese 45

Table 4.10: Color analysis of cheese subjected to baking conditions 47

Table 4.11: Color analysis of cheese subjected to broiling condition 48

Table 4.12: Cholesterol content of cheese samples 49

LIST OF FIGURES

Figure 2.1: Types of cyclodextrins 9

Figure 2.2: Types of phytosterols 14

Figure 2.3: Synthesis of β-sitosteryl oleate 15

Figure 4.1: Contour plot of synthesis of β-sitosteryl oleate. 35

Figure 4.2: HPLC chromatogram of Standard and Reaction product 36

Figure 4.3: DSC thermogram of fat extracts from different cream samples 37

Figure 4.4: Sensory results for milk samples 39

Figure 4.5: Scanning electron micrographs showing milk microstructure of milk samples 44

Figure 4.6: Cheese samples subjected to meltability test 46

COMMON ABBREVIATIONS

B-CD	Beta-cyclodextrin
BSO	Beta-sitosteryl oleate
RSM	Response surface methodology
SFC	Solid-fat content
FAME	Fatty acid methyl esters
DSC	Differential scanning calorimetry
SEM	Scanning electron microscopy
GC	Gas chromatography
HPLC	High performance liquid chromatography
TLC	Thin layer chromatography

CHAPTER 1

INTRODUCTION

Dairy foods such as milk, yogurt, butter and cheese are noted for their cholesterol contents, which has become a cause for concern to some consumers due to the positive association between plasma cholesterol levels and cardiovascular diseases. Thus to limit the consumption of foods rich in cholesterol, consumers have changed their dietary habits, leading to a steady decline in the consumption of dairy products, especially fluid milk over the last two decades (USDA, 2012). In order to avoid further decline in their consumption, several techniques were employed to reduce the cholesterol contents of the dairy products, of which the use of β-cyclodextrin has been the most successful. The cholesterol-content of dairy foods can be significantly reduced by about 95% with this technique (Kwak and others, 2001). However, the physical properties of the dairy products with reduced-cholesterol contents are not comparable to the control. This happens due to the significant loss of solid fat content as a result of cholesterol removal (Kim and others 2006; Jung and others 2005; Kwak and others 2001). Other parameters such as hardness, gumminess, chewiness and meltability were significantly different for a low-cholesterol cheese (Kwak and others 2001).

In order to improve the textural properties of reduced-cholesterol dairy products, phytosterols have been added to replace cholesterol due to their similar structures (Kim

and others, 2006). However, the finished products were harder due to the higher melting points of phytosterols (140°C). However, this can be solved by esterifying phytosterols with fatty acids. It is worth mentioning that cholesterol does not exist freely in fats, but rather as a cholesteryl ester of fatty acids. Therefore, the esterification of phytosterol with fatty acids is expected to result in phytosteryl ester with a significantly reduced melting point, thereby contributing to the viscoelastic properties of the finished product (Ostlund 2002; Mattson and others 1977). Phytosterols and their esters have the added benefit of interfering with the absorption of cholesterol in the gut (Duncan and Best 1956; Pollak, 1953).

The objective of this study was to synthesize and incorporate β-sitosteryl oleate, a phytosteryl ester, into reduced-cholesterol dairy products with the aim of improving solid-fat content and textural properties, thus resulting in products with physicochemical and sensory properties comparable or superior to regular milk and queso-fresco cheese.

Objectives

- Specific objective 1: Optimize the synthesis of β-sitosteryl oleate via chemical catalysis and incorporate β-sitosteryl oleate into reduced-cholesterol cream.
- Specific objective 2: Incorporate β-sitosteryl oleate into reduced-cholesterol milk and determine physicochemical and sensorial properties.

- Specific objective 3: Incorporate β-sitosteryl oleate into reduced-cholesterol queso fresco and determine physicochemical properties.

CHAPTER 2

LITERATURE REVIEW

2.1. Dairy

Milk is consumed not just in its basic or natural form, but the food industry today produces cultured, condensed, fermented, evaporated, baked and other forms of milk. Dairy products comprise many day-to-day foods such as butter, cheese, yogurts, ice-cream, and others. Food scientists have been working extensively with dairy and related products to bring to market premium quality end-goods, which are nutritious and also benefit physiological processes such as digestion.

Additions made to natural products modify their molecular structure and therefore affect their properties. Even characteristics, such as thickness, suitability and acceptance, can also be affected by the addition of various ingredients. Researchers constantly thrive to improve the nutritional value of the base product, while enhancing or not compromising on sensory properties.

2.2. Cheese

A large variety of cheeses are produced by coagulating and precipitating milk proteins, predominantly casein. Cheese making was originally considered as a means of conserving milk or milk constituents. Nowadays, cheese making is also a reason for increasing milk's economic and nutritional value. It is believed that cheese was first made

in Iraq about 8000 years ago (Fox 2004). Some cheeses are curdled by acidification with bacteria, which convert milk lactose into lactic acid. Sometimes vinegar or lemon juice is also used to curdle the milk. More than 2,000 varieties of cheeses are produced worldwide. They differ in their styles, flavors and textures, to name a few. These characteristics result from differences in processing techniques, fat content, the bacteria/mold used, and method of aging.

More than 200 million pounds of Hispanic-styled cheese were produced in the United States in 2010. One of such is called queso fresco. It is characterized by its fresh, creamy, moist, soft, mild-aged and crumbly nature, and its inability to melt. It is a favorite among other Hispanic-type cheeses and is frequently used as a topping or filling in dishes, crumbled over salads or soups, or served with fruits (Torres and Chandan 1981). Queso fresco cheese turns slightly brown and softens but does not melt when baked. Due to its weak protein structure, it crumbles when pressed (Hwang and Gunasekaran 2001; Guo 2011).

Dairy products are a source of dietary cholesterol. The cholesterol content in a whole milk is 10 mg per 100g (USDA MD 2009). Consumption of three servings of dairy products is recommended as part of a healthy well-balanced meal, owing to their richness in calcium, protein, vitamin A. However, the positive association between plasma cholesterol levels and cardiovascular disease has caused many to reduce their consumption of dairy products.

2.3. Impacts of cholesterol on human physiology

Cholesterol is a lipid molecule, making up approximately 20% of human cell membranes. It is required to build and maintain cell membranes and is also a precursor of bile acids, steroid hormones, and the vitamin D precursor, 7-dehydrocholesterol. In humans, low density lipoproteins (LDL) are the main carriers of cholesterol in blood from the liver to peripheral tissues. Cholesterol is returned from extrahepatic tissues to the liver in high density lipoproteins (HDL) by reverse cholesterol transport. Research in this area is continually uncovering the role of cholesterol on human physiology. The concentration of plasma cholesterol depends not only on endogenous biosynthesis (around 80% of cholesterol is made by the human body, depending on dietary consumption) but also on dietary intake. In spite of the vital functions, high levels of cholesterol contribute to various cardiovascular conditions.

According to the Center for Disease Control, cardiovascular disease is the leading cause of death in both men and women in the United States (http://www.cdc.gov/HeartDisease/facts.htm), developing due to inflammation of arteries and blood clotting. This inflammation of heart arteries begins with cholesterol plaque deposition and oxidation of low-density lipoproteins. Food consumption should be adapted such that nutrients having anti-inflammatory properties can prevent artery blockages and blood clotting. Nutrients, such as dietary fiber, fat-soluble vitamins A, D, and E, as well as water-soluble vitamins C, niacin, folate, vitamin B6, and B12, have exhibited these anti-inflammatory properties (Correa-Matos and others 2013).

Cholesterol, the prime cause of CVD, has been associated with high consumption of saturated fats and sodium. Consumption of foods containing phytochemicals, such as resveratrol, flavonoids, and anthocyanins, has a positive effect on cardiovascular health. In addition to endogenous synthesis of cholesterol, dietary intake has been shown to positively impact cardiovascular health as inferred from the Luxembourg survey (Alkerwi and others 2014). The correlation between cholesterol intake and risk for cardiovascular diseases has always been debated. The said survey examined data across 1352 participants, under 7 controlled metrics to arrive at what was called cardiovascular health score (CHS). Dairy food intake was found to be positively associated with the final CHS value of participants, irrespective of whether a dairy product had a low or high fat content. Jean Davignon and others (2010) also studied the association between dietary cholesterol (egg yolk consumption) and cardiovascular diseases. The study reported no significant association.

2.4. Beta-cyclodextrin

Cyclodextrins (CD) are basically cyclic glucose polymers produced by enzymatic modification of starch. They are often used by pharmaceutical, food, cosmetics and chemical industries (Menuel and others 2007; Thatiparti and others 2010). Normally, cyclodextrins have six to eight glucose monomer units in a ring. Alpha-cyclodextrin is comprised of six glucopyranose units, β-CD is made up of seven such units, and γ-CD contains eight units. The cyclic structures are cone shaped with two open ends: the narrow end of the C_6 hydroxyl groups and the wide end of C_3 and C_2 hydroxyl groups. The

outer surface of CD is hydrophilic, while its inner cavity is hydrophobic (Szejtli 1988; Easton and others 1999; Uekama 2004; Davis and others 2004).

Cyclodextrins are nontoxic, edible, non-hydroscopic and chemically stable (Nagamoto 1985; Makoto 1992). Of the other cyclodextrins, β-cyclodextrin has been used extensively in the food industries, specifically to reduce the cholesterol levels of dairy products such as milk, butter, and cheese (Lee 1999; Kwak 2002; Shim and 2003; Hwag and others 2005; Astray 2009; Dias 2010). Also, β-cyclodextrin has been used successfully to remove up to 90% of cholesterol in dairy products by forming a complex with cholesterol. Cholesterol, being hydrophobic, is hosted by the hydrophobic core of β-cyclodextrin. The resulting complex can then be removed by centrifugation.

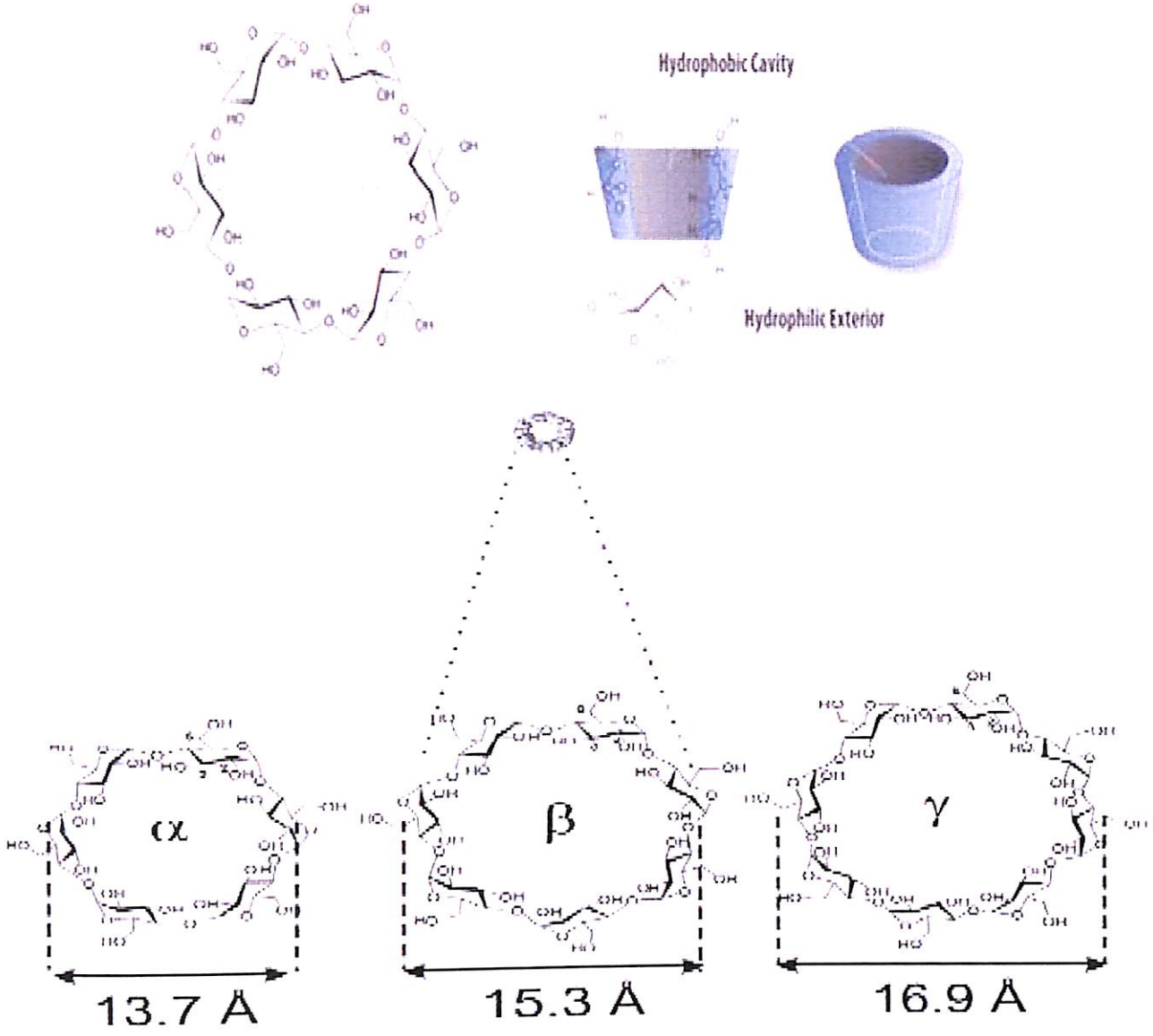


Figure 2.1. Types of cyclodextrins

2.5. Phytosterols

Phytosterols are plant sterols and stanols that are structurally similar to cholesterol found in plants. They only differ from cholesterol in the sense that they contain some substitutions at the C_{24} position of the sterol side chain. Most phytosterols have 28 or 29 carbons and one or two carbon-carbon double bonds, usually one in the sterol nucleus and the second in the alkyl side chain. Stanols are saturated sterols. Phytosterols are hydrophobic and soluble in alcohols and oils, but can also be dispersed in water after emulsification with lecithin. Of the more than two hundred sterols identified (Akhisa and others 1991), the most abundant phytosterols are β-sitosterol, campesterol, stigmasterol and ergosterol. Beta-sitosterol is widely distributed in plants such as avocados, nuts, and vegetable oils (USDA National Nutrient Database 2014). The basic function of phytosterols is to stabilize the phospholipid bilayers in plant cell membranes (Choudhary, 2011).

Plasma phytosterol levels in mammalian tissue are normally very low due to poor absorption from the intestine and faster excretion from the liver compared to cholesterol. They are metabolized in the liver into C_{21} bile acids. Even though absorbed only in trace amounts, they competitively inhibit intestinal absorption of dietary cholesterol including re-circulating endogenous biliary cholesterol, a key step in cholesterol elimination (Ostlund, 2002). Studies have shown that saturated phytosterols are more efficient than unsaturated ones in reducing cholesterol absorption. Natural dietary intake of phytosterols varies from about 167–437 mg/day. Approximately 170 mg/day of

phytosterols are consumed by people eating a typical western diet, whereas 360 mg/day are consumed on a vegetable-rich diet (Vries de 1997). The European Foods Safety Authority (EFSA) recommends consuming about 1.5 - 2.4 g/day of phytosterols and/or stanols in order to reduce blood cholesterol.

Nowadays, phytosterols are not only restricted to plant products but are also incorporated in dairy foods like milk, yogurt, and others. These products are designed to have higher levels of phytosterols or phytosteryl fatty acids, and are easily available on the global market (Laakso 2005). However, their low solubility in oils and their high melting points of about 140-150°C (Vu and others, 2004) restrict their application in foods to some extent. Esterification of phytosterols with long-chain fatty acids increases fat solubility by ten-fold (Ostlund, 2002). Phytosterols are safe, nontoxic with no obvious side effects, and are poorly absorbed (Ostlund, 2002). Additionally, research has demonstrated therapeutic benefits such as anti-tumor effects and it has also been shown experimentally to inhibit colon cancer development in rats (Awad and Fink, 2000). Thus, a significant reduction in plasma cholesterol level can be achieved with the incorporation of phytosterols into a variety of foods (Ling and others 1995; Moreau and others 2002).

2.6. Phytosteryl esters: -

Phytosteryl esters (PE) are a heterogeneous group of chemical compounds. They are structurally related to cholesteryl esters, but differ from cholesteryl esters in the structure of the side chain (fig 2.4). Phytosteryl esters are intended for use as a novel food ingredient with plasma cholesterol-lowering activity. Phytostanol and phytosterol esters

are produced via esterification of plant stanols or sterols with fatty acids from common vegetable oils. Thus, the fatty acid composition of the esters is similar to the parent vegetable oil used as a source of the fatty acids. Esterification of phytosterols or phytostanols modifies the physical properties from high-melting crystalline powders with low oil solubility into liquid or semi-liquid substances that can easily be incorporated into a variety of (fat containing) foods. The proportion of the phytosterol backbone is approximately 60 % by weight of the ester and that of the fatty acid tail approximately 40 % by weight.

Phytosteryl esters can be used as a fat replacer because the sterol moiety of the ester molecule does not provide any energy to the body. Moreover, phytosteryl esters may be used to modify the fatty acid composition of a fat blend and replace part of the hard fat in margarines and spreads (Cantrill, 2008). Furthermore, these esters can provide a crispy texture (prevents sogginess) to cereal products by coating the product surface. Both phytosteryl and phytostanyl esters give an enhanced creamy texture to low fat dairy products (yoghurt/ drinking yoghurt). They may also improve the taste of food products by masking bitterness and hence reducing the amount of sugar or other sweetener required to obtain a pleasant taste and mouth feel (e.g. in soy drinks). Phytosterols and their fatty acid esters are quite stable compounds and undergo only limited degradation during oil processing. Only under harsh conditions, such as high temperatures (>100°C) in the presence of oxygen, that oxidation of the phytosterol moiety may occur. Phytosterols and phytostanols are microbiologically largely inert as shown by the absence

of an effect during the fermentation process used to produce yoghurt. Furthermore, the ester added to various food products show excellent stability at different pH values during long term storage (up to at least a year) (Cantrill, 2008). Phytostanyl and phytosteryl esters are also stable in milk and fermented milk and products with viable bacteria like yoghurts and yoghurt drinks.

Even though current evidence suggests that dietary cholesterol has little effect on cardiovascular health, governmental dietary guidelines over the years advised consumers to limit their cholesterol intake. It was not until 2015 that government guidelines reversed course on the association between cholesterol and cardiovascular diseases. However, the prior guidelines had led consumers to change their dietary habits in order to limit the consumption of foods rich in cholesterol (Robinson, 2001). Dairy products probably suffered the most as a result of their high cholesterol contents. For example, milkfat contains about 310 milligrams total cholesterol per 100 grams (Christie, 1983). Moreover, the Economic Research Service of the United States Department of Agriculture (USDA) reported that the per capita consumption of some dairy products, especially fluid milk, has been on the decline, albeit slowly, since 1975 (ERS-USDA Report 1). It still remains unclear as to whether the new guidelines on dietary cholesterol will change consumers' perception on foods rich in cholesterol.

It is also worth noting that government guidelines with respect to saturated fats, which suggest that saturated fats are associated with high cholesterol levels in the blood and thus could promote cardiovascular heart diseases, remained unchanged. This is

important because a product can only be called a low-cholesterol product if it contains not more than 2 g of saturated fat per serving, meaning that producing reduced-cholesterol foods should go hand-in-hand with a reduction in saturated fat content.

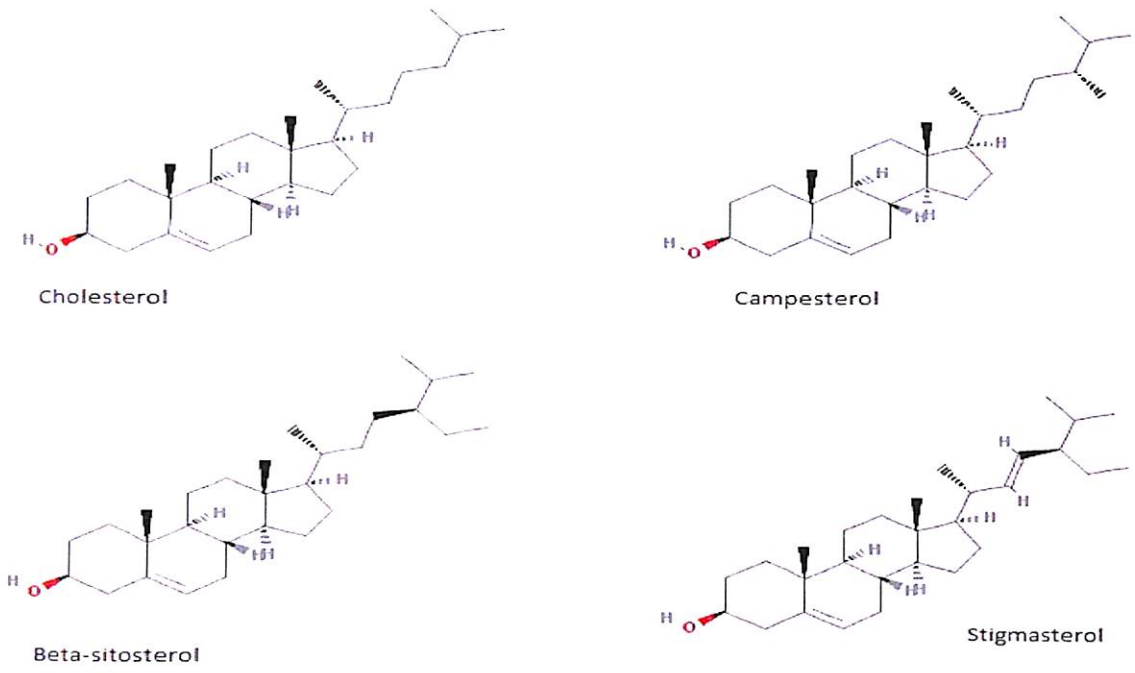


Figure 2.2. Types of phytosterol

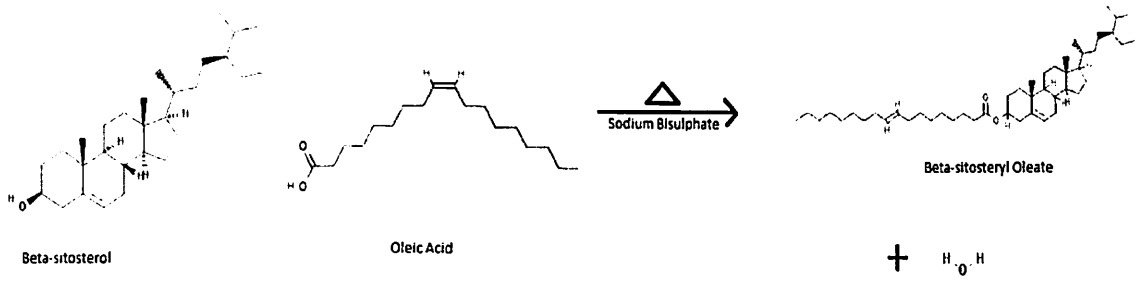


Figure 2.3. Synthesis of β-sitosteryl oleate

CHAPTER 3

MATERIALS & METHODS

3.1. Synthesis of β-Sitosteryl Oleate

Response surface methodology (RSM) was used to optimize the synthesis of β-sitosteryl oleate. The effects of the independent variables, time, temperature and molar ratios, on the esterification of oleic acid to β-sitosterol, which was calculated as degree of esterification were determined. Beta-sitosterol and oleic acid were dried under vacuum for an hour at 100°C. Two percent (w/w) Sodium bisulfate was added as catalyst (Qianchun 2011), after which the esterification reaction was carried out as specified by the RSM design (Table 3.1). The progress of the reaction was monitored by thin layer chromatography as previously described by Lumor and others (2005). The esterification reaction was stopped with the addition of cold water and an aliquot of hexane (100 mL) was added. The resulting reaction mixture was further purified by alkaline treatment to remove the unreacted oleic acid (Lumor and others 2007). The unreacted β-sitosterol was removed by dissolution in 100 mL methanol. The resultant organic layer was separated and filtered. The reaction product, β-sitosterol oleate, was recovered from hexane by means of a rotary evaporator.

3.2. Quantification of β-Sitosteryl Oleate

A Shimadzu LC-20AB HPLC system (Kyoto, Japan), equipped with SEDEX 90 ELSD detector, using a Kromasil ® 60-5-SIL (250×4.6 mm I.D., 5.0 μm particle size) was used for the quantification of β-sitosterol oleate. The mobile phase used was iso-octane:methyl tert-butyl ether (MTBE) (99.5: 0.5) (v/v). The column temperature was kept at 40°C with a flow rate of 1 mL/min.

The degree of esterification (%) of β-sitosterol and oleic acid to form the product β-sitosterol oleate was calculated from the HPLC results as given by equation 1:

Degree of Esterification (DE %) = BB + A × 100.....equation 1

(Where A= peak of β-sitosterol and β= peak of β-sitosterol oleate).

Table 3.1. Experimental settings for RSM study

Exp No.	Variables			Degree of Esterification (DE %)
	Temperature (°C)	Time (h)	Ratio	
1.	120	8	3	
2.	120	8	5	
3.	120	9	4	
4.	120	10	3	
5.	120	10	5	
6.	140	8	4	
7.	140	9	3	
8.	140	9	4	
9.	140	9	5	
10.	140	10	4	
11.	160	8	3	
12.	160	8	5	
13.	160	9	4	
14.	160	10	3	
15.	160	10	5	

3.3. Cholesterol Removal from Cream

Bulk ultra-pasteurized cream (80 mL) was stirred (200 rpm) with 15% (w/w) β-cyclodextrin (β-CD) on a plate stirrer at 35°C for 45 min. The β-CD-treated cream was then centrifuged at 444 x g (1500) for 10 min to remove the β-CD:cholesterol complex. The resulting reduced-cholesterol cream was used for further analyses.

3.4. Treatment of reduced-cholesterol cream with β-sitosteryl oleate (BSO)

Different percentages of β-sitosteryl oleate were incorporated into reduced cholesterol cream. This was followed by the extraction of fat from the reduced-cholesterol cream as described by Stefanov and others (2010) in order to determine cholesterol content and fat melting profile. Briefly, the cream (10 g) was treated with 16 mL dichloromethane-ethanol solution (DME) (2:1 v/v) in a 50 mL plastic centrifuge tube. The mixture was vortexed for 90 s and then centrifuged at 2500 x g for 10 min. The aqueous layer was carefully removed and 3 mL of DME solution was added to the remaining organic layer. The mixture was vortexed again for 90 s and centrifuged at 2500 x g for 6 min. The centrifugation resulted in a white protein precipitate and an organic layer. The organic layer was further removed and filtered by passage through an anhydrous sodium sulfate column to remove residual moisture. Finally, the fat was recovered from dichloromethane by means of a rotary evaporator.

3.5. Cholesterol content determination

Saponification was performed by weighing 0.1 g of the extracted fat into a 50 mL screw-cap test tube; followed by the addition of 50 µL of internal standard (5 mg/mL of

β-sitosterol in hexane) and 10 mL of saponification reagent (a mixture of ethanol and 33% (w/v) KOH solution in a ratio of 94:6). It was then refluxed for 1 h at 50°C. After cooling, 5 mL of 2 M KCl solution and 5 mL of hexane were added. The tubes were then capped tightly and thoroughly vortexed. After phase separation, the hexane layer containing unsaponifiable matter was passed through a column of anhydrous sodium sulfate to remove residual moisture, followed by drying under a stream of nitrogen. The cholesterol content of the unsaponifiable matter was then determined by GC-MS as previously described by (Du & Ahn, 2002).

3.6. Differential Scanning Calorimetry

The phase behavior or melting profiles of fat samples from reduced-cholesterol cream treated with BSO were determined by DSC on a Perkin-Elmer model DSC 4000 equipped with an intercooler (Norwalk, CT). Regular cream served as positive control, and reduced-cholesterol cream as negative control. Analysis was performed using a modification of AOCS recommended procedure Cj 1-94 (AOCS Official Methods, 1989). Briefly, samples were held for 2 min at 25°C, followed by rapid heating to 80°C at 10°C/min, and held for 10 min. The samples were then cooled to -80°C at 5°C/min, and held for another 10 min. Finally, samples were heated to 80°C at 5°C/min. Normal standardization was performed with cyclohexane (2 thermal transitions at -87.06 °C and 6.54 °C) and indium (thermal transition at 156.6 °C). The melting profiles of fat from cream treated with different amounts of β-sitosteryl oleate were compared, and the treatment, herein called cream optima, with fat melting profile similar to that of regular

cream (positive control) was used to constitute 3.5% milk for making reduced-cholesterol cheese for further analyses.

3.7. Preparation of Reduced-Cholesterol Milk & Queso Fresco Cheese

Cream optima, the treated cream that was treated with β-sitosteryl oleate to produce a milk fat melting profile similar to that of regular cream, was used to constitute 3.5% milk from skim milk. This was followed by pasteurization (72°C for 15 s) and homogenization (500/1000 psi). The pasteurized milk was collected and chilled. The treated milk sample (20 kg) was poured into a table-top stainless steel vat, and heated to 32°C, and queso fresco cheese was prepared as previously described (Tunick and others 1993; Van Hekken and others 2012, 2007). This was followed by compositional analyses and physicochemical characterization. The control cheese was made with untreated milk in the same manner.

3.8. Fatty Acid Profile Analysis

The fat from the milk samples were converted into fatty acid methyl esters (FAME) (AOAC official method, section 996.01). The FAME was analyzed on GC-2010 plus system (Shimadzu Scientific Instruments, Inc., Columbia, MD) using a SP-2560, 100mm x 0.25 mm i.d., 0.2 μm film column equipped with flame an ionizing detector (FID) in parallel with a with FAME standard (Supelco 37 component FAME mix, Supelco®, Bellafonte, PA) (Lumor and others 2008).

strip, Hach Co., Loveland, CO, USA, AOAC Official Method 971.19; AOAC International 1998), and titratable acidity (titration, AOAC Official Method 920.124; AOAC International 1998) and total plate count (AOAC Official Methods of Analysis, sec. 966.23).

3.12. Color and Meltability

A Hunter Lab ColorQuest XE 2382 colorimetric spectrophotometer (Hunter Associate Laboratory, Reston, VA) was used to determine the color of the cheese samples. Six disks (5 mm thick, 38 mm in diameter) were prepared from each sample and initial color values for L*, a*, and b* were determined. The three disks for each sample were then heated to 232°C for 5 min and cooled, after which color measurements were collected again and the data was used to calculate total color change (ΔE) (Guo and others 2011). Meltability was determined using the Schreiber Melt Test (Kosikowski and others 1997).

3.13. Statistical analysis

Minitab version 17.0 was used to perform statistical analysis for all samples. Analysis of variance was used to determine significant differences between the samples. Statistically significant p-value ≤ 0.05 with 95 % confidence level.

CHAPTER 4

RESULTS & DISCUSSION

Specific objective 1: Optimize the synthesis of β-sitosteryl oleate via chemical catalysis and incorporate β-sitosteryl oleate into reduced-cholesterol cream.

4.1.1. Synthesis of β-sitosteryl oleate

Response surface methodology was used to obtain different experimental settings for the synthesis of β-sitosteryl oleate to obtain the degree of esterification (DE %). The different experimental settings are shown in Table 3.1. The obtained result shows that the highest degree of esterification (93.86 %) was achieved by experimental setting 9 (140°C, 9h and 1:5 substrate ratio) and the least degree of esterification (32.82 %) was obtained with the experimental setting 3 (120°C, 9h and 1:4 substrate ratio). Further, the results obtained (**Table 4.1**) were then fitted to a second-order polynomial equation using Minitab 17.0.

Interestingly, from the regression analysis which was carried out at the significance level of $p \leq 0.05$, only temperature (X_1) and its square term (X_1^2) were significant. Time (X_2), substrate ratio (X_3) and their squares were insignificant. (**Table 4.2.**). Thus, temperature was the only independent factor which had effects on the degree of esterification. This result was in accordance with a previous study in which the production of a phytosteryl ester increased with increasing reaction temperature (Meng and others 2011). This was due to the increased energy of the reactants as temperature

increased, the effect of temperature (X_1) was positive while the square of temperature (X_1^2) was negative. Hence the model equation for degree of esterification (DE %) can be written as follows:

DE (%) = -1447 + 19.22 (X_1) - 0.0529 (X_1^2).

The formation of the reaction product, i.e., β-sitosteryl oleate was confirmed by thin layer chromatography (TLC). Cholesteryl linoleate standard was used to compare and confirm the formation of BSO as both have similar polarities. Silica gel was the stationary phase while the mobile phase was non-polar. The mobile phase used was petroleum ether: ethyl ether: acetic acid (90:10:1, v/v/v) (Lumor and Akoh, 2005). The β-sitosterol band travelled the least because of its high polarity. This was followed by oleic acid band which is less polar. Cholesteryl linoleate and β-sitosteryl oleate bands appeared at the top (i.e., the most travelled bands) about same height since they were the least polar. This was a confirmation that β-sitosteryl oleate was in fact synthesized. The formation of the reaction product, β-sitosteryl oleate, was also determined by HPLC using cholesteryl linoleate as an external standard. **Figure 4.2.** Shows the chromatogram (A) of the standard cholesteryl linoleate in comparison with the chromatogram (B) of the reaction product consisting of β-sitosteryl oleate (peak 1) and the starting material, β-sitosterol (peak 2).

4.1.2. Differential scanning calorimetry analysis

Fats extracted from regular cream (control) and reduced-cholesterol cream treated with different amounts of β-sitosteryl oleate were subjected to differential scanning

calorimetry, and their respective melting profiles obtained (**Figure 4.2.**). The thermograms showed one major and three minor peaks. However, there was one peak missing from the regular cream thermogram which justifies the fact that we added β-sitosteryl oleate to cream, thus, signifying that the said peak was due to the presence of β-sitosteryl oleate. Additionally, the thermogram pattern of the cream treated with 3% β-sitosteryl oleate was very similar to that of regular cream, indicating that both may have comparable melting and textural properties.

Specific objective 2: Incorporate β-sitosteryl oleate into reduced-cholesterol milk and determine physicochemical and sensorial properties.

4.2.1. Fatty acid profile analysis

The fatty acid profile of regular milk (untreated) and milk treated with β-sitosteryl oleate is shown in **Table 4.4** Even though some of the fatty acids varied slightly between the treated and un-treated samples, there was no general trend. There was a slight decrease in the percentages of palmitic and palmitoleic acids, whereas, the percentage of oleic acid increased. The increase of oleic acid is indicative of the fact that β-sitosteryl oleate was incorporated into milk. Therefore, it can be concluded that the processes used in the treatment of the milk did not impact its fatty acid profile as there were no significant changes to its the fatty acid content.

4.2.2. Scanning electron microscopy

Micrographs of the scanning electron microscope showed fat globules of regular milk (control) and milk treated with β-sitosteryl oleate. The micrograph showed fat (**Figure 4.5.**) as spherical globules with an oval shape, irregularly arranged and surrounded by a lipoprotein membrane. It is evident that the treatment of milk with 3% BSO did not result in significant changes in the sizes of the globules. However, the fat globules of the control milk were clustered, whereas, fat globules of treated milk were sparsely distributed with almost no cluster formation. This may be due to the reduction in the viscosity between the control and treated milk samples as discussed in the next section. Hence, we can conclude that there is a slight difference between the microstructures of the control and treated milk samples.

4.2.3. Color & viscosity

The changes in color of the treated milk is shown in Table 4.5. Values of L*, a* and b* were recorded. There were no significant differences between the L* and b* values of both the control and the treated milk. But there was a significant difference in their a* values. The treated milk showed more greenness (Table 4.5), and this may be attributed to the addition of β-sitosteryl oleate. This is in accordance with a previous study (Kim and others, 2006).

The viscosities of the regular milk (control) and treated milk were analyzed to compare the changes in their flow properties. The viscosity values were 26.7cp and 23.5cp for the control and treated milk samples, respectively. The difference was significant, and this may be due to the processes employed for the treatment of the milk.

4.2.4. Sensory analyses

A total of 26 participants' evaluated regular milk (control) and milk treated with β-sitosteryl oleate with respect to viscosity, color and aroma (**Figure 4.4.**). A triangle test was used to analyze the attributes. For aroma, 85 % of the participants were not able to differentiate between the control and treated milk sample. A similar trend was observed for color with 58%, and viscosity with 62%. The chi-squared test was used to analyze the results. The research hypothesis proposed was that there would be no differences between the control and treated milk samples with respect to aroma, color and viscosity. From the chi-squared distribution chart, $\chi^2_{1,\ 0.05} = 3.84$, while the χ^2 values for aroma, color and viscosity were 30.79, 6.95 and 9.32, respectively. These were greater than 3.84,

indicating the rejection of the hypothesis, leading to the conclusion that there was no significant difference between the control and the treated milk sample. The instrumental test results for viscosity and color showed some differences between the control and treated milk samples. However, the sensory tests showed no significant differences. The results could be explained that perhaps the panelists could not detect the differences in the samples, but the instrumental analysis could detect it. Instrumental analyses are more sensitive than sensory evaluation involving the use of humans (Fox and others, 2004). Moreover, instrumental tests are more objective than sensory tests using humans (Przemysław and others, 2015).

Specific objective 3: Incorporate β-sitosteryl oleate into reduced-cholesterol cheese and determine physicochemical properties.

4.3.1. Compositional, physicochemical and microbiological analyses

The 22.3% fat, 6.9 pH, 0.21 titratable acidity and 0.18 % NaCl content of the treated queso fresco cheese sample containing 3% β-sitosteryl oleate did not show any significant differences when compared to the regular queso fresco cheese (control) (**Table 4.6.**). However, moisture, ash, and lactose content varied slightly, though significantly. This result was in accordance with a previous study (Kim and others 2006) in which moisture content was higher in the treated samples and no differences were found in other chemical components with the addition of phytosterols. Interestingly, microbial colonies were observed in the control cheese whereas none was observed in the treated cheese. These results indicate that the addition of β-sitosteryl oleate did not significantly affect the chemical composition of the queso fresco cheese.

4.3.2 Color & meltability

The color of both regular queso fresco cheese (control) and the treated queso fresco cheese changed significantly after heating. There was significant difference in the L*, a* and b* values between the control and the treated sample before and after being subjected to baking (130°C for 30 min) and broiling (232°C for 5 min) conditions (**Table 4.7.-4.8.**). This significant differences in the L*, a* and b* values between the control and treated sample were in accordance with a previous study (Kim and others 2006), which attributed the change in color to the addition of β-sitosteryl oleate. Besides there was a large decrease in the whiteness (L*) of both the control and the treated samples when

baked compared to when broiled. The ΔE values for the control cheese under baking conditions were at least 1.5 times higher than under broiling conditions, whereas in the treated cheese, the ΔE values under baking conditions were almost twice the value under broiling condition. The a* value for the control cheese was positive (magenta), whereas that of treated cheese was negative (green).

Both the cheese samples did not melt when subjected to heat, which is expected with these kind of cheese. This may be due to its high pH levels, which ensured that the colloidal calcium phosphate remained in the casein matrix, thus preventing melting (Choi and others 2008). The change in the disc area was insignificant between the control and the treated cheese samples (**Figure 4.6.**).

Table 4.1. Synthesis of β-sitosterol oleate

Exp No.	Variables			Degree of Esterification (DE%)
	Temperature (°C)	Time (h)	Ratio	
1.	120	8	3	32.90 ± 21.27
2.	120	8	5	52.22 ± 33.14
3.	120	9	4	32.83 ± 13.76
4.	120	10	3	64.07 ± 24.89
5.	120	10	5	75.98 ± 18.39
6.	140	8	4	74.77 ± 27.16
7.	140	9	3	92.14 ± 7.09
8.	140	9	4	78.41 ± 22.05
9.	140	9	5	93.86 ± 0.01
10.	140	10	4	83.39 ± 0.02
11.	160	8	3	83.65 ± 7.03
12.	160	8	5	91.63 ± 3.69
13.	160	9	4	82.62 ± 5.84
14.	160	10	3	83.64 ± 16.73
15.	160	10	5	90.96 ± 1.08

Table 4.2. Regression analysis for the synthesis of β-sitosterol oleate

Terms	Regression Coefficients	P-value
Constant	78.80*	0.000
Temperature (X_1)	17.45*	0.000
Time (X_2)	6.29	0.083
Ratio (X_3)	4.82	0.176
X_1^2	-21.18*	0.005
X_2^2	0.18	0.979
X_3^2	14.10	0.051
X^1X^2	-6.95	0.086
X^1X^3	-1.99	0.610
X^2X^3	-1.01	0.796
R^2	69.72%	-
R^2 (adj)	56.10%	-
R^2 (pred)	27.75%	-

*Significant at $p \leq 0.05$

Table 4.3. Analysis of Variance for the Synthesis of β-sitosteryl Oleate

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Regression Model	9	10915.0	1212.78	5.12	0.001
Linear	3	7345.7	2448.58	10.33	0.000
Square	3	2715.8	905.25	3.82	0.026
2-Way Interaction	3	853.5	284.49	1.20	0.335
Lack-of-Fit	5	386.9	77.37	0.27	0.924

DF – Degree of freedom
Adj SS – Adjusted sum of squares
Adj MS - Adjusted mean squares

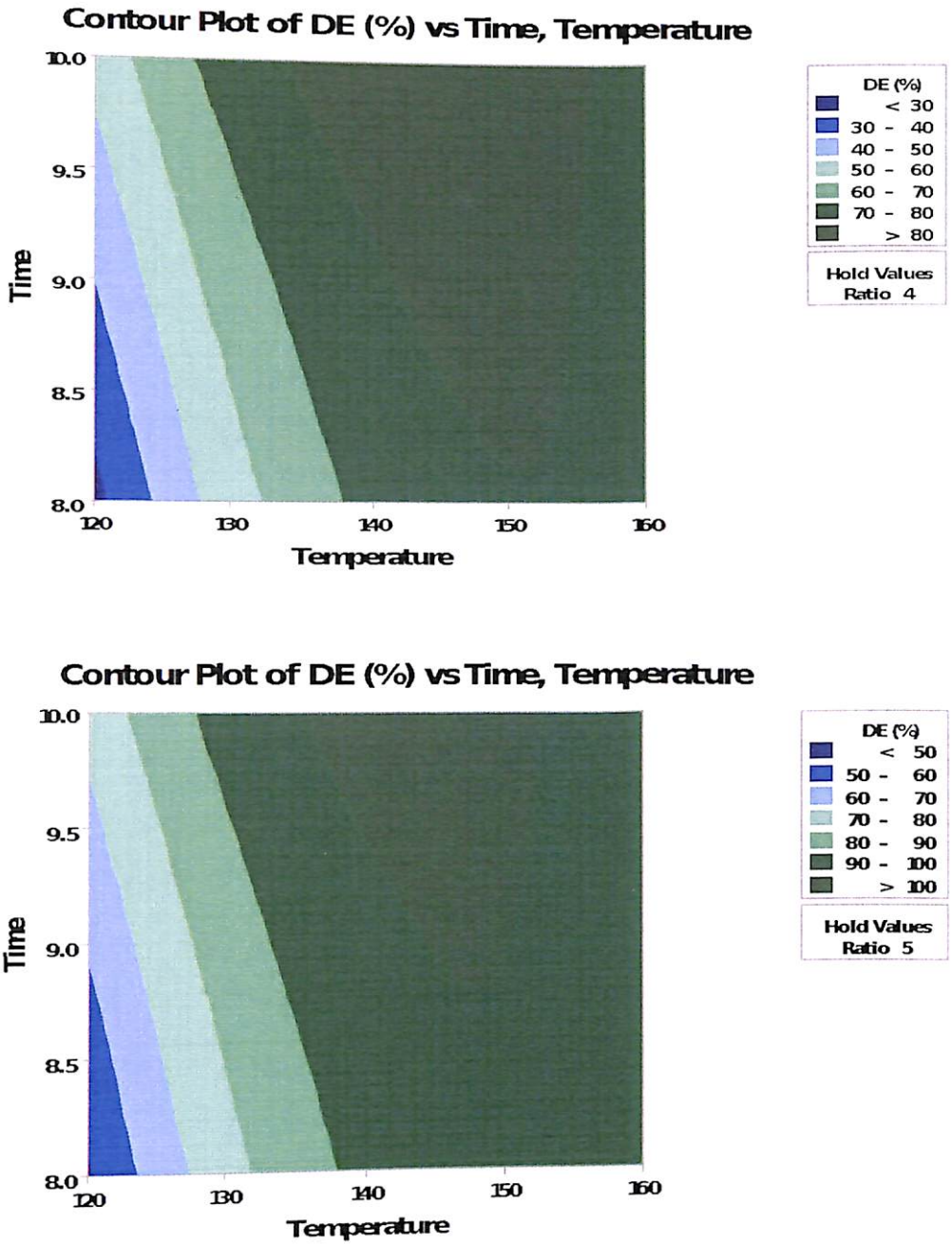
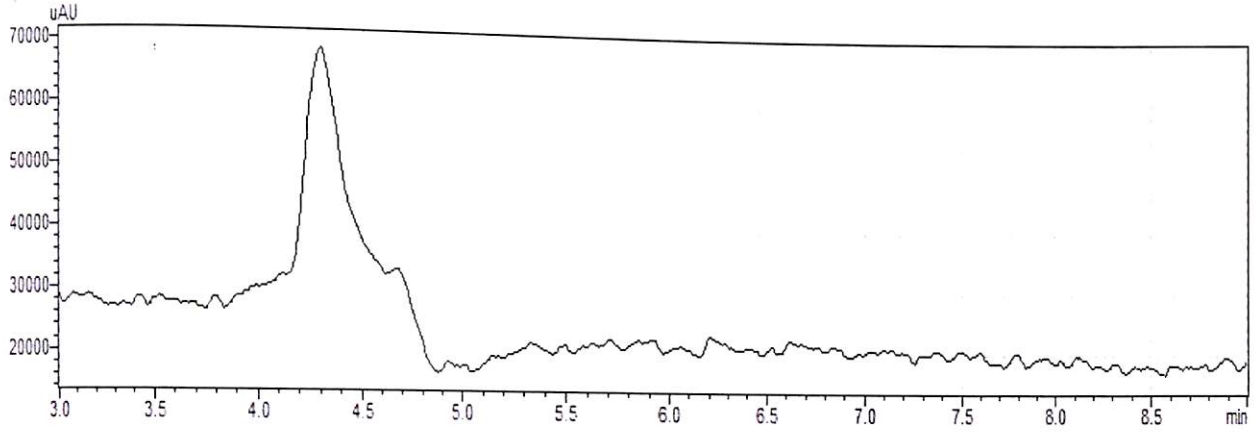


Figure 4.1. Contour plot showing the effects of temperature (°C), time (h) and molar ratio on the synthesis of β-sitosteryl oleate. Each contour represents the degree of esterification of oleic acid to β-sitosterol.

a)



b)

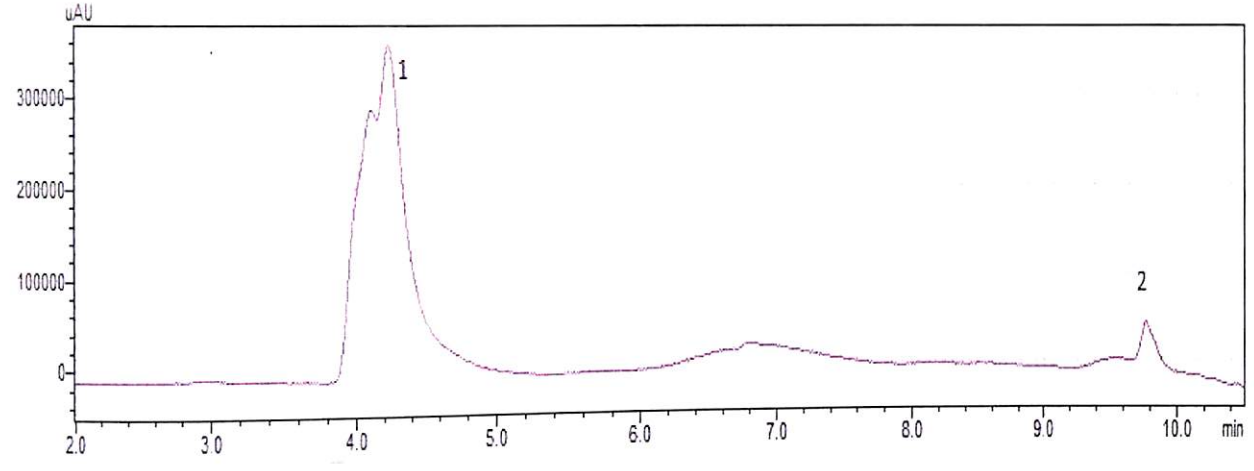


Figure 4.2. Reversed-phase HPLC chromatogram of (a) Cholesteryl Linoleate Standard, (b) Reaction product (Peak 1= β-sitosteryl oleate, Peak 2= β-sitosterol)

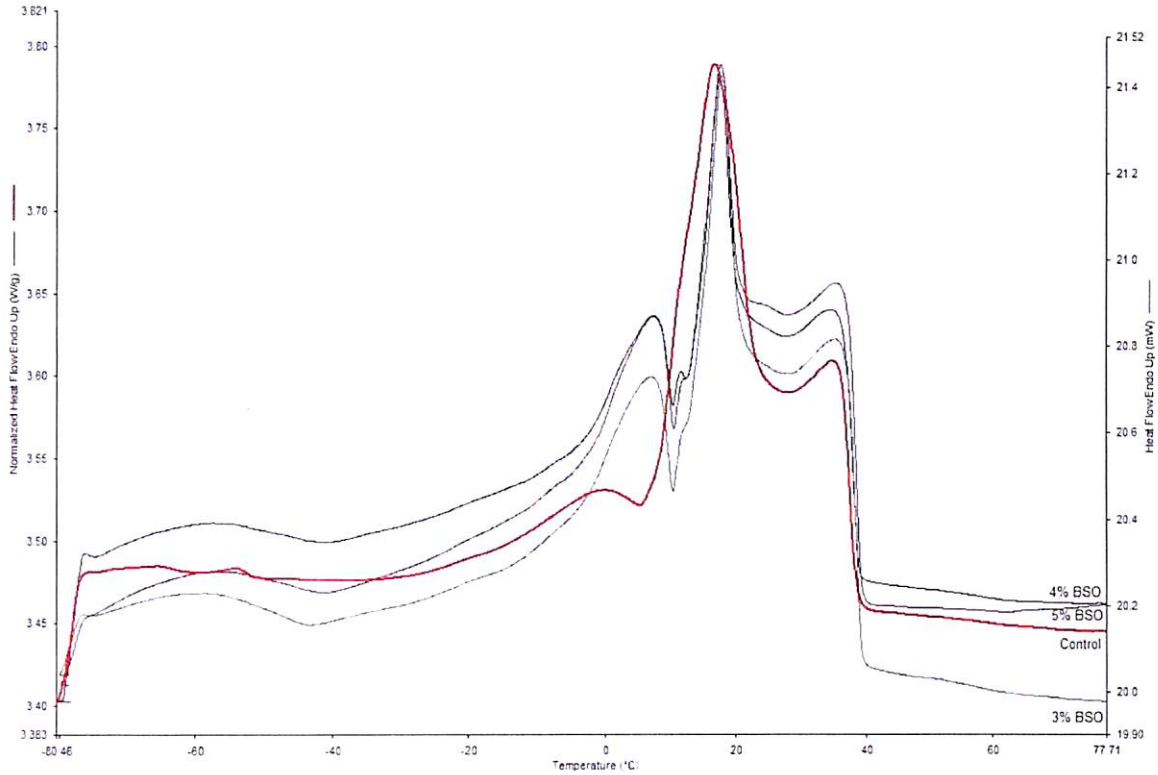


Figure 4.3. DSC thermogram of fat extracts from cream (control) and reduced cholesterol cream treated with 3, 4 and 5% β-sitosteryl oleate (BSO)

Table 4.4. Fatty Acid Profile of Reduced-Cholesterol Milk Fortified with β-Sitosteryl Oleate

Fatty Acid		Fatty Acid Content (%)
	Regular Milk	Treated Milk
Caproic acid (C6:0)	1.68 ± 0.01	1.61 ± 0.06
Caprylic acid (C8:0)	0.96 ± 0.01	0.94 ± 0.01
Capric acid (C10:0)	3.14 ± 0.01	3.12 ± 0.02
Lauric acid (C12:0)	3.63 ± 0.02	3.58 ± 0.03
Myristic acid (C14:0)	12.32 ± 0.01	12.22 ± 0.12
Myristoleic acid (C14:1)	0.94 ± 0.03	0.93 ± 0.06
Pentadecanoic acid (C15:0)	1.16 ± 0.01	1.11 ± 0.02
Palmitic acid (C16:0)	32.54 ± 0.01*	32.20 ± 0.06*
Palmitoleic acid (C16:1)	2.01 ± 0.01*	1.95 ± 0.02*
Stearic acid (C18:0)	11.78 ± 0.01	11.74 ± 0.03
Trans oleic acid (C18:1)	2.16 ± 0.05	2.15 ± 0.04
Oleic acid (C18:1)	24.36 ± 0.02*	25.18 ± 0.09*
Linoleic acid (C18:2)	2.36 ± 0.03	2.35 ± 0.02
Alpha linolenic acid (C18:3n3)	0.52 ± 0.02	0.52 ± 0.02
Eicosadienoic acid (C20:2)	0.44 ± 0.01	0.42 ± 0.02

*Significantly Different at $p \leq 0.05$

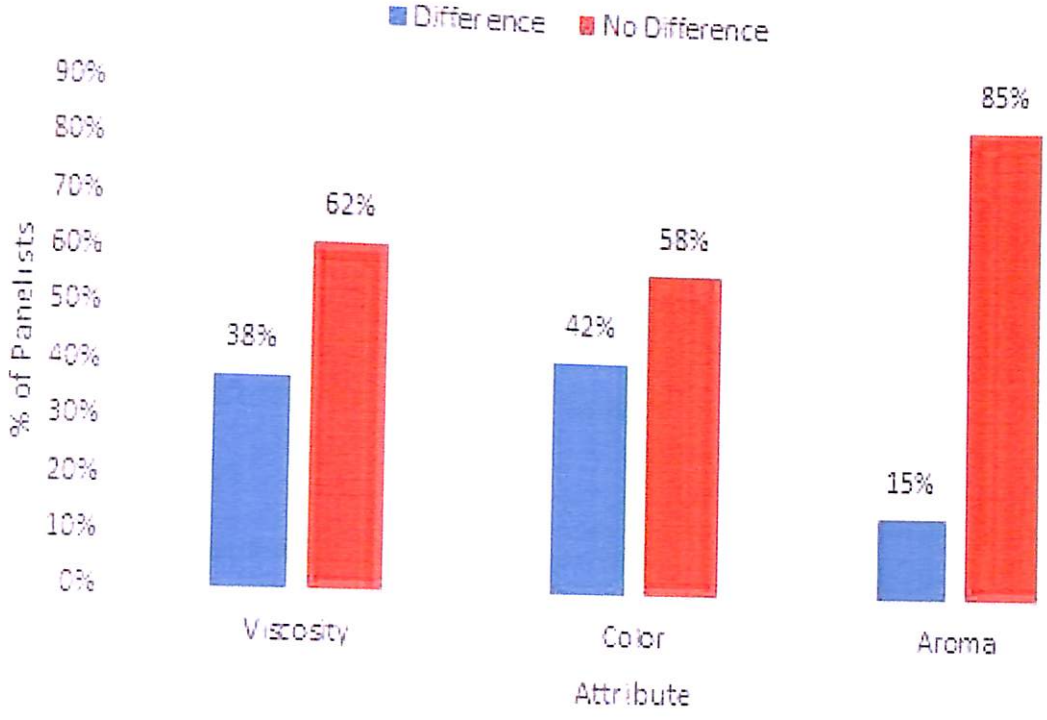


Figure 4.4. Sensory results showing the percentages of panelists who were able to discriminate between the treated and control milk samples.

Table 4.5. Chi-squared results for aroma

Responses	O	E	(O-E)	$(O-E)^2$	$X^2=(O-E)^2/E$
Correct	22	8.66	13.34	177.96	20.54
Incorrect	4	17.33	-13.33	177.69	10.25
Total					30.79

A= Control milk samples, B= Treated milk samples

H_a: A=B; H_0: A≠B

From chi-square distribution chart, $\chi^2_{1,\,0.05} = 3.84$

Since $\chi^2 = 30.79 > 3.84$, therefore, we reject the null hypothesis and conclude there was no significant difference between control and treated milk samples with respect to aroma.

Table 4.5. Chi-squared results for aroma

Responses	O	E	(O-E)	$(O-E)^2$	$X^2=(O-E)^2/E$
Correct	22	8.66	13.34	177.96	20.54
Incorrect	4	17.33	-13.33	177.69	10.25
Total					30.79

A= Control milk samples, B= Treated milk samples

H_a: A=B; H_0: A≠B

From chi-square distribution chart, $\chi^2_{1.\,0.05} = 3.84$

Since $\chi^2 = 30.79 > 3.84$, therefore, we reject the null hypothesis and conclude there was no significant difference between control and treated milk samples with respect to aroma.

Table 4.5. Chi-squared results for aroma

Responses	O	E	(O-E)	$(O-E)^2$	$X^2=(O-E)^2/E$
Correct	22	8.66	13.34	177.96	20.54
Incorrect	4	17.33	-13.33	177.69	10.25
Total					30.79

A= Control milk samples, B= Treated milk samples

H_a: A=B; H_0: A≠B

From chi-square distribution chart, $\chi^2_{1, 0.05} = 3.84$

Since $\chi^2 = 30.79 > 3.84$, therefore, we reject the null hypothesis and conclude there was no significant difference between control and treated milk samples with respect to aroma.

Table 4.6. Chi-squared results for color: -

Responses	O	E	(O-E)	$(O-E)^2$	$X^2=(O-E)^2/E$
Correct	15	8.66	6.34	40.20	4.64
Incorrect	11	17.33	-6.33	40.07	2.31
Total					6.95

A= Control milk samples, B= Treated milk samples

H_a: A=B; H_0: A≠B

From chi-square distribution chart, $\chi^2_{1,\,0.05} = 3.84$

Since $\chi^2 = 6.95 > 3.84$, therefore, we reject the null hypothesis and conclude there was no significant difference between the control and treated milk samples with respect to color.

Table 4.7. Chi-squared results for viscosity: -

Responses	O	E	(O-E)	$(O-E)^2$	$X^2=(O-E)^2/E$
Correct	16	8.66	7.34	53.88	6.22
Incorrect	10	17.33	-7.33	53.73	3.10
Total					9.32

A= Control milk samples, B= Treated milk samples

H_a: A=B; H_0: A≠B

From chi-square distribution chart, $\chi^2_{1,\,0.05} = 3.84$

Since $\chi^2 = 9.32 > 3.84$, therefore, we reject the null hypothesis and conclude there was no significant difference between the control and treated milk samples with respect to viscosity.

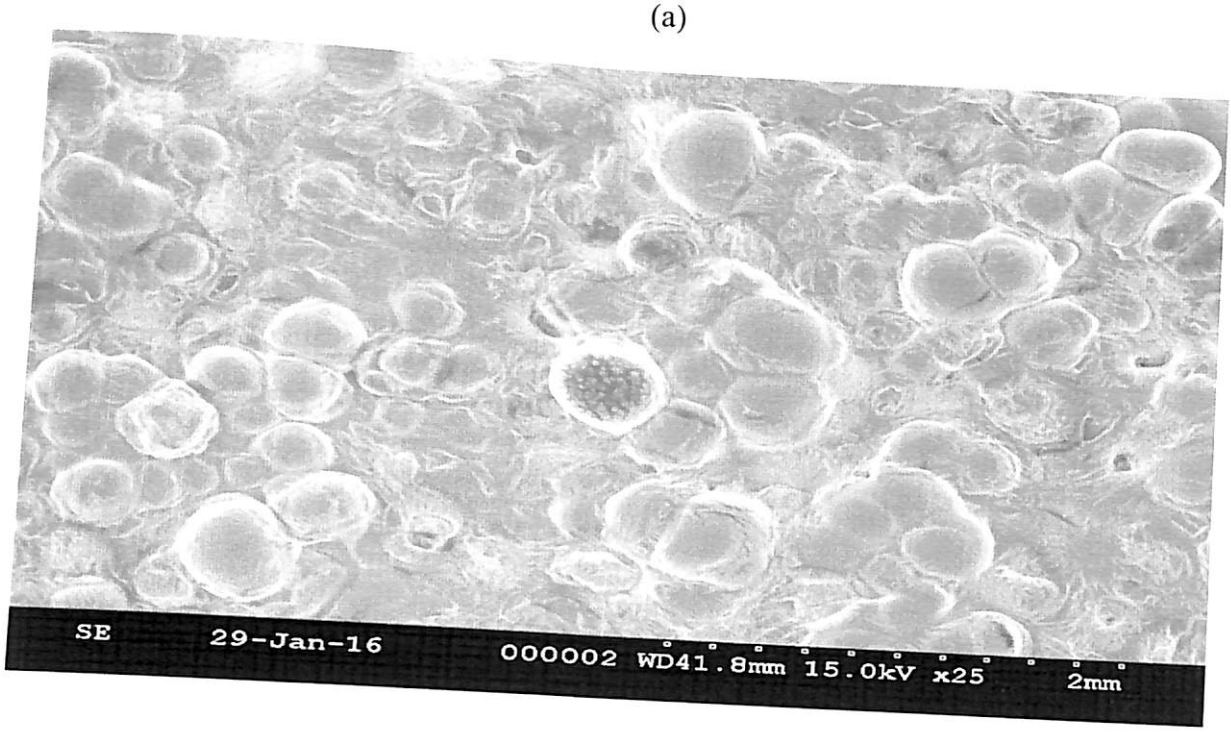
Table 4.8. Color & viscosity of milk

Color Properties	Control	Treated
L*	86.94±0.1[a]	87.28±0.5[a]
a*	-1.56±0.005[a]	-1.74±0.03[b]
b*	8.68±0.02[a]	8.73±0.06[a]

	Control	Treated
Viscosity (cp)	26.7[a]	23.5[b]

[ab]Values with different superscript letters within the same group are significantly different ($P < 0.05$)

(a)



(b)

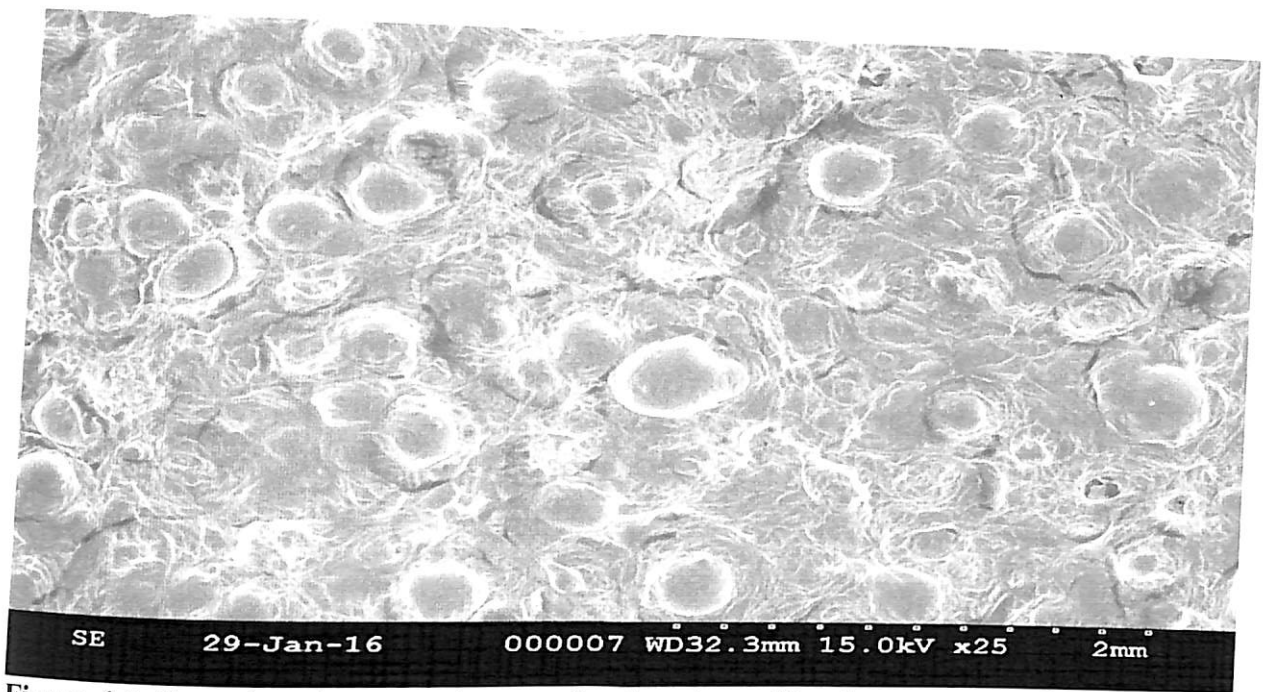


Figure 4.5. Scanning electron micrographs showing milk microstructure of (a) control(b) treated milk.

Table 4.9. Compositional, physicochemical and microbiological analyses of cheese

	Control Cheese	Treated Cheese
Moisture (%)	44.11 ± 0.1^{a}	42.78 ± 0.3^{b}
Ash (%)	4.13 ± 0.1^{a}	3.81 ± 0.0^{b}
Fat (%)	21.7 ± 0.6^{a}	22.30 ± 0.6^{a}
pH	6.80 ± 0.03^{a}	6.90 ± 0.01^{a}
Titratable Acidity	0.15 ± 0.00^{a}	0.21 ± 0.02^{a}
NaCl (%)	0.19 ± 0.01^{a}	0.18 ± 0.0^{a}
TPC (cfu)	3.0×10^{2}	0
Lactose (%)	2.12 ± 0.17^{a}	2.73 ± 0.10^{b}

[ab]Values with different superscript letters within the same row are significantly different ($P < 0.05$).
TPC = total plate count.

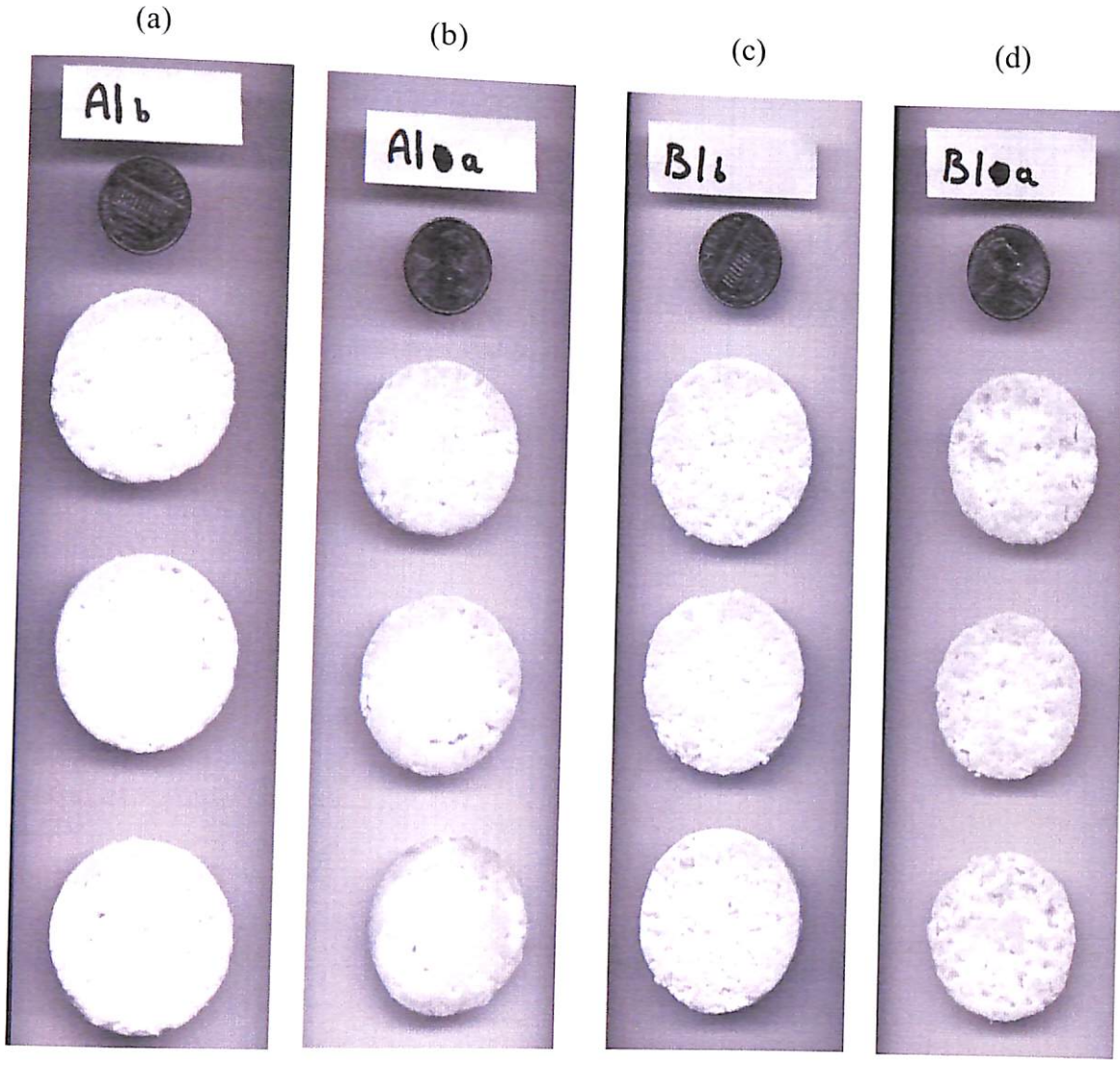


Figure 4.6. Cheese samples before (a&c) and after (b&d) of heating at 232°C for 5 min, (a-b) control, (c-d) treated.

Table 4.10. Color analysis of cheese subjected to baking conditions (130°C for 30 min)

Color Properties	Before Heating		After Heating	
	Control	Treated	Control	Treated
ΔE	-	-	17.63	14.71
L*	95.08	93.07	79.55	76.45
A*	0.51	-0.13	-1.23	-0.54
B*	10.73	9.01	18.03	15.91

ΔE = total color change, L* = whiteness, a* = magenta/green, b* = yellow/blue

Table 4.11. Color analysis of cheese subjected to broiling condition (232°C for 5 min)

Color Properties	Before Heating		After Heating	
	Control	Treated	Control	Treated
ΔE	-	-	10.07	7.98
L*	95.55	94.43	89.05	86.88
A*	0.56	-0.47	-0.23	-0.69
B*	10.62	15.96	18.23	13.59

ΔE = total color change, L* = whiteness, a* = magenta/green, b* = yellow/blue

Table 4.12. Cholesterol content of regular queso-fresco cheese (RQFC) and reduced cholesterol queso fresco cheese containing 3% β-sitosteryl oleate (RCQFC-BSO)

	RQFC	RQFC-BSO
Cholesterol content (mg/g)	19.68 ± 1.8^{a}	4.71 ± 0.8^{b}

[ab]Values with different superscript letters within the same row are different ($P < 0.05$)

CHAPTER 5

CONCLUSION

β-sitosteryl oleate was synthesized successfully via chemical catalysis by using 2% sodium bisulfate (w/w). The maximum degree of esterification (93.8 %) for the synthesis of β-sitosteryl oleate was achieved with a parameter combination of 140°C for 9 h with 1:5 molar ratio of β-sitosterol and oleic acid. Among the three experimental variables, temperature, time and molar ratio, only the effect of temperature was statistically significant. Furthermore, of all the levels of BSO incorporated into the cream, only 3% β-sitosteryl oleate showed melting profiles similar to that of regular cream.

When this treated cream was used to constitute 3.5% milk, the percentages of oleic, palmitic and palmitoleic acids decreased, and consequently may have impacted its viscosity. Sensory analysis predominantly showed that the experimental milk was highly comparable to regular milk. There was a significant reduction in the cholesterol content of the experimental cheese. Cholesterol content of the treated cheese was reduced by 76% relative to regular queso fresco cheese. The treated cheese also showed results comparable to that of the control cheese with respect to pH, fat content, titratable acidity, NaCl content, meltability and microbial load. However, it showed significant differences in ash, moisture, lactose contents and color.

This study has shown that the synthesis and incorporation of β-sitosteryl oleate into cream to constitute 3.5% milk and consequently queso fresco cheese with reduced cholesterol contents was successful. In general, our work has shown no significant differences between the experimental milk and cheese and their respective controls.

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CURRICULUM VITAE

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PROFILE SUMMARY

- Seeking an opportunity in food industry focused on quality assurance, safety, sensory and R&D.

EDUCATION

Delaware State University, Dover, DE *Jan 2014 - May 2016*
Master of Food Science and Biotechnology, Concentration: Food Chemistry
Thesis Title: Optimization of Physicochemical and Sensory Properties of Reduced-Cholesterol Milk & Cheese

Veermata Jijabai Technology Institute (V.J.T.I), Mumbai, India *Jun 2009 - Jun 2011*
Post Graduate Diploma in Chemical Technology, Focus in: Food, Drugs & Cosmetics

University of Mumbai, Mumbai, India *Jun 2005 - May 2008*
Bachelor of Science, Focus in: Biotechnology

EXPERTISE

Food Chemistry • Food Toxicology • Food Analysis • Food Technology • Food Microbiology • Food Science & Nutrition • Food Additives & Nutraceuticals • Pharmaceutical Technology • Advanced Biochemistry

RESEARCH EXPERIENCE

Graduate Research Assistant - Delaware State University, Dover, DE
Specialization: Dairy Products Development

- **Research Project:** Effect of Free Fatty Acids on Lipid Oxidation
- Responsible for operating, managing and maintaining all lab equipment used for analysis
- Conduct experiments in the area of lipid chemistry (Application of gas chromatography, high performance liquid chromatography, differential scanning calorimetry, spectrophotometry, etc)
- Analyze, record and track the observations of the experiments under various parameters for the conclusion

PROFESSIONAL EXPERIENCE

Research Analyst -Dolcera Corp. (Patent and Market Research Services), *Aug 2011 – Oct 2011*
Hyderabad, India

- Researched patents on Thomas Reuters database for packaging of perishable food products and patent infringement
- Segmented, classified data, developed landscape and created report based on the multiple parameters

Analytical Trainee - Sitec Labs Pvt Ltd. [Contract Research Organization *Jan 2011- Jun 2011*
(CRO)], Mumbai, India

- Performed qualitative analysis and developed reports for client validation of active and inactive pharmaceutical ingredients, chemical intermediates and finished products, elemental impurities and assay in pharmaceutical industry

SKILLS AND CERTIFICATES

- Completed HACCP Manager's Workshop by American Food Safety Services & Technical and Business Services, LLC
- Completed Implementing SQF Systems Practitioner Workshop by American Food Safety Services & Technical and Business Services, LLC *Jan 2016*
- Trained on Camag HPTLC system at CMAG Switzerland recognized Application Research Laboratory, *Jan 2016*
- Overview of production process during industrial visit to Cipla, Emami and Worli Dairy, Mumbai
- Technical Skills: MS Excel, MS PowerPoint, MS Word

AWARDS

USDA Capacity Building Grants Program (Department of Human Ecology, DSU)

- Graduate Assistantship for Master's Degree program of $33,000 per academic year to cover tuition and stipends

The National Society of Leadership and Success

- Selected as a Member of honor society for Leadership Program at National Level

PRESENTATIONS

- Abaidoo-Ayin H.K., Jadhav P.S., Lumor S.E. ***Do Free Fatty Acids Promote Lipid Oxidation?*** 106th AOCS Annual Meeting, Orlando, FL *May 2015*
- Jadhav P.S., Lumor S.E. ***Optimization of the synthesis of beta-sitosteryl oleate via chemical catalysis,*** Graduate Research Symposium, Dover, DE *Apr 2015*
- Abaidoo-Ayin H.K., Jadhav P.S., Lumor S.E. ***Do Free Fatty Acids Promote Lipid Oxidation?*** Graduate Research Symposium, Dover, DE *Apr 2015*

PROFESSIONAL MEMBERSHIPS

- The National Society of Leadership and Success *Sep 2015 - Present*
- Institute of Food Technologists (IFT) *Jan 2014 - Present*
- American Oil Chemists' Association (AOCS) *Jan 2014 - Present*

PROBONO VOLUNTEERING

- Organized and managed a two weeks' summer camp of "Food Science" for high school students *Jul 2015*