Development of liposomal formulation containing phytosterols and tocopherols with the aim of reducing low density lipoprotein cholesterol (LDL-C)

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By Asmita Poudel

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ABSTRACT

Background and Rationale: Phytosterols can reduce low-density lipoprotein cholesterol (LDL-C) in serum from the range of 8-14% and have been approved by FDA and Health Canada for their cholesterol-lowering abilities. They can be obtained from vegetable oilseeds and their deodorizer distillates. Canola oil deodorizer distillate (CODD) contains 15-25% of phytosterols, making it an excellent underutilized source of these bioactives. Phytosterols obtained from the CODD can be developed into nutraceutical products in order to attain their commercial potential. However, there are three major challenges in utilizing phytosterols: 1) their lipophilic nature; 2) their thermo-sensitivity leading to the generation of phytosterol oxidation products (POPs); and 3) their formulation-dependent therapeutic efficiency. Nanotechnology-based liposomal formulation can possibly address all these challenges. In addition, the incorporation of antioxidants, namely tocopherols in phytosterol formulation, can enhance phytosterols' oxidative stability. This study aims to develop liposomes containing phytosterols along with antioxidants into a nutraceutical formulation. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods have been developed to assess liposomal formulations. Furthermore, the LDL-C lowering efficiency of the developed formulation was assessed using the hamster animal model. For the first time, the efficacy of brassicasterol, a phytosterols unique to canola oil, in combination with campesterol and β-sitosterol will be assessed.

Methods: Three different approaches were utilized for the preparation of liposomes containing phytosterols and tocopherols, namely, thin layer hydration homogenization, thin layer hydration ultrasonication, and the Mozafari method. Two LC-MS/MS methods were developed to determine the entrapment efficiency of phytosterols and tocopherols inside the liposomes as well as POPs

present in liposomal formulations. Reversed-phase chromatography with isocratic elusion and atmospheric pressure chemical ionization (APCI) was used in LC-MS/MS method. The methods were validated as per the ICH and FDA guidelines to ensure the accuracy of analytical measurements. An accelerated stability study (microwave heating) was conducted for three different products: crude phytosterols, liposomal phytosterols, and liposomal phytosterols containing tocopherols to monitor the presence of POP during long term storage. The optimized formulations were orally fed to diet-induced hyperlipidemic hamsters for four weeks to assess the extent of LDL-C reduction by the liposomal phytosterols compared to control and marketed phytosterols-containing product.

Results: Liposomal vesicles prepared via homogenization and ultrasonication methods were significantly lower in size (<200 nm) compared to the ones produced by the Mozafari method (>200 nm). All three methods showed comparable zeta potential values (-9 to -14 mV), which was adequate for the physical stability of the vesicles. LC-MS/MS method developed for the determination of phytosterols, and tocopherols had a run time of only seven minutes with excellent linearity (R^2 = 0.998). An entrapment efficiency of >89% was obtained for target analytes. LC-MS/MS method for the determination of POPs had a run time of five minutes which is the shortest run time among reported methods for the determination of POPs. Only one POP (7-ketobrassicasterol) was in the quantifiable range in the liposomal formulations indicating that the preparation method results in negligible levels of POPs. In fact, the quantified value for 7-ketobrassicasterol was too low to cause cytotoxicity. Microwave heating (i.e., accelerated stability study) showed the presence of various POPs with the following ascending order for their generation: liposomal formulation of phytosterol and tocopherols < liposomal formulation of phytosterols < crude phytosterols. This confirms that the generation of POPs is prevented by the

protective action of liposomes as well as antioxidants. Finally, animal testing showed a significant reduction of LDL-C by the liposomal phytosterols compared to the control.

Conclusion: In conclusion, liposomes are an excellent carrier to deliver phytosterols as they imparted high entrapment efficiency, prevented phytosterols' oxidation in the presence of tocopherols, and showed a significant effect in reducing LDL-C in animals.

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Dedicated to

My parents, Damaru Dev Poudel and Sita Sharma Poudel,

My Brother, Apar Poudel,

My Sister-in-Law, Nisha Kadel Poudel,

My Niece, Charvi Poudel.

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LIST OF ABBREVIATIONS

LDL-C	Low density lipoprotein cholesterol
PS	Phytosterols
CODD	Canola oil deodorizer distillate
FDA	Food and Drug Administration
RESS	Rapid expansion of supercritical solutions
GC	Gas chromatography
LC	Liquid chromatography
FID	Flame ionization detection
LC-MS/MS	Liquid chromatography tandem mass spectrometry
ELSD	Evaporative light scattering detection
UV	Ultraviolet
DAD	Photodiode array detection
MSTFA	N-methyl-N-(trimethylsilyl)-trifluoroacetamine
BSTFA	N-O-bis- (trimethylsilyl)-trifluoroacetamine
MTBSTFA	N-methyl-N-tert-butyldimethylsilyltrifluoroacetamide
NIST	National Institute of Standards and Technology
ESI	Electrospray ionization
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photoionization
SUV	Small unilamellar vesicles
LUV	Large unilamellar vesicles

MLV	Multilamellar vesicles
GUV	Giant unilamellar vesicles
MVV	Multivesicular vesicles
EE	Entrapment efficiency
POPs	Phytosterol oxidation products
COPs	Cholesterol oxidation products
CVD	Cardiovascular disease
PC	Phosphatidylcholine
ICH	International Council for Harmonization
LOD	Limit of detection
LLOQ	Lower limit of quantification
LQC	Low quality control
MQC	Middle quality control
HQC	High quality control
RT	Retention time
TEM	Transmission electron microscopy
B/L	Bioactives:Lipid
PDI	Polydispersity index
DLS	Dynamic light scattering
HTST	High temperature short time
RPM	Revolution per minute
TOF	Time of Flight
S/N	Signal to noise

ME	Matrix effect
CID	Collision induced dissociation
MRM	Multiple reaction monitoring
EVOO	Extra virgin olive oil
NPLC	Normal phase liquid chromatography
T-C	Total cholesterol
VLDL-C	Very low density lipoprotein cholesterol
L-PS-OJ	Liposomal phytosterols in orange juice
L-PS-W	Liposomal phytosterols in water
M-PS-OJ	Marketed phytosterols in orange juice
OJ	Orange juice
W	Water

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error. Symbols (*, $) show the presence of statistical significance at P <0.05. "*" above the dotted line represents the statistical significance of the treatment groups with their respective control group at a constant time point. OJ group is control of treatment groups L-PS-OJ and M-PS-PJ, whereas W group is control of the treatment group L-PS-W. "$" above the solid line represents the statistical significance between treatment groups at a constant time point. N values: i) week 0; L-PS-OJ (N=12), L-PS-W (N=12), M-PS-OJ (N=12), OJ (N=12), and W (N=12); ii) week 2; L-PS-OJ (N=12), L-PS-W (N=12), M-PS-OJ (N=10), OJ (N=12), and W (N=12) and; iii) week 4; L-PS-OJ (N=11), L-PS-W (N=8), M-PS-OJ (N=9), OJ (N=9) and W (N=7), Abbreviations : L= Liposomal, PS = PS, M = Marketed, OJ = Orange juice, and W = Water. The decrease in N value in week 2 and week 4 are due to, i) presence of outlier, ii) death of animals, iii) exclusion of animals with diarrhea with significant weight reduction…….199

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were used to express statistical significance at $P<0.05$. The different letters in each column denotes the statistical significance. No significant differences were observed between treatments relative to their respective control. Abbreviations: L= Liposomal, PS = PS, M = Marketed, OJ = Orange juice, and W = Water. N values: i) week 0; L-PS-OJ (N=12), L-PS-W (N=12), M-PS-OJ (N=12), OJ (N=12), and W (N=12); ii) week 2; L-PS-OJ (N=12), L-PS-W (N=12), M-PS-OJ (N=10), OJ (N=12), and W (N=12) and; iii) week 4; L-PS-OJ (N=11), L-PS-W (N=8), M-PS-OJ (N=9), OJ (N=9) and W (N=7), Abbreviations: L= Liposomal, PS = PS, M = Marketed, OJ = Orange juice, and W = Water. The decrease in N value in week 2 and week 4 are due to, i) presence of outlier, ii) death of animals, iii) exclusion of animals with diarrhea with significant weight reduction....196

CHAPTER 1

Literature review

1.1. Introduction

Nutraceuticals have gained popularity in the last decade due to their potential role in disease prevention [1]. Health Canada defines nutraceuticals as *" a product isolated or purified from food that is generally sold in medicinal forms and is demonstrated to have a physiological benefit or provide protection for chronic disease*"[2]. Health benefits of the nutraceuticals lie in their ability to prevent or mitigate diseases such as diabetes [3], hypercholesterolemia [4, 5], hypertension [6], and cancer [7, 8]. Nutraceuticals are mostly plant-derived such as phytosterols (PS) [9], tocopherols [10], ascorbic acid [11], niacin [12], and carotenoids [13, 14].

PS and tocopherols are nutraceuticals that have demonstrated health and nutritional benefits. PS reduce low-density lipoprotein cholesterol (LDL-C) [15], whereas tocopherols are antioxidants [16]. Tocopherols can be obtained from both natural (olive oil, sunflower oil, canola oil, palm oil, and nuts) and synthetic sources [17, 18]. Regarding PS, canola, the most abundant oilseed crop of Canada, is an excellent source [19-21]. During the refining process, canola oil loses some of its nutritional components, such as PS which are transferred to the waste stream, termed canola oil deodorizer distillate (CODD) offering an ideal source for their extraction [22]. Therefore, there are unexplored health and economic gains from extracting PS and incorporating them into a nutraceutical formulation. However, the commercial potential can only be achieved if these compounds are properly formulated into food products.

Formulating PS and tocopherols in food products has always been challenging due to their lipophilicity as well as heat and light sensitivity as they are prone to oxidation [23]. Also, the requirement of food-grade solvents and emulsifiers makes the formulation task more challenging due to the availability of a low number of Food and Drug Administration (FDA)-approved food-grade solvents/emulsifiers. The poor solubility of these compounds can be addressed by applying encapsulation techniques, widely used in the pharmaceutical and food industry [24, 25]. However, not all the encapsulation techniques are suitable for formulating thermo-sensitive compounds because some of the methods utilize high temperature, leading to their oxidation [26, 27]. In addition, some of the encapsulation techniques use a large amount of emulsifiers, which can have deleterious effects on human health [28].

Nanotechnology-based liposomal entrapment is a promising approach for incorporating PS and tocopherols into food products due to their potential ability to overcome solubility issues without compromising stability [29, 30]. Liposomes can be prepared from biocompatible and biodegradable natural phospholipids, such as phosphatidylcholine. Apart from addressing physicochemical challenges mentioned above, the optimum formulation must enhance the therapeutic efficiency of the active ingredients. It is evident from the literature that the cholesterol-lowering efficiency of PS depends largely on the formulation in which it is delivered [31, 32]. Therefore, it is important to evaluate the efficacy of new formulations by performing animal trials. In this thesis, we are developing a liposomal formulation containing PS obtained from CODD and commercially available tocopherols. The formulation will be incorporated into food products. I am also developing two different analytical methods to determine encapsulation of PS and tocopherols into liposomes, and to monitor harmful oxidation products of PS during preparation and storage of the formulations. Cholesterol-lowering efficiency of PS will be evaluated in animals.

1.2. Phytosterols

PS (**Figure 1.1**) are plant-derived sterols having structural resemblance with cholesterol (**Figure 1.1**). They are secondary plant metabolites belonging to the triterpene family. PS are present in high concentrations in nuts (pistachio, pine nut, and almond) and vegetable oil seeds, such as canola, soybean, sesame, and sunflower [14, 33]. The most abundant PS found in oilseeds are brassicasterol, campesterol, stigmasterol, and β-sitosterol (**Figure 1.1**).

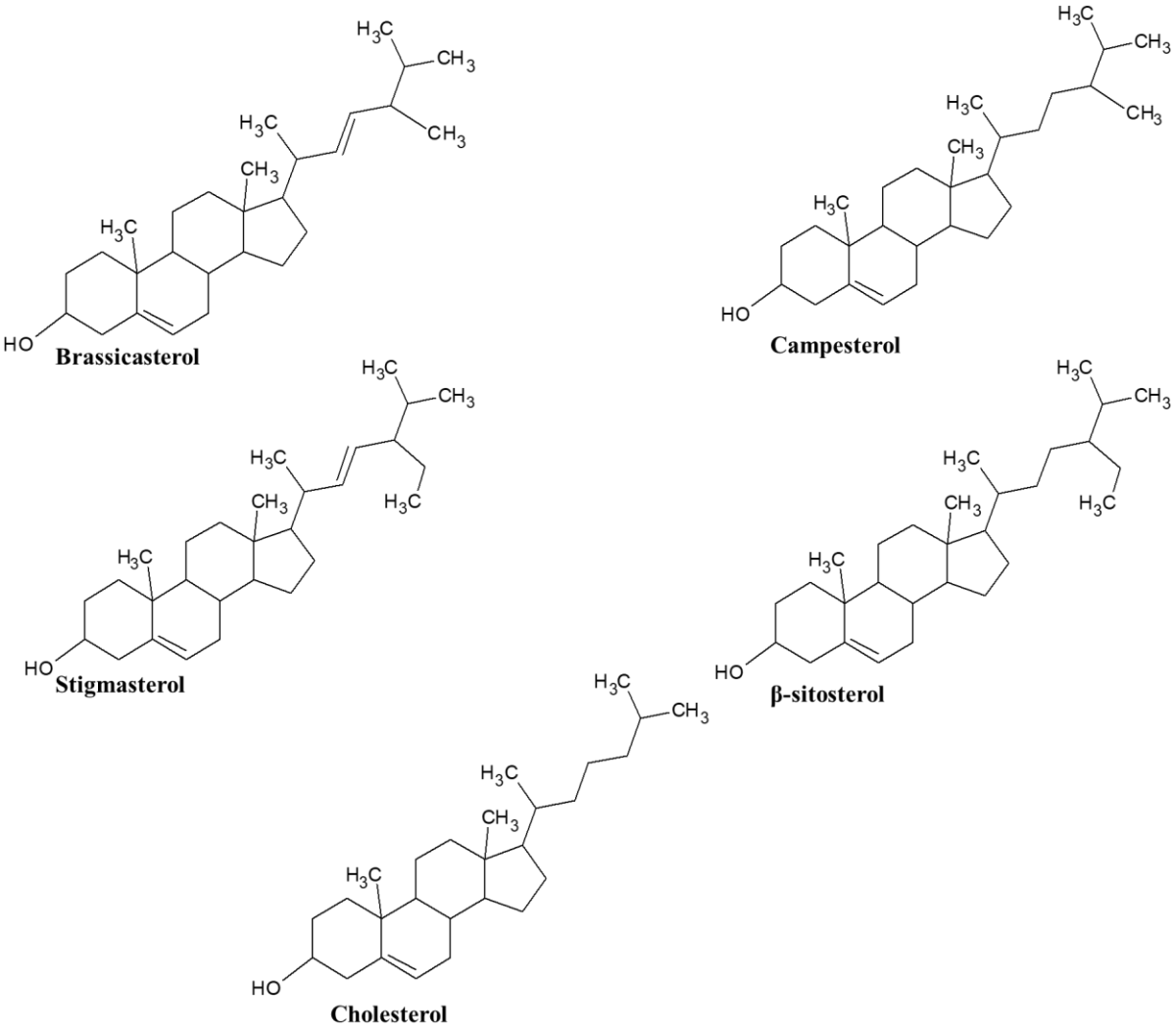


Figure 1.1. Structure of four abundant phytosterols; brassicasterol, campesterol, stigmasterol, and β-sitosterol, along with cholesterol.

PS can reduce the serum LDL-cholesterol in the range of 8-14% at a daily dose of 2 grams [31, 34-37]. The primary mechanism of action of PS is to compete with cholesterol for the solubilization in bile salts micelles [37-39], thereby inhibiting its intestinal absorption. Unlike

cholesterol, whose absorption is about 50% in the intestinal tract, PS are much less absorbed, around 2-8% [40, 41]. **Figure 1.2** summarizes the difference in intestinal fate of cholesterol and PS. Other secondary mechanisms, such as the inhibition of the action of the enzyme acyl CoA acyltransferase (an enzyme that is required to esterify cholesterol to get it absorbed) and the efflux of cholesterol back into the intestinal lumen in the presence of PS have also been reported in the literature [42].

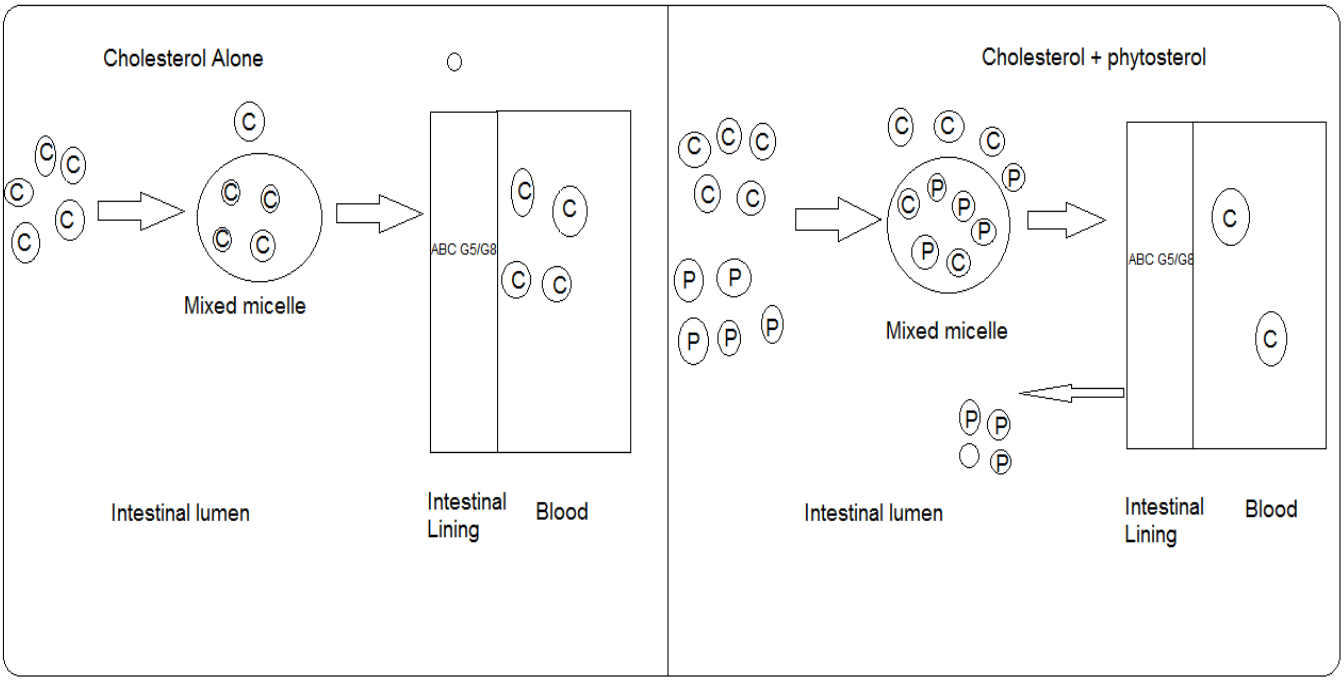
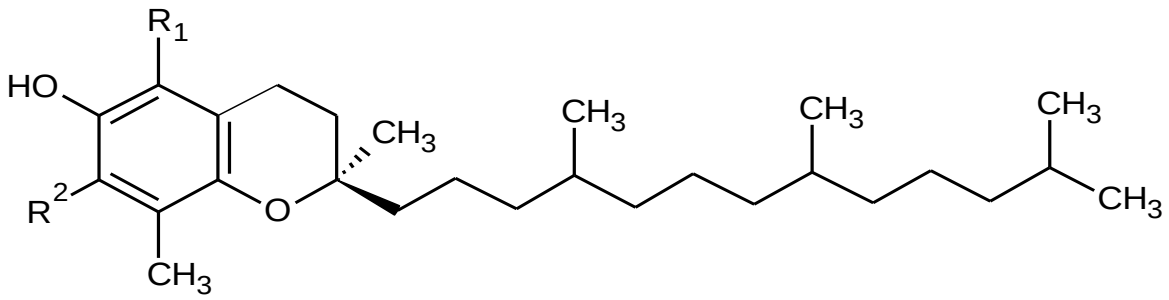


Figure 1.2. Absorption of cholesterol in the blood in the presence (right panel) and absence of phytosterols (left panel). Cholesterol is denoted by "C," and PS is denoted by "P." i) P competes with C for the solubilization in mixed micelles, ii). P present in mixed micelles gets absorbed by enterocytes in the intestinal lining, and iii). P gets released back to the intestinal lumen by the action of ATP binding cassette (ABC) G5/G8 transporter [42].

1.3. Tocopherols

Tocopherols (**Figure 1.3**), or vitamin E, are lipid-soluble methylated phenols consisting of chromane ring linked with saturated isoprenoid C16 side chain. They are naturally present in oilseeds such as sunflower, canola, and palm [43]. The most abundant tocopherols found in plants are alpha, beta, gamma, and delta tocopherols (**Figure 1.3**). Although alpha tocopherol alone is widely used as an anti-oxidant, a mixture of tocopherols (alpha, beta, gamma, and delta)

demonstrated higher potency compared to alpha tocopherol alone [44]. They are antioxidants that act as peroxyl radical scavengers, disabling the production of damaging free radicals in cells [45]. Tocopherols donate the hydrogen from their hydroxyl group to the reactive free radicals, breaking the chain propagation, thus neutralizing the effect of the radicals [46, 47]. In addition to natural tocopherols, synthetic derivatives are equally popular as anti-oxidants [48]. Synthetic tocopherols are present as the racemic mixtures of all eight stereoisomers (RRR, SRR, RRS, RSS, RSR, SSR, RSS, and SSS), unlike natural tocopherols, which are only present in a single form (RRR) [49]. Both *in vitro* and *in vivo* antioxidant activity of tocopherols have been demonstrated. [50, 51].



Tocopherols	**R1**	**R2**
Alpha	CH3	CH3
Beta	CH3	H
Gamma	H	CH3
Delta	H	H

Figure 1.3. Structures of the four most abundant tocopherols; alpha-tocopherol, beta-tocopherol, gamma-tocopherol, and delta-tocopherol.

1.4. Canola oil deodorizer distillate (CODD) - source of phytosterols

Canola is an abundant oilseed crop produced in Canada [19]. The most notable bioactives in canola oil are PS [20, 22]. However, a significant fraction of PS is lost during the oil refining process of

canola, ending up in the waste stream (i.e., the deodorizer distillate) **(Figure 1.4)**. Therefore, the canola oil deodorizer distillate (CODD) is a potential natural source of PS [22]. It is reported that the percentage of PS in CODD is about 21-25% [52]. Other components of CODD includes to squalene, phytol, and tocopherols [53]. The CODD is currently treated as a waste or low-value animal feed product. PS can be extracted from the CODD and be designed into a nutraceutical formulation. This way both economic and health benefits can be attained.

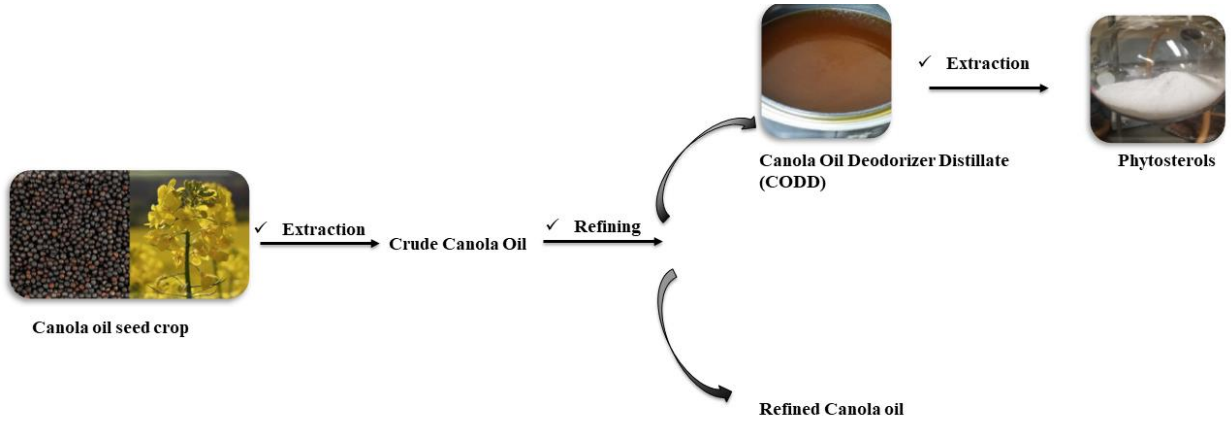


Figure 1.4. Schematic diagram showing canola oil deodorizer distillate (CODD) as a source of phytosterols.

1.5. Challenges of development of nutraceuticals containing phytosterols and tocopherols

Lipophilicity and thermo-sensitivity are the two major challenges in the development of nutraceutical formulations containing PS and tocopherols. Both PS and tocopherols are poorly soluble in water, making them difficult to be incorporated in foods and beverages, such as fruit juice and milk. In addition, PS can undergo oxidation in the presence of heat and light (detailed in section 2.9)

Nevertheless, various encapsulation techniques have been utilized in the food industry to overcome lipophilicity challenges and to enhance the compounds' oxidative stability [24, 54]. However, not all encapsulation techniques are suitable for formulating thermo-sensitive compounds. Therefore, it is crucial to thoroughly investigate the suitability of the encapsulation techniques prior to their application. Along with encapsulation, the presence of antioxidant tocopherols can impart additional oxidative stability of PS.

1.6. Encapsulation techniques used in the food industry and their suitability for liposomal formulations of phytosterols and tocopherols

Encapsulation is defined as the process to entrap active compounds within inactive ingredients such as wall materials, nano-carriers (nanoparticles, liposomes), coating materials, and capsules [55, 56]. Encapsulation techniques like spray drying [24, 57], spray chilling [58, 59], micro emulsification, inclusion entrapment, rapid expansion of supercritical solutions (RESS), and liposomal entrapment have been employed in the food industry [24].

Spray drying is widely popular in the food industry with around 80-90% of total encapsulated bioactives being entrapped using this effective and low-cost method [24, 57]. The basic principle of spray drying involves dissolving active components and wall material such as sodium alginate, hydroxypropylmethyl cellulose, inulin, and methyl β-cyclodextrin in water. The resulting mixture in the liquid form is fed at an optimum flow into a spray dryer, maintained at a temperature around 100-300 °C. The spray-dried products are mostly powdered form, or agglomerated particles, depending on the nature of the active ingredients and the wall materials [57]. The major disadvantage of spray drying is the need for high temperature, which can cause oxidation/degradation of thermo-sensitive compounds. For instance, oxidation products were identified in spray-dried quercetin, a polyphenol, and lycopene, a carotenoid [60, 61].

Unlike spray drying, spray chilling requires low temperature, thus can maintain oxidative stability of thermo-sensitive compounds. In spray chilling, the liquid mixture is atomized at refrigeration, where liquid droplets convert into solid powdered particles [58, 59]. The challenge with the spray chilling process is the requirement of special handling and storage conditions of the finished product [24]. Thus, this methodology is not advisable from an economic point of view. However, when particles in the nanometer range are desired, both spray drying and spray chilling are unsuitable because the particle size obtained with these techniques ranges from 10-400 micrometer [62].

To address the particle size issue, other techniques such as inclusion encapsulation, RESS, and micro emulsification can be adopted. These three techniques do not require the utilization of higher temperatures [62]. Inclusion encapsulation involves the complexation of active compounds inside the inner hydrophobic cavity of cyclodextrins, a cyclic oligosaccharide composed of glucopyranose [63, 64]. RESS involves two major steps; i) dissolving the active ingredients and coating materials in supercritical fluid (CO2), and ii) expanding the resulting solution (maintained at high pressure) through orifice nozzle, which leads to desorption of solvent, thus forming a layer of coating materials around the active ingredients [65, 66]. Unfortunately, both inclusion encapsulation and RESS can be costly for scale-up procedures [62]. Micro-emulsification involves the encapsulation of active compounds within self-assembled surfactant molecules [67]. It is economical for scale-up processes; however, it requires large amounts of emulsifiers, which can lead to adverse effects in humans [68]. In addition, numerous surfactants are prohibited in the food industry as per the FDA guidelines [69]. In sum, despite the wide use of the above-mentioned techniques in the food industry, they are not appropriate for encapsulating PS and tocopherols due

to various shortcomings, such as need for high temperature, cost for scale-up, and the requirement of emulsifier that could be harmful for health.

In addition to the need for optimum encapsulation techniques, there is a requirement for robust analytical platforms for analyzing PS and tocopherols in the developed formulation.

1.7. Challenges in the analysis of phytosterols and tocopherols

Gas chromatography (GC) and liquid chromatography (LC) coupled with detectors such as flame ionization detector (FID) [70], mass spectrometry [71, 72], ultraviolet (UV) [73], evaporative light scattering detection (ELSD) [74], and photodiode array detection (DAD) [75] have been routinely utilized for the determination of PS.

1.7.1 Gas chromatography (GC)

GC involves the physical separation of analytes based on their volatility and their interaction with stationary phase in the presence of inert gas as a carrier. PS have low volatility (due to the presence of a hydroxyl group), thus require high temperature for their determination using GC system [76]. However, usage of high temperature may degrade such sensitive compounds. Therefore, PS can usually derivatized to increase their volatility using commonly used silylating agents such as N-O-bis- (trimethylsilyl)-trifluoroacetamine (BSTFA), N-methyl-N-(trimethylsilyl)-trifluoroacetamine (MSTFA), and N-methyl-N-tert-butyldimethylsilyltrifluoroacetamide with 1% tert-butyldimethylchlorosilane (MTBSTFA). In addition to enhancing volatility, derivatization also increases the sensitivity in PS determination [77]. For example, PS in rapeseed oil analyzed in both the underivatized and derivatized forms showed around ten times higher content in the case of derivatized form compared to non-derivatized counterparts [77]. GC system can be coupled with FID [70, 78] and MS detectors (**Table 1.1**) [72]. For instance, GC-FID was employed

to determine brassicasterol, campesterol, and β-sitosterol fortified in food products such as cheese, milk, fat spread and milk powder with the method with a total run time of 15 min [70]. Similarly, GC-FID was used for the analysis of PS present in five different vegetable oils namely- rapeseed, sunflower, linseed, sesame, and maize were determined [78]. However, GC-FID method does not provide selectivity for co-eluting PS or the ones imparting poor baseline separation. On the other hand, GC-MS (as shown in **Table 1.1**) can be employed to overcome these shortcomings, as selectivity for co-eluting analytes is attained by tandem mass spectrometry (MS/MS) since analytes are identified based on their diagnostic product ions. GC-MS widely utilizes electron impact (EI) as the ionization source [79]. EI is a harsh ionization method where compounds being bombarded with highly energetic electron beam held at a voltage around 70 eV [79]. A constant voltage is used in order to match the obtained mass spectra with the global spectral database library of the National Institute of Standards and Technology (NIST). A main drawback associated with EI is the possibility in the degradation of sensitive compounds.

Table 1.1. GC and LC methods utilized for the determination of phytosterols in various samples.

Analytical method	**Phytosterols**	**Chromatographic conditions**	**Sample**	**Run time (min)**	**Shortcomings**	**Ref**
GC-FID	Campesterol Brassicasterol Stigmasterol β-Sitosterol δ5-Avenasterol δ7-Avenasterol	30 m DB-5MS column	PS in vegetable oil such as canola, sunflower, maize, linseed, and sesame oil	22.5	Requirement of derivatization of PS Longer run time	[78]
GC-MS	Campesterol Stigmasterol β-Sitosterol	Capillary column Restek RTX-5 coated with 95% dimethyl and 5% diphenyl polysiloxane	PS enriched milk and yogurt.	10	Requirement of derivatization of PS	[72]
LC-ELSD	Campesterol Stigmasterol β-Sitosterol	Reversed-phase XTerra phenyl column Isocratic elution using acetonitrile:methanol:water (48:22.5:29.5 v/v/v)	PS in soybeans	61	Longer run time Less sensitive detector	[80]

LC-UV	Stigmasterol β-Sitosterol	Reversed-phase C8 narrow bore column Isocratic elution using acetonitrile:water (86:14)	PS in soybean oil	14	Less sensitive detector	[73]
LC-ESI-MS	Ergosterol Stigmasterol β-Sitosterol	Reversed-phase BEH Phenyl column Gradient elution using methanol (0.1% formic acid) and water (10mM ammonium acetate)	PS in tobacco leaves	6	PS ionizes poorly in ESI[81] .	[82]
LC-APCI-MS	Brassicasterol Campesterol β-Sitosterol	Normal phase chromatography Gradient elution using n-hexane and ethyl acetate	PS in canola oil	30	Longer run time. Longer equilibrium time	[83]
LC-APPI-MS	Brassicasterol Campesterol Stigmasterol β-Sitosterol	Reversed-phase Chromolith SpeedRod RP-18e monolithic column Isocratic elution using methanol and water	PS in human serum	6	Baseline separation was not obtained for stigmasterol and β-sitosterol	[84]
LC-APCI-MS	Brassicasterol Campesterol Stigmasterol β-Sitosterol Ergosterol Fucosterol	Reversed-phase ZORBAX Eclipse XBD-C18 Column Gradient elution using water (0.1% formic acid), acetonitrile, and methanol	PS in olive oils.	~25	Baseline separation was not obtained for stigmasterol and β-sitosterol Longer run time	[85]

Apart from conventional GC-MS method, two dimensional GC methods, GC X GC-MS are being developed in order to enhance separation as well as sensitivity of PS [86]. GC X GC utilizes two columns in tandem resulting in a powerful separation resolution [87]. It is well-suited for determining analytes in complex matrices or the analytes mixtures of diverse volatility [87]. To sum, even the newer GC based approaches are showing excellent separation of PS as well as better sensitively, however, complexity still lies in the requirement of derivatization of molecules which makes GC methods tedious and time consuming.

1.7.2. Liquid chromatography (LC)

Unlike GC, LC does not require the derivatization of analytes, thus is a simpler analytical approach. Both normal and reversed-phase liquid chromatography has been explored for PS analysis [80, 83, 85]. However, reversed phase is advantageous as it can provide a shorter run and equilibrium time (**Table 1.1**). In addition, unlike normal phase chromatography, reversed phase uses mobile phases with decreased volatility and toxicity, in general. Detectors such as UV, ELSD, and MS have been coupled with LC system for determining PS in various matrices [73, 80, 83]. For instance, LC-UV was employed to determine stigmasterol, and β-sitosterol present in soybean oil using C8 column at a wavelength of 208 nm [73]. The method developed method had a total run time of 12 min [73]. On the hand, LC-ELSD was adopted to quantify campesterol, stigmasterol, and β-sitosterol present in vegetable oil [80]. Unfortunately, one of the major drawbacks of UV and ELSD is the lack of ability to distinguish between for co-eluting PS. LC-MS can be utilized to overcome this shortcoming (**Table 1.1**).

Soft ionization methods such as electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) have been explored for the determination of PS using LC-MS. In case of ESI, sample gets introduced in capillary at a flow rate of 1-1000 µL/min. The voltage in the range of 5

to 6 kV is applied in the capillary and can be either positive or negative depending on the nature of analytes. ESI involves ionization of analytes by two steps: i) nebulization of a sample to produce charged droplets of analytes, and ii) solvent evaporation of droplets using curtain gas to produce ions in a gaseous state [88] (**Figure 1.5**).

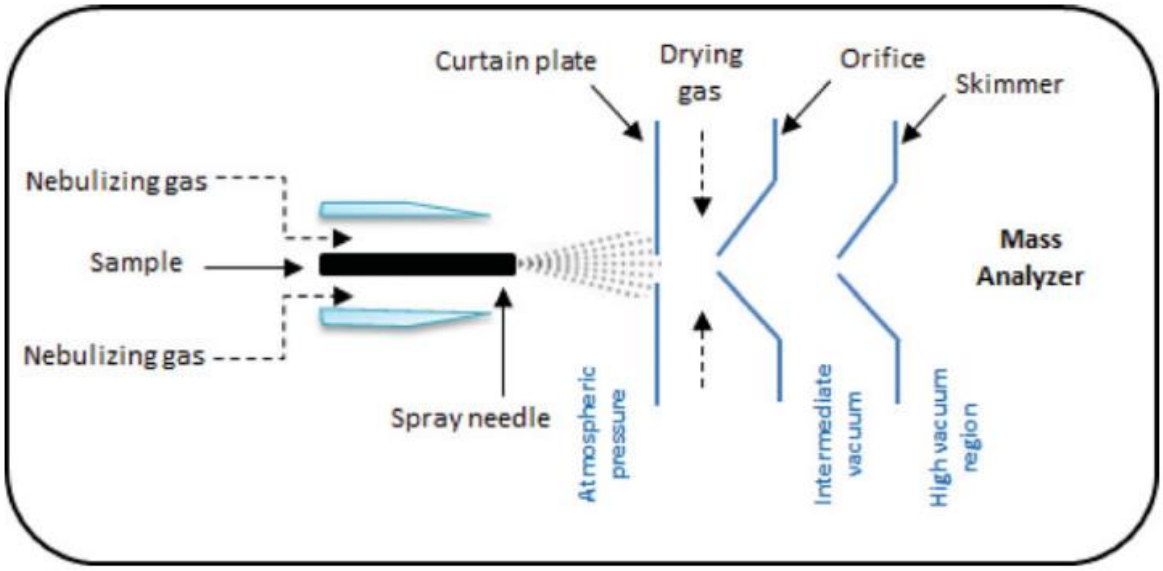


Figure 1.5. Schematic diagram of an ESI source adapted from reference [88].

LC-ESI-MS/MS was employed to determine many sterols, namely ergosterol, stigmasterol, and β-sitosterol present in tobacco leaves [82] as well as in camellia and olive oil [89]. However, the major challenge with ESI is its low ionization efficiency for non-polar compound, such as PS [81]. APCI, therefore, is an excellent alternative in ionizing such compounds. In fact, APCI has been widely used to determine PS in both vegetable oils and biological samples (**Table 1.1**). APCI involves the formation of sample droplet (neutral ones unlike ESI) through nebulization which gets evaporated in heated quartz tube to obtain both the analytes and solvent in a gaseous state (**Figure 1.6**). Finally, the solvent gets ionized by corona discharge electrode or needle which in turn ionizes analytes by charge exchange or proton transfer [88]. Additional advantage of APCI

compared to ESI is tolerance to matrix interferences [88]. This is because in the case of APCI, ionization occurs in the fully gaseous state, unlike ESI where analytes ionize in the liquid state in which non-volatile components present in liquid tend to interfere with the ionization of analytes. In fact, previous reports that compared the analysis of PS using ESI and APCI, demonstrated a reduced sensitivity in the case of former [20, 90].

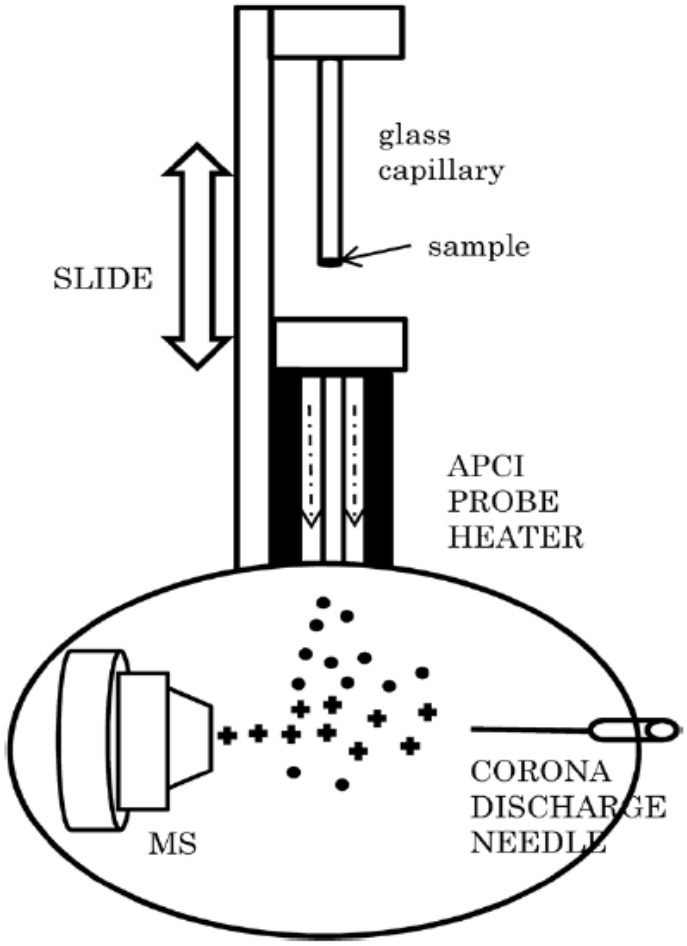


Figure 1.6. Schematic diagram of an APCI source adapted from reference [91].

In addition to ESI and APCI, few of reported methods utilized atmospheric pressure photoionization (APPI) for PS analysis [84, 92]. Similar to APCI, APPI is suitable for ionizing

lipophilic compounds and shows enhanced sensitivity compared to ESI [84]. PS ionize by forming $[M+H-H_2O]^+$ in the case of APCI and APPI. However, in the case of ESI both $[M+H-H_2O]^+$ and the protonated species $[M+H]^+$ has been reported in literature [76, 90].

Similarly to PS, tocopherols have been determined using GC and LC-based approaches. **Table 1.2** details various analytical methods for tocopherols determination. Unlike PS, all three ionization sources, discussed above, were well suited for analysis [93].

Table 1.2. GC and LC methods utilized for the determination of tocopherols in various samples.

Analytical method	**Tocopherol type**	**Chromatographic conditions**	**Sample**	**Run time (min)**	**Shortcomings**	**Ref**
LC-UV-vis	γ-tocopherol α-tocopherol	Reversed-phase Pinnacle C18 column Isocratic elution with acetonitrile:methanol: dichloromethane	Human milk	6 min	Less sensitive detector	[94]
GC-FID	γ-tocopherol α-tocopherol β-tocopherol δ-tocopherol	30 m DB-5MS column	Tocopherols in vegetable oil such as canola, sunflower, maize, linseed and sesame oil	22.5	Requirement of derivatization of tocopherols Longer run time	[78]
GC-MS	γ-tocopherol α-tocopherol β-tocopherol δ-tocopherol	DB-1HT column	Human Serum	~5 min	Requirement of derivatization of tocopherols	[95]
LC-ESI-MS	α-tocopherol γ-tocopherol	Reversed-phase chromatography C18 column Gradient elution with water (0.1% formic acid) and methanol (0.1% formic acid)	Tocopherols in human serum and plasma	15 min	Longer run time	[96]

LC-APCI-MS	γ-tocopherol α-tocopherol β-tocopherol δ-tocopherol	Reversed-phase chromatography Polymeric YMC C30 column Gradient elution using methanol and water.	Tocopherols in infant formula and dietary supplements	15 min	Baseline separation was not obtained for β and γ tocopherol Longer run time	[97]

1.8. Formulation-dependent efficiency of phytosterols: evidence from clinical studies

Various clinical studies on PS have investigated their therapeutic efficacy [39, 98, 99]. Results from these studies have provided strong evidence for the cholesterol-lowering abilities of PS. For instance, yogurt enriched with PS emulsion reduced serum LDL-C by 12% relative to control in moderately hypercholesterolemic individuals [100]. Similarly, PS-fortified orange juice (formulation, not known) reduced LDL-C by 12.4% during eight weeks of supplementation in mildly hypercholesterolemic individuals [101]. PS have been approved by both the FDA and Health Canada as an LDL-C lowering compound [102, 103]. This has led to the emergence of PS-enriched food and supplements as adjuvant therapy in treating hypercholesterolemia. Food matrices, such as margarine [104-106], salad dressing [106], orange juice [107], and chocolate bars [108] have been explored for PS enrichment. Among these foods, the use of fat-rich matrices is not favorable for healthy living and is not suitable to people on a low-fat diet. Thus, non-fat food matrices, such as granola bars, orange juice, and other non-fat beverages are more desirable. However, incorporation of the hydrophobic PS in low-fat matrices is challenging.

Both *in-vitro* and clinical studies have shown the impact of adopting the correct formulation approach to enhance the efficiency of PS [109, 110]. Failure to design optimum formulations has, in fact, led to a failed clinical trial [109]. PS delivered in capsules did not reduce LDL cholesterol significantly in the treatment group compared to the control [109]. On the other hand, three studies where PS were formulated in lecithin micelles were able to significantly reduce total cholesterol by up to 32% [111-113]. Similarly, an *in vitro* study conducted using a lipolysis model also highlighted the importance of optimum formulation in order to enhance the micellization of PS, a critical step for its efficacy [110]. Thus, the formulation plays a pivotal role in enhancing the cholesterol-lowering ability of PS.

1.9. Liposomal entrapment: a novel approach for formulating phytosterols and tocopherols

Liposomes are artificial membranes of colloidal particles comprised of phospholipids, consisting of one or more lipid bilayers enclosing an aqueous core (**Figure 1.7**) [114]. Due to the presence of aqueous and lipid phases, both lipophilic and hydrophilic molecules can be incorporated in liposomal formulations[115]. Liposomes can be prepared from natural ingredients, such as soy or egg phosphatidylcholine (**Figure 1.8**) and thus are biocompatible and biodegradable [116]. This is the main reason behind the wide acceptance of liposomes in the food industry.

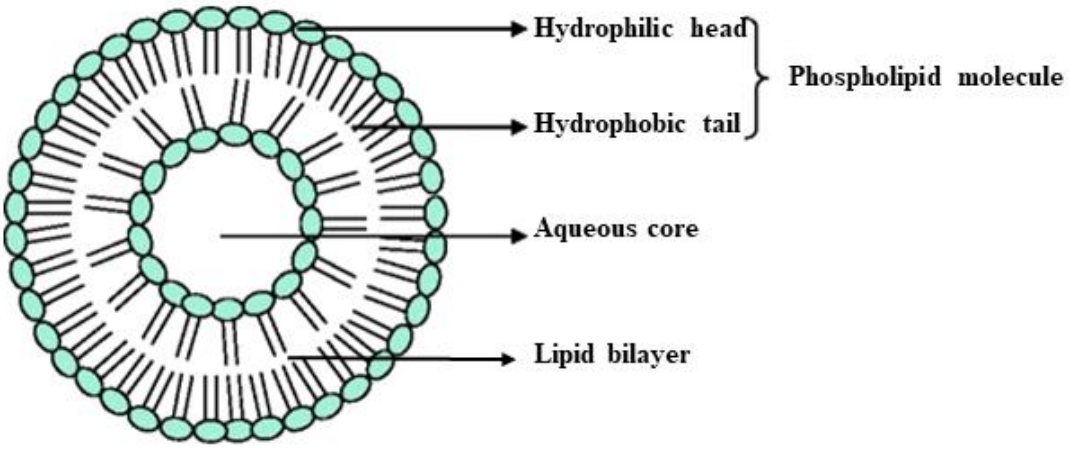


Figure 1.7. Schematic diagram of liposomes showing aqueous core and lipid bilayer adopted from reference [117].

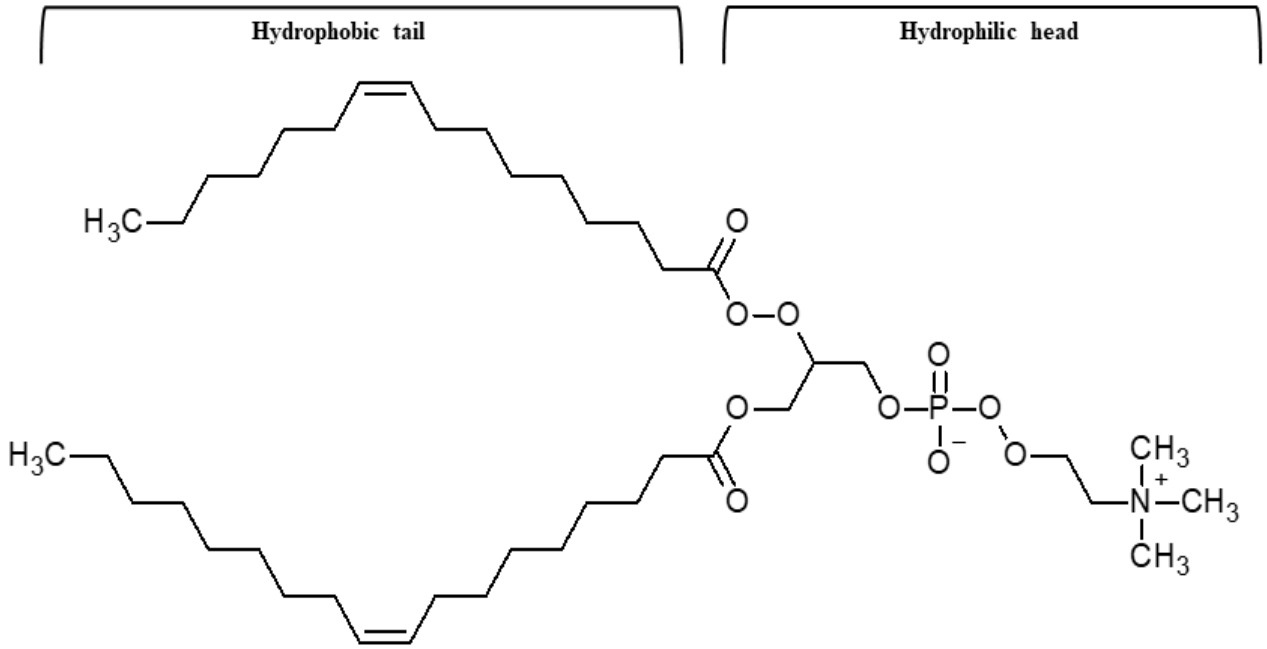


Figure 1.8. Structure of phosphatidylcholine showing a hydrophilic head and hydrophobic tail.

In an aqueous medium, phospholipids tend to minimize unfavorable interactions between their hydrophobic tails and the aqueous phase, thus forming a stable bilayer sheet where polar heads are exposed to an aqueous medium and non-polar tails interact with each other. Upon energy input, this bilayer sheet forms spherical vesicles, liposomes in order to minimize the free energy on its surface [118]. The size of liposomes ranges from 20 nm to several micrometers [119]. Liposomes can be classified on the basis of size and lamellarity (i.e., number of lipid bilayers) as small unilamellar vesicles (SUV), large unilamellar vesicles (LUV), multilamellar vesicles (MLV), giant unilamellar vesicles (GUV), and multivesicular vesicles (MVV) [120], as shown in **Table 1.3.** Unilamellar and multivesicular vesicles accommodate a larger volume of aqueous core, thus are

favorable for encapsulating hydrophilic compounds; on the other hand, MLVs favor the entrapment of lipophilic compared to hydrophilic compounds [114].

Table 1.3. Sizes and lamellarity number of various liposome types.

Liposome type	**Size (nm)**	**Lamellarity (# of the lipid bilayer)**
SUV	20-100	1
LUV	100-1000	1
GUV	>1000nm	1
MLV	>500nm	At least 2
MVV	>1000nm	At least one vesicle inside liposome

Liposomes are widely utilized in both the pharmaceutical and the food industry. Nutraceuticals are encapsulated/entrapped inside liposomes to increase their oral bioavailability, enhance oxidative stability (i.e., extending shelf life), and attain control or sustained release of the compounds for therapeutic purposes [121-123]. For instance, nutraceuticals such as quercetin and thymoquinone entrapped in unilamellar liposomal vesicles showed enhanced bioavailability (using *in vitro* model) as well as enhanced their oxidative stability [121, 122]. Other examples of liposomal nutraceuticals and its applications are shown in **Table 1.4**.

Table 1.4. Nutraceuticals incorporated in liposomes along with their applications.

Liposomal bioactives	Application	References
Quercetin liposomes	Enhanced stability Controlled release Two-five folds enhanced anti-angiogenic activity	[121]
Thymoquinone liposomes	Enhanced bioavailability Enhanced oxidative stability	[122]
Curcumin liposomes	Enhanced oral bioavailability	[123]
Betanin liposomes	Two-fold enhanced antioxidant activity Enhanced stability	[124]
Nisin liposomes	Controlled release Enhanced antimicrobial activity	[125]
Vitamin A	Enhanced oxidative stability	[126]

Interestingly, PS have been previously utilized in liposomal formulations as additives to improve vesicle stability and the encapsulation efficiency of drugs [127, 128]. To the best of my knowledge, there is no study in which PS are designed in liposomes in order to be develop into nutraceutical formulations to enhance its LDL-C reducing ability. Unlike PS, there are several studies where tocopherols have been formulated using liposomes in order to develop its nutraceutical formulations [129, 130].

1.9.1. Criteria for the selection of the preparation method for liposomes

A liposomal preparation method should lead to high entrapment efficiency, avoid the usage of toxic solvents, and produce well-defined, uniform, and reproducible particle size [131]. Further, it should be easy to scale up. Two parameters entrapment efficiency and drug/lipid ratio are critical factors to be evaluated before selecting a preparation method [131].

1.9.1.1. Entrapment efficiency and drug/lipid ratio

The entrapment efficiency (%EE) of the liposomes is defined as the percentage of a drug that is entrapped within the lipid bilayer or encapsulated inside the aqueous core of the liposomes. It is calculated using equation (1.1).

$$\%\text{Entrapment efficiency} = \frac{\text{Concentration of trapped bioactives}}{\text{Total concentration of bioactives}} 100 \qquad (1.1)$$

An optimum liposomal formulation exhibits an entrapment efficiency of 90% or more for lipophilic compounds [132, 133]. For example, resveratrol liposomes exhibited entrapment efficiency of 92% [132]. Similarly, retinol liposomes showed an entrapment efficiency as high as 94% [133]. High trapping efficiency precludes the need to remove the non-entrapped drug by separation procedures, such as dialysis or passage through an exclusion column. Such methods are usually tedious and expensive [134]. Thus, a method exhibiting high entrapment efficiency should be selected for formulation development [134]. Isailovic *et al*. conducted a comparative evaluation of various liposomal formulation methods such as thin-film hydration, pro-liposome formation, extrusion, and sonication in terms of their entrapment efficiency for a phenolic compound, resveratrol [132]. The entrapment efficiency of resveratrol for all the methods was in the range of 92-95% except for sonication, which was 44-55%. The authors rationalized that the high shear force involved in sonication might have led to the disruption of liposomes, eventually leading to leakage of the encapsulated drug, associated with low entrapment efficiency [132]. Similarly, low entrapment efficiency (%EE = 35) was observed in salidroside (glucoside) liposomes prepared by sonication in comparison to the one prepared by freeze-thaw (%EE = 40) and reverse phase evaporation methods (%EE = 40) [135]. These studies demonstrate that preparatory techniques affect the entrapment efficiency of liposomes. In addition, the highest possible value of the drug-to-lipid ratio in liposomal formulations is always desirable from a commercial point of view. In

sum, a preparatory method exhibiting high entrapment efficiency, optimum drug/lipid ratio needs to be employed for the preparation of liposomal formulation [131].

1.9.2. Preparation methods for liposomes

Numerous preparation methods have been developed for the preparation of liposomes. Commonly used methods include thin layer hydration [136], ultrasonication [136], extrusion [136], homogenization [137] and Mozafari method [138].

A common step in most preparation techniques is the hydration of a solid lipid film [139]. In the case of thin layer hydration, phospholipids and lipophilic drug(s) are dissolved in organic solvents, such chloroform or chloroform/methanol mixture. It is then followed by evaporation of the solvent using a rotary evaporator. A thin lipid film containing the active compounds will be formed around the vessel, which is then lyophilized for twenty four hours, then hydrated with an aqueous solution maintained at the temperature above the phase transition temperature of lipids to obtain multi-lamellar vesicles (MLV) [140]. Mechanical energy provided by vortexing facilitates the dispersion of film in the aqueous medium. The major advantage of this method is the easy solubilization of the phospholipid and bioactive together. However, its major drawback is the failure to achieve a small unilamellar liposomal vesicles.

Multi-lamellar liposomes, prepared by the thin-film method, are converted into SUVs using ultrasonication and extrusion techniques. Ultrasound produced by sonic bath or probe ultrasonication provides the required energy to break MLVs to unilamellar vesicles (ULV). The main advantage of ultrasonication is its ability to produce SUV of size 20-50 nm [141]. However, the major drawback of this technique is the possible disruption of the liposomal vesicle due to high shear force [132]. Also, the use of a probe sonicator may contaminate the formulation due to the

release of metal particles from the probe [140]. On the other hand, extrusion involves the passing of MLV, obtained from thin layer hydration, through a polycarbonate membrane with a well-defined pore diameter. Usually, 5-10 passages of preparation through the membrane are required to obtain homogenous and small vesicles [142]. The mean diameter of the membrane is close to that of the final liposomal vesicle obtained after extrusion [140]. Despite their advantages in producing SUV, these methods use organic solvents like chloroform, methanol that are not permitted in the food industry. Nevertheless, these solvents can be replaced by food-grade ethyl acetate, which is enlisted as class 3 solvent (lower risk to human health) by ICH guideline

[143].

The Mozafari method is more popular in the food industry as it does not require the use of organic solvents [138]. Liposomes can be prepared at a temperature ranging from 40-120 $^{\circ}$C by using solvents such as water, glycerol, and ethanol. Only low shear forces are required to form the vesicles [144]. Curcumin loaded liposomes prepared with the Mozafari method exhibited narrow particle size of 323 nm, polydispersibility index of 0.397, and high entrapment efficiency (around 84%) [145]. Similarly, liposomal omega-3 fatty acid prepared using Mozafari method showed size <200 nm, PDI of 0.216, and entrapment efficiency >90% [146]. Therefore, the Mozafari method can be a good choice for preparing liposomes of uniform size and high entrapment efficiency under low shear stress conditions without using organic solvents. However, the main drawback of the Mozafari method is the possibility of the oxidation of thermo-liable compounds.

In addition to the Mozafari method, homogenization is another good approach for preparing liposomal formulations for bioactives within food [137, 147]. The lipid film obtained by evaporating solvent is hydrated using water maintained at phase transition temperature of lipids. The formed MLVs are homogenized at the optimum pressure and time in order to obtain

homogenous ULV [137]. The major advantage of this method is that it is easy to scale-up. However, the drawback is the difficulty with the cleaning the homogenizer [137].

In sum, the optimum preparatory method for liposomes should yield well-defined liposomes with rapid and user-friendly scale-up procedures. Also, the method should be able to exhibit high entrapment efficiency.

1.9.3. Characterization of liposomes

The basic characterization of liposomes entails particle size and surface charge measurements. Particle size and size distribution influence the physical stability of liposomes. Particle size measurement is based on dynamic light scattering principles [148]. Particle size of 100-200 nm is commonly reported for liposomes intended for oral delivery [149, 150]. Zeta potential predicts the stability of the formulations, and optimum values are required to enhance liposomal stability [151]. The zeta potential of liposomes is measured using laser Doppler electrophoresis [152]. Based on the DLVO theory, liposomes with zeta potential outside of the range of ± 30 mV are highly stable; ± 20-30 mV is moderately stable; ± 10-20 mV is relatively stable; and ± 0 to 10 mV is theoretically considered to be unstable [153]. Both particle size and zeta potential of liposomes can be measured using the same instrument [154].

1.10. Assessment of the oxidation of phytosterols in liposomal formulations

PS, similar to cholesterol, are prone to autoxidation leading to the generation of phytosterols oxidation products (POPs) [155, 156]. Several studies have postulated the association between the absorption of POPs and the development of cardiovascular disease, primarily due to their possible pro-atherogenic effects that can possibly lead to atherosclerosis [157, 158]. In addition, the prevalence of POPs in various PS-enriched food is in the range of 0.03-5 mg/100g food [159, 160].

As such, there are real possible health risks associated with consuming PS-enriched products on a regular basis [161].

1.10.1. Description of POPs

Primarily, POPs are formed by the oxidation of the steroid ring of PS. As a result, POPs of diverse polarity are generated; however, the most abundant POPs are the polar ones such as 7-keto, 7-hydroxy, and 5, 6 epoxy (**Figure 1.9**) [162, 163] . Non-polar POP derivatives such as 3,5 dienes and 4,5 diene-3-one [164] are also formed, albeit at a lower extent (**Figure 1.9**). Apart from ring oxidation, the generation of POPs by the oxidation of the side chain has also been reported [155, 165]. However, the presence of side-chain oxidized PS in food products is low [166]. This is probably due to the fact that side-chain oxidation of PS is mediated by enzymatic reactions *in vivo* rather than by auto-oxidation [167].

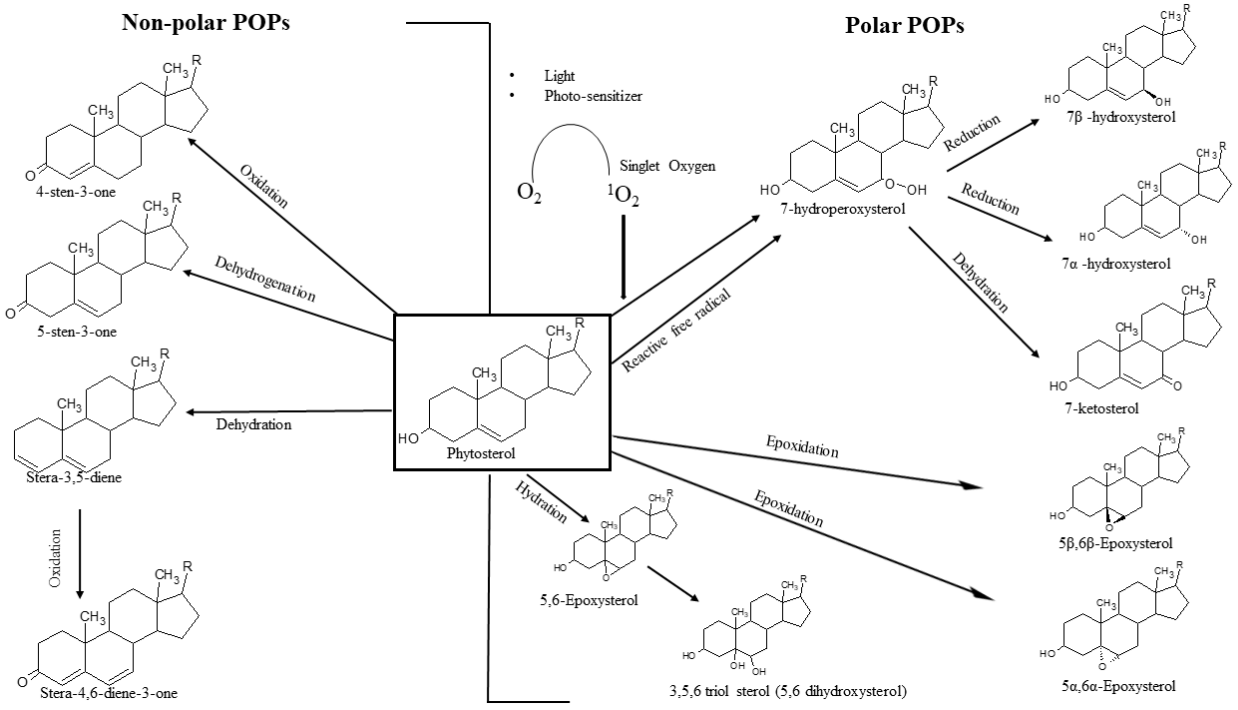


Figure 1.9. Auto-oxidation of phytosterols forming various polar and non-polar phytosterols oxidation products [168]

1.10.2. Factors affecting POP generation

There are three primary factors responsible for the generation of POPs that can be categorized into i) effect of heat; ii) effect of light, and iii) chemical structure of PS

1.10.2.1 Effect of heat

PS in food products undergo autoxidation due to the free radicals and reactive oxygen species (ROS) generated at high temperatures during thermal processing or heat treatment [169, 170]. Heat-mediated PS' oxidation has been well studied in the literature [169, 170]. Liquid margarine heated at 150 °C to 210 °C at a constant time (5 minutes) led to more than two-fold increase in POP contents at 210 °C in comparison to 150 °C [169]. Similarly, POP levels in PS-enriched rapeseed oil evaluated at 160 – 200 °C showed a similar trend [170]. Both studies indicate the negative association between stability of PS and temperature, demonstrating that a temperature between 150 – 200 °C is detrimental to the stability of PS. On the other hand, a study conducted at three different temperatures 4 °C, 24 °C, and 37 °C for 2 months in a PS-enriched fruit juice showed no significant increase in POP levels during storage [171]. As such, temperatures between 4 – 37 °C do not affect the stability of PS.

In sum, the stability pf PS was assessed at both high (150 – 200 °C) and low (4 – 37 °C) temperatures in two different matrices [170-172]. The assessment of low and high temperatures was done with the aim of monitoring POP occurrence during storage and cooking procedures, respectively [170, 171]. However, what is currently lacking is the evaluation of POP production upon exposure to a wide range of temperatures, namely 0 – 200 °C, in a single comparative experiment. Such a study can guide the selection of the ideal temperature in designing and storing PS-enriched foods.

In addition to temperature, several experiments have been conducted to investigate the impact of regular cooking procedures on the generation of POPs [161, 173]. The cooking procedures, such as microwave heating, pan-frying, and oven heating, were evaluated on PS-enriched margarine [173]. POPs were generated in the following order, microwave heating (780 mg/kg) <pan frying (1079 mg/kg) <oven heating (2903 mg/kg) [173]. All of these heating procedures led to generation of POPs of similar profile. In another study, shallow frying (in potatoes), produced a greater amount of POPs than food cooked by stir-frying and microwaving [174]. Similar POP profile was obtained in all these heating methods. Higher heat exposure in the case of shallow fried potatoes is most likely the reason for the observed higher contents of POPs. Overall, these results elucidate the association between the cooking procedure and the extent of the generation of POPs. It can be concluded that PS-enriched food products subjected to any form of cooking procedure lead to the generation of POPs, albeit at varying levels.

The time of exposure to heat also affects the extent of POP formation [169, 170]. For example, liquid margarine heated at a constant temperature of 150 °C led to the production of 2 fold higher POPs at 16 minutes compared to heating for 8 minutes [169]. Similarly, increasing pan frying time from 5 to 10 minutes led to an increase in the oxidation of β-sitosterol from 0.2% to 0.4% [170]. Based on the above discussions, it is recommended to fortify PS in food products that do not undergo heating or require minimal heating.

1.10.2.2. Effect of light

There are only a few studies dealing with PS' photo-oxidation [175-177]. Both natural sunlight and artificial light have been utilized to investigate the photo-oxidation effects [175-177]. PS in vegetable oil, upon exposure to natural light, produced β-sitosterol oxidation products (633 µg/g of oil) [176]. Similarly, PS-enriched triolein was exposed to the artificial light of the intensity of

20,000 and 30,000 Lux (unit of illuminance) for 14 days showing a reduction in PS' content by 60 and 65%, respectively; whereas, when the same product was kept in the dark place for 14 days, only 17% reduction was observed [175]. This indicates that photo-oxidation is affected by the intensity of the light. It was also found that the presence of a photosensitizer (e.g., chlorophyll) in the sample accelerates the photo-oxidation process [175]. Isoalloxazine moiety present in chlorophyll and riboflavin (naturally present in fruits and juices) tend to have high oxidation potential for phytochemicals, thus acts as a photosensitizer [178].

Similar to heating duration, photo-oxidation of PS is affected by exposure time [175, 176]. PS added to triolein were exposed to light for 12 days producing 2-fold higher POPs than triolein exposed for six days [175]. On the other hand, PS in spreads, exposed to sunlight for 35 days, did not undergo oxidation [177]. The conflicting findings suggest that PS' photooxidation is affected by the food matrices in which PS are present. Nonetheless, additional research is needed to test such a hypothesis. Equally important is to evaluate the detailed mechanism of PS' photooxidation, which is currently lacking, as it may possibly provide an explanation for the contradictory results.

1.10.2.3. Chemical structure of phytosterols

The extent of autoxidation of PS is dependent on their structure as variable oxidation rates were reported among the different PS [169, 179]. Campesterol was significantly more susceptible to oxidation than β-sitosterol in PS-enriched liquid margarine and chocolate [169, 180]. On the contrary, β-sitosterol present in sunflower oil was more susceptible to oxidation than campesterol during deep and pan-frying [179]. The reason for such inconsistent results can be attributed to the combined effects of the structure of the PS, the utilized food matrix, and the temperature. Apart from these conflicting results, the comparative studies were done among campesterol, β -sitosterol, and stigmasterol have shown the following order for their susceptibility to oxidation: campesterol

≈ β-sitosterol > stigmasterol [181]. However, the reason behind this order of susceptibility to oxidation is still unknown.

PS also show discrepancy in the produced POP profile [174, 182]. For example, PS-enriched margarine, containing campesterol, and sitosterol, subjected to various cooking procedures, showed the production of POPs in the following descending order 5,6 epoxy sterol >7-keto sterol > 7-hydroxy sterol [174]. In contrast, the POP profile in thermally processed food in vegetable oil showed an abundance of 7-keto sterol followed by 5,6 epoxy sterol [182]. The possible reason for such varying POP profiles could be attributed to the discrepancy in the type and nature of food matrices in which PS were enriched or naturally present. In sum, existing studies are insufficient to compare the oxidative stability among various PS. It is highly important to investigate the oxidative potential among the various PS in order to enrich the food product with the most stable PS.

Even though liposomal formulations and tocopherols can prevent the generation of POPs, it is still crucial to screen the prevalence of POPs in PS encapsulated within liposomal formulations. Screening and quantifying POPs can be attained using LC-MS/MS and gas chromatography-mass spectrometry (GC-MS) [163, 164].

1.10.3. Challenges in the analysis of phytosterols oxidation products (POPs)

The major challenge in the determination of POPs is the lack of commercially available reference standards [164]. Most published methods utilized cholesterol oxidation products (COPs) as a reference standard as they have a similar response factor as that of POPs [164, 183]. On the other hand, few studies synthesized POPs standards in-house [184]. For instance, stigmasterol oxidation products were synthesized using stigmasterol as a precursor followed by acetylation, hydroxylation and in turn saponification [184].

In the same way as PS, POPs have been routinely analyzed using GC and LC-based methods (**Table 1.5**). LC methods are advantageous over GC as derivatization of POPs is omitted [185, 186]. LC system coupled with detectors such as UV, fluorescence, and MS has been utilized [185, 186]. However, fluorescence and UV do not provide specificity for co-eluting POPs. In addition, POPs do not absorb sufficient UV due to a lack of chromophore within their structure, thus reducing sensitivity during UV detection [187]. On the other hand, LC-MS is ideal for POP determination as it provides adequate selectivity and sensitivity for the target analytes. Normal phase LC-MS was developed to quantify polar and non-polar POPs using a diol column. However, the method had a long run time of around thirty minutes, which is one of the major drawbacks of normal phase liquid chromatography [164]. Reversed phase can be utilized to decrease the run time [184]. For instance, the reversed-phase LC-MS method developed to determine stigmasterol oxidation products showed a run time of only eleven minutes [184]. For the ionization method, APCI or APPI techniques is ideal in ionizing POPs (due to their hydrophobicity) compared to ESI [168]. Hydroxy, triol, and epoxy derivatives of PS ionize by forming $[M+H-H_2O]^+$ or $[M+H-2H_2O]^+$ by the loss of one or two water molecules [164]. On the other hand, keto derivatives ionize, forming $[M+H]^+$ ions. **Table 1.5** details various analytical methods utilized to quantify POPs.

Table 1.5. GC and LC methods utilized for the determination of phytosterols oxidation products in various samples.

Analytical method	**Phytosterols oxidation products (POPs)**	**Chromatographic conditions**	**Sample**	**Run time (min)**	**Shortcomings**	**Ref**
LC-GC-FID	5,6 Epoxy and 7-hydroxy derivative of sitosterol and stigmasterol, 7-keto derivative of stigmasterol	Eurospher-100 si column (for LC) n-hexane/methyl-tert-butyl-ether/isopropanol Fused silica capillary column coated with trifluoropropylmethylpolysiloxane (GC system)	PS enriched food	~ 14 min	Non-specific detector	[188]
GC-MS	7-hydroxy and 7-keto derivatives of campesterol, stigmasterol and β-sitosterol	HP-5 MS capillary column	Human plasma	~ 35 min	Derivatization of analytes Longer run time	[163]
LC-UV-Fluorescence	Stigmasterol oxidation products	Silica supercosil column 1 butanol: methanol (1:1)	Photo-oxidized POPs	~ 14 min	Non-specific detector	[185]

LC-APCI-MS	Stigmasterol oxidation products	Silica Supercosil column Heptane and Isopropanol	Thermo-oxidized PS	~ 30 min	Longer run time	[186]
LC-APPI-MS	Non-polar and polar oxidized derivative of campesterol, stigmasterol, and β-sitosterol	Normal Phase Chromatography Diol column Hexane and Isopropanol	Forced oxidized PS	~ 32 min	Longer run time	[164]
LC-ESI-MS	Stigmasterol oxidation products	Reversed-phase Waters Atlantis 5 µm column 150 mm x 4.6mm Acetonitrile (0.1% formic acid) and chloroform	Synthetic POPs	~ 11 min	Lower ionization efficiency of ESI	[184]

1.11. Incorporation of bioactives into orange juice

Numerous food products have been used for the incorporation of bioactive, including orange juice [101], chocolate bars [108], milk [189], and yogurt [190]. Since the aim of adding PS and tocopherols in food products is to lower cholesterol and provide anti-oxidant properties linked to better cardiovascular health [191], using low fat or non-fat food such as orange juice, apple juice can be more appropriate to obtain the desired therapeutic outcome. Specialized techniques need to be used for incorporating liposomes in order to ensure the uniform distribution of vesicles throughout the food matrix.

1.12. Rationale for research

Hypercholesterolemia, or increased LDL-C, is one of the major risk factors of cardiovascular disease (CVD). PS can reduce LDL-C in the range of 8-14%, thus they have been approved by both the FDA and Health Canada as an adjuvant therapy along with statins [102, 103]. Oilseed crops and their waste streams are the main sources for PS. Canola oil deodorizer distillate (CODD) is an excellent underutilized source of PS [52]. PS from CODD can be used to develop a nutraceutical formulation with a commercial potential. To achieve their full potential three major limitations, need to be addressed: i) lipophilic PS are difficult to incorporate in food products, ii) PS can undergo auto-oxidation generating PS oxidation products (POPs), and iii) formulation dependent efficiency of PS. Nanotechnology-based liposomal formulation can address the lipophilicity, while the addition of antioxidant tocopherols can prevent the formation of POPs. Regarding efficacy, it is evident from the literature that micellar PS can reduce LDL-C by approximately 14%. Thus, liposomes made up of the same backbone as micelles can possibly

enhance the efficiency of PS. In addition, liposomes are superior compared to micelles in terms of stability and entrapment efficiency [192, 193].

Furthermore, the FDA and Health Canada only specify β-sitosterol, stigmasterol, and campesterol, in their health claim related to PS [102, 103]. Unfortunately, one of the abundant PS in canola oil, brassicasterol, has not yet been studied for their therapeutic efficiency.

This research aims to develop a novel liposomal formulation containing CODD phytosterols composed of brassicasterol, campesterol, and β-sitosterol along with tocopherols (alpha, gamma and delta) to enhance LDL-C lowering efficiency of PS and to maintain their oxidative stability. Herein, nanotechnology is used to develop the liposomal formulation. In addition, robust LC-MS/MS methods were developed for the analysis of tocopherols, PS, and POPs. Finally, a pre-clinical study is conducted to assess the effect of the developed nutraceutical formulation on LDL-C.

1.13. Research Hypotheses and Objectives

1.13.1. Hypothesis I

Phytosterols and tocopherols extracted from the CODD can be entrapped at high efficiency (above 85%) using an optimized liposomal formulation comprised of soy phosphatidylcholine (PC).

1. **Objective 1:** To formulate liposomes containing phytosterols and tocopherols using thin-layer hydration homogenization, thin- layer hydration ultrasonication, and Mozafari method.

2. **Objective 2:** To develop and validate LC-MS/MS method for comparative evaluation of the entrapment efficiency of the active ingredients in the liposomes prepared by the above-mentioned techniques.

1.13.2. Hypothesis II

Liposomal phytosterols and tocopherols incorporated into model orange juice will maintain chemical and physical stability after pasteurization (heat treatment at 72°C for 15 sec) during 1-month storage

1. **Objective 1:** To incorporate liposomal formulations into food product (model orange juice)
2. **Objective 2:** To determine the physical stability (size and zeta potential of liposomes) for a month and chemical stability of the bioactives before and after pasteurization.

1.13.3. Hypothesis III

Tocopherols (antioxidants) formulated in liposomes along with phytosterols will prevent the formation of phytosterols oxidation products (POPs)

1. **Objective 1:** To develop and validate LC-MS/MS method for quantifying phytosterols oxidation products present in liposomal formulation prepared with and without tocopherols.

1.13.4. Hypothesis IV

Liposomes containing phytosterols and incorporated in orange juice (OJ) will have significantly higher LDL-C lowering ability relative to control and marketed product.

1. **Objective 1:** To measure the serum lipid concentrations (total cholesterol, HDL-C, LDL-C, VLDL-C, and triglyceride) in hamsters, fed with a) orange juice fortified with liposomal phytosterols, b) water fortified with liposomal phytosterols, c) marketed orange juice with fortified phytosterols, and d) control (both orange juice and water).
2. **Objective 2:** To statistically determine and compare the extent of reduction in serum lipid concentration among the hamsters.

1.14. References

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CHAPTER 2

The simultaneous quantification of phytosterols and tocopherols in liposomal formulations using validated atmospheric pressure chemical ionization- liquid chromatography –tandem mass spectrometry

Asmita Poudel[1], George Gachumi[1], Ildiko Badea[1], Zafer Dallal Bashi[1] and Anas El-Aneed[1]

[1]Drug Design and Discovery Group, College of Pharmacy and Nutrition, University of Saskatchewan, 107 Wiggins Road, Saskatoon, SK S7N 5E5, Canada

[*]Corresponding author: Anas El-Aneed (anas.el-aneed@usask.ca)

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Rationale

There is always a need to assess the interactions among components during the formulation development. In our research, liposomal formulations need to be evaluated for their efficiency in entrapping phytosterols and tocopherols. Thus, a robust and sensitive analytical platform is required to measure these compounds. In Chapter II, we developed and validated a sensitive liquid chromatography tandem mass spectrometry (LC-MS/MS) method to simultaneously quantify phytosterols and tocopherols entrapped in liposomes.

Contribution statement:

Asmita Poudel contributed to this paper by designing and conducting experiment, data acquisition, data analysis and writing the manuscript

Highlights

- LC-MS/MS method was developed for simultaneously quantifying phytosterols, and tocopherols entrapped in liposomal formulation.
- For the first time, these analytes are simultaneously quantified in pharmaceutical formulation using LC-MS/MS.
- The shortest run time has been achieved for these analytes in comparison to reported methods.
- Unlike complex gradient elution reported in literature, simple isocratic elution was adopted to separate these analytes.
- A unique chromatographic phenomenon for α-tocopherol was observed upon liposomal entrapment which showed the separation of its enantiomers.

2.1. Abstract

A novel liquid chromatography tandem mass spectrometry (LC-MS/MS) method was developed and validated to simultaneously quantify phytosterols (brassicasterol, campesterol, stigmasterol and β-sitosterol) and tocopherols (alpha, beta, gamma and delta) entrapped in the lipid bilayer of a liposomal formulation. Apart from liposomes (a pharmaceutical product), the developed method was able to quantify target analytes in agricultural products, thus showing wide applications. Atmospheric pressure chemical ionization (APCI) was employed due to the enhanced ionization of PS and tocopherols in comparison to electrospray ionization. Unlike published work, the chromatographic conditions were modified to simplify the analytical approach. For the first time, a simple isocratic elution (acetonitrile:methanol 99:1 v/v) was utilized for the separation of four PS and four tocopherols in a single run. A substantially better baseline separation of PS was obtained in comparison to reported methods by using poroshell C18 column. The method has a total run time of 7 minutes, which is the shortest run time among all reported quantitative methods for the simultaneous determination of four phytosterols and four tocopherols. Calibration curves for all PS were linear in the range of 0.05-10 µg/mL. In the case of tocopherols, alpha tocopherol showed linear response in the range of 0.25-10 µg/mL. However, gamma and delta tocopherols exhibited quadratic relationships in the same concentration range (0.25-10 µg/mL). Validation parameters met the International Conference on Harmonization (ICH) guidelines in terms of selectivity, accuracy, precision, repeatability, sensitivity, matrix effects, dilution integrity and stability. The method was, for the first time, successfully applied for the quantifying PS and tocopherols entrapped inside liposomes. An interesting chromatographic phenomenon was observed during sample analysis. Alpha tocopherol (entrapped in the liposomal lipid bilayer) was found to elute at two retention times, 2.53 minutes and 3.60 minutes. Such dual separation was

not observed in calibration standards and quality controls. It was concluded that the chiral recognition ability of liposomes made up of phosphatidylcholine separated the enantiomers of alpha tocopherol, giving rise to two peaks at two different retention time. To sum, the reported novel LC-MS/MS method addresses three major analytical shortcomings, namely i) longer run time, ii) complex gradient elution and iii) poor baseline separation of phytosterols and tocopherols.

2.2. Introduction

Phytosterols (PS) and tocopherols are plant bioactives mostly derived from vegetable oil seeds such as canola, sunflower and soybean [1]. The health benefits of PS are linked to their ability to reduce low density lipoprotein (LDL) cholesterol levels in plasma by 6-12 % [2]. Tocopherols (i.e. vitamin E), on the other hand, are methylated phenols that act as peroxyl radical scavengers and are used as anti-oxidants [3]. Due to these health benefits, naturally obtained PS and tocopherols can be highly valuable for formulation development in pharmaceutical and food industries. However, effective formulation strategies are crucial to formulate these bioactives.

Formulation of PS and tocopherols has always been challenging due to their lipophilicity and heat / light sensitivity. Fortunately, nanotechnology-based liposomal entrapment could address the lipophilicity issue without compromising stability. Liposomes are colloidal structures, consisting of a self-assembling lipid bilayer enclosing an aqueous core. There are numerous methods that have been adopted for liposomal preparation by the pharmaceutical and food industries, such as microfluidization , thin film hydration, and heating method [4]. Quantifying liposomal entrapped bio-actives is an integral part of optimizing a formulation method. Thus, there is a necessity for developing an analytical method that can simultaneously quantify PS and tocopherols in a liposomal formulation.

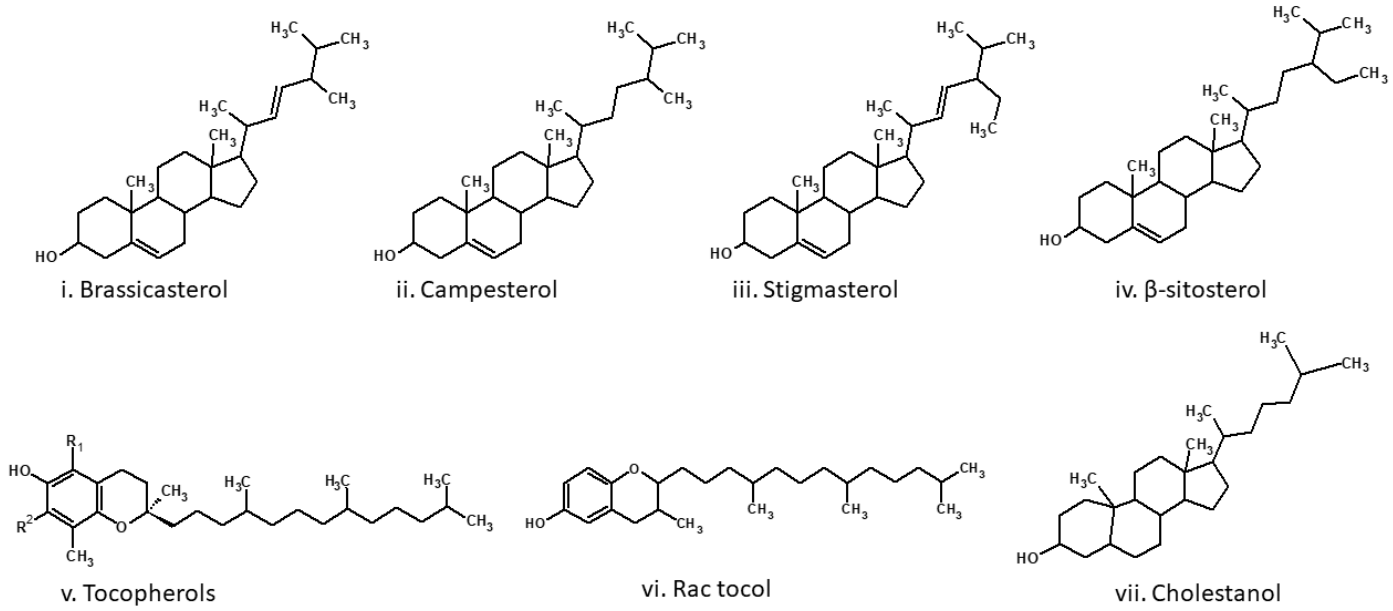
Gas chromatography (GC) coupled to Flame ionization detector (FID)[5] and mass spectrometry[6] have been widely used for the quantification of PS and tocopherols. Unfortunately, GC-based approaches require the derivatization of target analytes, making the analysis tedious and time consuming.

To avoid derivatization, liquid chromatography (LC) based analytical platforms have been developed using diode array detector (DAD) [7], evaporative light scattering detector (ELSD) [8] and mass spectrometer (MS) [9]. LC coupled to ELSD has been used for the quantification of three PS (campesterol, stigmasterol and β-sitosterol), three tocopherols (alpha, gamma and delta), and lutein in soybeans [8]. Similarly, LC-DAD have been developed for the determination of PS and tocopherols in sunflower seed and nuts [7]. However, these methods could not achieve good resolution for campesterol and stigmasterol [7, 8].

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been adapted by various researchers for the simultaneous analysis of PS and tocopherols [9-17]. Both electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) were used for the ionization of these analytes [10, 16]. However, APCI is superior to ESI in terms of imparting higher ionization efficiencies of these lipophilic compounds [10, 18]. A number of methods have been developed using LC-APCI-MS/MS for analyzing PS and tocopherols either separately or simultaneously [9-11]. However, most of these methods require long run times along with gradient elution [9-11], which adds complexity to the analytical approach. In addition, some of the reported methods did not show a good baseline separation for brassicasterol, campesterol, sitgmasterol and β-sitosterol [9, 11]. Baseline separation is sometimes not required in highly selective MS/MS; however, analytes sharing similar MRM transitions need to be separated for their proper

identification. A recently published work have utilized isocratic elution for quantification of PS and tocopherols [12]. However, only two PS i.e, stigmasterol and β-sitosterol were targeted by this method [12]. Apart from this, most of the reported methods have quantified PS and tocopherols either in food or oil samples [9-11]. None of the reported method simultaneously quantifies these analytes in pharmaceutical formulations. In sum, there is a need for a simple and fast LC-MS/MS method with good baseline separation that can simultaneously quantify four major PS and tocopherols in pharmaceutical formulations

In this work, a novel LC-MS/MS method is developed for the quantification of PS and tocopherols (**Figure 2.1**) entrapped in liposomal formulation. Further, the developed method was applied in quantifying target analytes in agricultural products. The method has a total run time of 7 minutes that is, to the best of our knowledge, the shortest run time reported in the literature for the simultaneous quantification of target analytes. The new method used isocratic elution, simplifying the analytical procedure, while achieving better baseline separation for PS in comparison to reported methods.



Tocopherols	**R1**	**R2**
Alpha	CH_3	CH_3
Beta	CH_3	H
Gamma	H	CH_3
Delta	H	H

Figure 2.1. Structures of abundant PS (i.Brassicasterol, ii. Campesterol, iii. Stigmasterol, iv. β-sitosterol) and tocopherols (v. alpha, beta, gamma and delta tocopherols) found in CODD and internal standards (vi. Rac tocol, vii. Cholestanol).

2.3. Materials and Methods

2.3.1. Reagents and Chemicals

β-Sitosterol, Campesterol, Stigmasterol, and Brassicasterol at 98 % purity were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Cholestanol (95 %), α-tocopherol (99.9 %), γ-tocopherol (96.8 %), δ-tocopherol (94 %), and acetic acid were purchased from Sigma Aldrich (Oakville, Ontario, Canada) while rac tocol (95 %) was obtained from Abcam (Toronto, Ontario, Canada). Acetonitrile (LC-MS grade), methanol (LC-MS grade), chloroform (analytical grade) were obtained from Fisher Scientific (Ottawa, ON, Canada). Phosphatidylcholine (PC) was purchased from Avanti polar lipids (Alabaster, Alabama, USA). PS were extracted from canola oil deodorizer distillate (CODD)obtained from Louis Dreyfus Company (LDM foods) [19].

2.3.2. LC conditions and MS Parameters

Chromatographic separation of the analytes was obtained on an Agilent Acquity UPLC (Agilent Technologies, Mississauga, Ontario, Canada) system with an Agilent Poroshell C18 column (2.1 mm × 150 mm, 5µm) protected by a guard column (2.1 mm × 5 mm, 2.7 µm) of the same packing material. The column temperature was set at 30°C and the injection volume was 2.5 µL. An isocratic elution of acetonitrile: methanol (99:1 v/v) with 0.1% acetic acid was optimized at a flow rate of 0.8 mL/min. AB Sciex 6500 QTRAP® quadruple-linear ion trap (QqQ-LIT) mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source (AB Sciex, Concord, Ontario, Canada) was used for detection and quantification. Positive ion APCI tandem mass spectrometric analysis was employed for ionization. Direct infusion of individual standards were done to optimize source and compound dependent parameters. **Table 2.1** shows the collision energy of analytes and internal standards.

Table 2.1. Retention time, precursor ion, MRM transitions, collision energy, LOD, LLOQ and calibration range of phytosterols and tocopherols where all PS and alpha tocopherols were quantified using a linear calibration curve, whereas delta and gamma tocopherols were quantified using quadratic calibration curve.

Compound		Retention time (min)	Precursor ion (*m/z*)	Monitored fragment ions MRM (*m/z*)	CE (V)	LOD (µg/ml)	LLOQ (µg/ml)	Calibration range (µg/ml)
Phytosterols	Brassicasterol	4.05	**381.4**	**297.4** 147.1	22 30	0.005	0.05	0.05-10 Linear
	Campesterol	4.9	**383.4**	**161.1** 147.1	30 30	0.005	0.05	0.05-10 Linear
	Stigmasterol	5.1	**395.4**	297.4 **83.2**	23 26	0.01	0.05	0.05-10 Linear
	β-Sitosterol	5.8	**397.4**	**161.2** 134.9	27 28	0.005	0.05	0.05-10 Linear
Tocopherols	Delta Tocopherol (δ)	2.65	**402.4**	**177** 137	32 38	0.01	0.5	0.25-10 Quadratic
	Gamma Tocopherol (γ)	3.15	**416**	**151.1** 191.2	37 34	0.01	0.25	0.25-10 Quadratic
	Alpha Tocopherol (α)	3.6	**430**	**165.3** 205.2	38 36	0.05	0.25	0.25-10 Linear
Internal Standards	Cholestanol	5.3	**371.4**	**95.2** 109.1	32 30	NA	NA	NA
	Rac Tocol	2.05	**388.4**	**163.2** 122	30 40	NA	NA	NA

2.3.3. Preparation of liposomes (Method application in pharmaceutical products)

Thin layer film hydration-homogenization approach was used for the formulation of liposomes [19]. Briefly, 20 mg of phosphatidylcholine, 10 mg of PS (extracted from CODD containing brassicasterol, campesterol and β-sitosterol) and 1 mg each of alpha, gamma and delta tocopherols were solubilized together in food grade ethyl acetate in a round bottom flask. Ethyl acetate was then evaporated using rotary evaporator for 15 min leading to the formation of thin film layer at the bottom of the flask. The thin film was rehydrated with 20 mL of purified water (Millipore, Bedford, MA, USA) maintained at 55 °C with occasional vortexing for 3 hours, leading to the formation of the liposomes.

Liposomes were then size-reduced using a recirculating high fluid pressure homogenizer (Microfluidic Corporation, Westwood, United States) at 60-psi pressure for 20 mins. Using an ultracentrifuge (SW 60T1 Beckman rotor) (Indianapolis, USA), free bio-actives were separated from entrapped bioactives from 5 mL of liposomes. The process was optimized for G-force of 138,000 for 60 mins [19]. The sedimented liposomes were then lyophilized.

For LC-MS/MS analysis, the lyophilized liposomal sediment was dissolved in chloroform. The desired concentrations were then obtained by spiking aliquot of mixed internal standards, followed by diluting with acetonitrile (described in section 2.3.5).

2.3.4. Extraction of phytosterols and tocopherols from canola waste or CODD (Method application in agricultural and food products)

PS and tocopherols are concentrated in unsaponifiable fraction of CODD [20]. Thus, unsaponifiable fraction of CODD was extracted to quantify its analytes [20]. Briefly, 5g of CODD was dissolved in 50mL of 1M KOH solution prepared in 95% ethanol and was refluxed for 1 hour at 80°C. The mixture was then cooled down to room temperature and 50 mL of distilled water

was added. Unsaponifiables were extracted three times with 50mL hexane and the combined organic phase was washed with 10% Ethanol until the washings were neutral to phenolphthalein. This organic phase containing the unsaponifiables was dried by passing it through anhydrous sodium sulfate followed by hexane evaporation using a Buchi rotary evaporator R200 (Buchi corp., DE, and USA). The residue was further dried under high vaccum using Trivac vacuum D4A (Leybold vacuum products Inc., PA, USA) overnight and weighed. A blank was prepared as described above but without the CODD. For LC-MS/MS analysis, the sample was prepared in the same way as described in section 2.3.5.

2.3.5. Calibration Standard preparation

Stock solution of PS and tocopherols were prepared by dissolving standard of each compound in chloroform at concentration of 1mg/mL. Beta and gamma tocopherols which are positional isomers (**Figure 2.1)** have similar physicochemical properties, thus, tend to elute as one peak on a C18 stationary phase [9, 21]. As such, they were collectively quantified using gamma tocopherol as analyte standard. A mixed stock solution containing all 7 standards were prepared at concentrations of 50 µg/mL by pipetting the required volume of the standard stock and diluting it with acetonitrile. Similarly, stock solution of internal standards (cholestanol for PS and rac tocol for tocopherols) were prepared by dissolving each at 1,000 µg/mL in chloroform. A mixed internal standard stock solution was prepared by pipetting the required volume of each and diluting with acetonitrile for a final concentration of 30 µg/mL and 100 µg/mL for rac tocol and cholestanol, respectively. Empty liposomes without analytes were prepared using the thin-layer hydration homogenization method, as described in section 2.3.3 and a 5 mL was aliquoted and freeze-dried.

Calibration standards were prepared by spiking mixed stock of analytes in liposomal matrix in the presence of internal standards followed by diluting with acetonitrile. Calibration standards of concentrations 0.01, 0.05, 0.25, 0.5, 1, 3, 5, 7.5, 10 µg/mL were prepared.

2.3.6 Method validation

International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidance for bioanalytical method validation were used as a guideline to validate the LC-MS/MS method (http://www.gmp-compliance.org/guidemgr/files/Q2(R1).PDF). The method was validated for linearity, selectivity, accuracy, precision, repeatability, sensitivity, matrix effects, dilution integrity and stability.

2.3.6.1 Calibration curve and Sensitivity

Calibration curves were plotted by measuring analytes in liposomal matrix at concentration ranges from 0.01 µg/mL to 15 µg/mL. The ratio of peak area of analyte to peak area of internal standard were plotted against the concentrations of analyte. A least square regression with a weighting factor of 1/x was applied for calibration curve construction. The calibration curve is only accepted when all the points in the curve lies within ± 15% standard deviation at nominal value except for lower limit of quantitation (LLOQ) that can be ± 20% standard deviation.

(LOD) was determined based on a signal to noise (S/N) ratio, and the lowest detectable concentration was chosen if the criteria S/N ≥ 3 was met. LOD were tested in the concentration range of 0.005-0.05 µg/mL. LLOQ was set at the lowest concentration that showed accuracy within standard deviation of ± 20% from the nominal value and coefficient of variation (CV) of ± 20%.

2.3.6.2 Interday and intraday accuracy and precision.

Accuracy was calculated by running quality control samples. In addition, accuracy of calibration standards was determined by comparing the measured values to know concentration. Quality controls (QCs) at four different concentrations were prepared to determine interday and intraday accuracy and precision. LLOQ, low quality control (LQC), middle quality control (MQC) and high-quality control (HQC) were prepared in liposomal matrix. LQC was three times the LLOQ; MQC was concentration in the middle of calibration curve; and HQC was 90% of the upper limit of quantification (ULOQ). Accuracy for QC samples should lie within ± 15% of nominal value except for LLOQ, which can be within ± 20%, whereas precision or relative standard deviation standards should be ± 15% except for LLOQ which can be ± 20%. Intraday accuracy and precision were calculated using QC replicates (n=4) in a single analytical run whereas interday accuracy and precision measurements were conducted using QC replicates (n=4) prepared on three consecutive days.

2.3.6.3 Dilution integrity

Dilution integrity was evaluated by preparing a mixed stock solution of 50 µg/mL (described in section 2.4) by pipetting the required volume of each analyte from an individual stock solution of 1,000 µg/mL of analytes. Determination was done at both low and high concentration as follows: a concentration of 12.5 µg/mL was prepared by pipetting the required volume from mixed stock solution of 50 µg/mL into liposomal matrix and diluting it with acetonitrile in the presence of internal standard. This was followed by further dilutions to achieve a concentration of 2.5 µg/mL (5 times dilution) and 7.5 µg/mL (1.7 times dilution). The tested dilutions were chosen to match the expected dilution during liposomal sample analysis.

2.3.6.4 Selectivity and Matrix effects

Selectivity was assessed to investigate the presence of any interference from other components of the liposomal matrix. Blank liposomal samples were prepared (see section 2.3) and analyzed.

To determine matrix effect, quality controls (LQC, MQC and HQC) in liposomal matrix and a neat solution (without liposomal matrix) were prepared in the absence of internal standard. The analytes response in liposomal matrix were compared to analytes response in neat solution to evaluate the matrix effect. The matrix effect was calculated using the following equation (2.2):

$$\text{Matrix effect} = \frac{\text{Response of analytes in liposomal matrix}}{\text{Response of analytes in neat solution}} \times 100 \qquad (2.2)$$

2.3.6.5 Stability

Freeze-thaw, bench top, autosampler and short-term stability of PS and tocopherols were conducted using LQC, MQC and HQC. All the measurement for stability studies were conducted using freshly prepared calibration standards. In case of freeze thaw cycle, QCs were frozen at -80 °C for 12 hours and was thawed to room temperature followed by refreezing. For bench top stability, QCs were kept on bench top at room temperature for 10 hours prior to sample analysis. For autosampler stability, QCs were kept in the autosampler for 24 hours at 10 °C prior to analysis. One month stability was conducted for QCs stored at -80 °C every 7 days for a month.

2.4. Results and Discussions

2.4.1. Method development

2.4.1.1 Choice of the Internal Standard

LC-MS/MS method was developed for the simultaneous quantification of four PS along with four tocopherols (**Figure 2.1**). Cholestanol and rac tocol (structural analogues) were used as internal standards for PS and tocopherol respectively (**Figure 2.1**). Ideally, the use of isotopically labeled internal standards is the gold-standard approach in quantitative analysis as they co-elute with the target analytes, especially if labeled with C13 [22]. Such internal standards can compensate for variations in ionization and can correct for matrix effects [23].

However, in a multi-analyte analysis, the use of isotopically labelled internal standards will render the analytical method costly for routine analysis. Thus, the use of structural analogues can be adopted as internal standards since their ionization behaviors closely mimics that of the analytes of interest. It is an approach that have been successfully used for the quantification of eribulin in various biological samples[24]. Similarly, internal standard structural analogue have been used for the quantification of fluroquinolones in cattle manure-based biogas residue [25]. Both methods were proven to be robust and reliable as bioanalytical method validation was attained [24, 25].

2.4.1.2 Optimization of the chromatographic conditions

PS and tocopherols are lipophilic compounds; hence, the use of a reverse phase chromatography with a C18 column is suitable for their separation. Qiang *et al* used an LC-MS method with a C18 column for the separation of PS, erthrodiol, uvaol, tocopherol and lutein [9]. Similarly, it has been used for the separation of PS and tocopherols along with triterperic acid [21]. One of the most commonly used solvent systems, acetonitrile and methanol at different ratios were evaluated to

achieve adequate chromatographic separation for similar target analytes [9, 18]. As such, we tested varying ratios of acetonitrile and methanol during method development. As shown in **Appendix I, Figure S2.1** methanol showed a stronger elution profile and its increment led to decrease in the run time but at the expense of good analyte separation. Although LC-MS-MS does not necessarily require complete analyte separation, it is needed in the case of analytes that share similar MRM transitions.

Both 99:1 and 100:0 solvent ratio of acetonitrile to methanol showed good baseline separation of PS. However, solvent ratio at 100:0 required slightly longer run time than that observed at ratio 99:1. Therefore, 99:1 of acetonitrile: methanol was selected as a mobile phase that allowed for baseline separation with a relatively short run time. Acetic acid was also evaluated as an additive to the mobile phase at 0.01 % to 0.1 % to enhance ionization. The latter resulted in the best ionization and was, therefore, selected (data not shown). In sum, C18 column along with a mobile phase of acetonitrile/methanol 99:1 (0.1% acetic acid) was optimized for the simultaneous separation of PS and tocopherols **(Appendix I, Figure S2.1)**.

Most of the reported methods have overlapping PS peaks [8, 9, 11]. However, the developed method illustrates baseline separation for all target analytes, including PS (**Appendix I, Figure S2.1**). It should be noted that beta and gamma tocopherols, which are positional isomers, co-eluted with identical MRMs; they will be, therefore, quantified collectively. Similar elution pattern of beta and gamma tocopherols has been observed in other quantification methods reported in the literature and were collectively quantified [9, 21].

2.4.1.3. MS detection

The lipophilicity of PS renders them difficult for ionization using ESI. As such, APCI may serve as an alternative suitable ionization method in which the ionization is facilitated with a corona

discharge. While tocopherols are ionized easily using ESI, they are also effectively ionized using APCI [26]. Thus, APCI in the positive ion mode was selected for the simultaneous MS analysis of both analyte groups.

The ion source and compound dependent parameters were optimized where peak intensity and signal stability were the basis for the selection of the optimum values. Following interface parameters were employed: curtain gas 35 psi, nebulizer current 2.5 µA, an ion source gas1 30 psi and source temperature 380°C. Detailed information regarding ionization and product ion formation of PS and tocopherols have been demonstrated on our recently published paper[27]. PS ionized as $[M+H-H_2O]^+$, whereas tocopherols showed abundant molecular ion $M^{+\bullet}$ as shown, for example, in the full scan for stigmasterol and alpha tocopherol in **Figure 2.2,** respectively. Multiple reaction monitoring (MRM) mode was selected for quantification as it enhances the selectivity of the analytical method. Two MRM transitions were monitored to ensure the identity of target analytes as well to monitor ion ratios (**Table 2.1**). The site of cleavage yielding the monitored qualifier and quantifier ions for the analytes and internal standards are shown in **Appendix I, Figure S2.2. Table 2.1** shows retention time, precursor ion along with MRM transitions of PS and tocopherols.

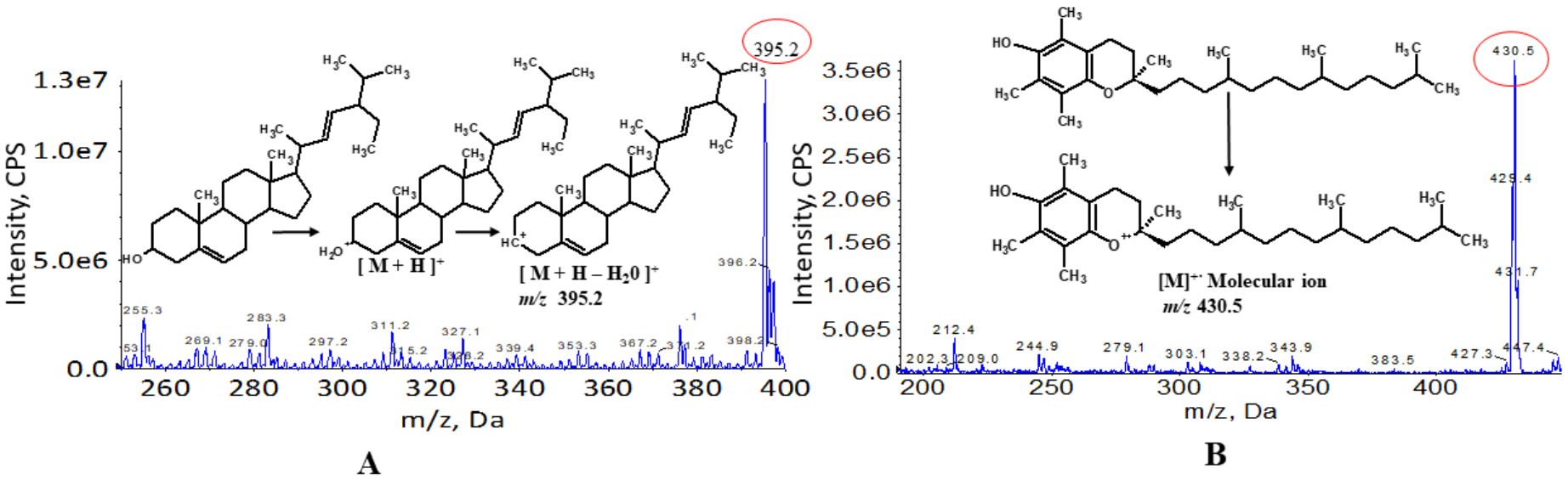


Figure 2.2. General MS ionization pattern of PS and tocopherol (A) stigmasterol and (B) α-tocopherol as representative structures. The charge in tocopherol was proposed to be localized on the oxygen atom within the chromane ring.

2.4.2. Application of the method for the quantification of phytosterols and tocopherols in liposomal formulation

The LC-MS/MS method was applied for the quantification of liposomal entrapped PS (except stigmasterol, which was not present in CODD) and tocopherols. As expected, the LC-MS/MS chromatogram of the liposomal samples **(Figure 2.3 I)** was identical to that of the standard mixture (**Appendix I, Figure S2.1**). However, unlike the standard mixture, an unknown peak was observed at a retention time of 2.5 min in the liposomal analytes (labeled as peak X). Peak X shared similar MRM transitions with that of alpha tocopherol **(Figure 2.3 II)**. Therefore, a third transition m/z 430 → 137.1 was monitored for alpha tocopherol. Surprisingly, the unknown peak X was still observed, sharing the same three MRM transitions (data not shown). MS/MS analysis was then performed on peak X and was compared with the MS/MS spectrum of alpha tocopherol (**Figure 2.4)**. Surprisingly, the MS/MS behavior of peak X was identical to that of alpha tocopherol. It can, therefore, be concluded that alpha tocopherol elutes at two different time points.

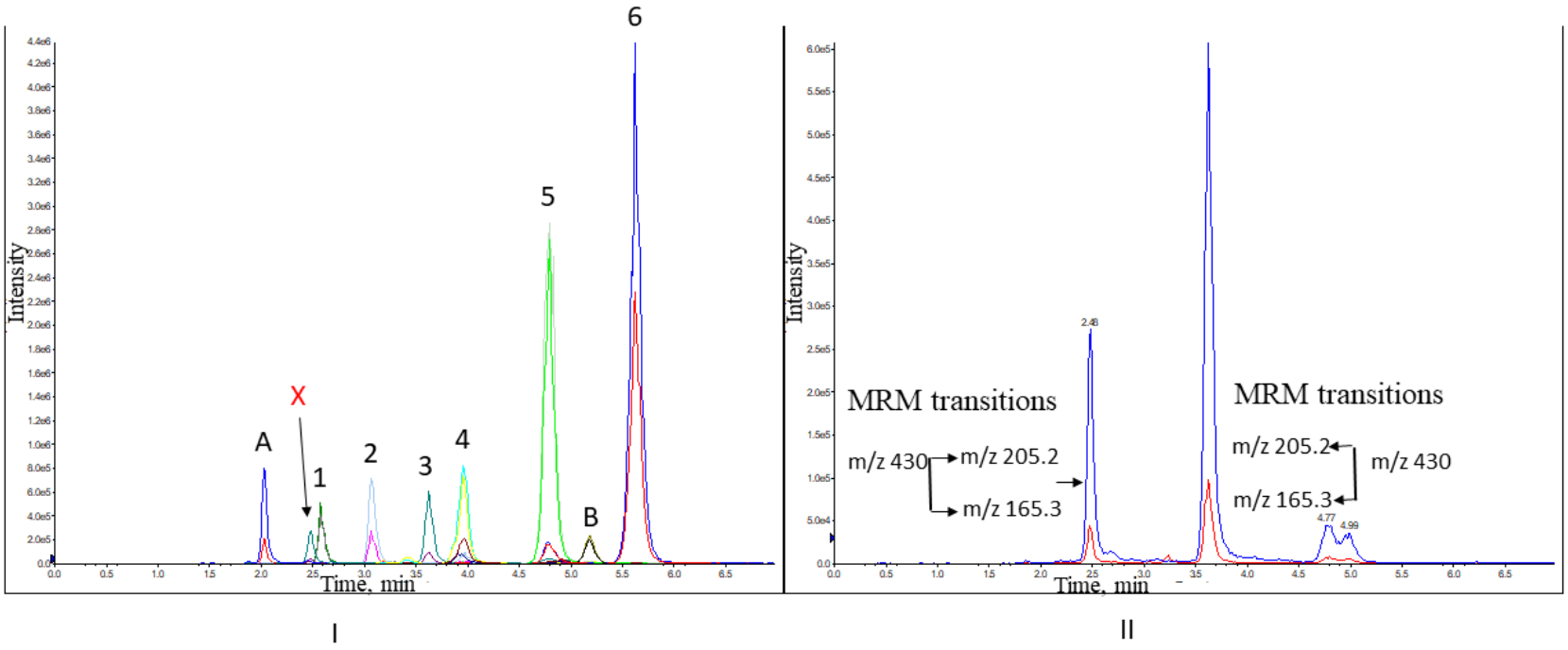


Figure 2.3. I- LC-MS/MS chromatograms of bio-actives entrapped in liposomes showing analytes and internal standard (1-δ tocopherol, 2- γ-tocopherol, 3-α-tocopherol, and PS 4-brassicasterol, 5-campesterol and 6- β-sitosterol) A-Rac tocol and B-cholestanol, II- Extracted unknown peak (RT=2.5min) sharing same transitions with alpha tocopherol (RT=3.6min)

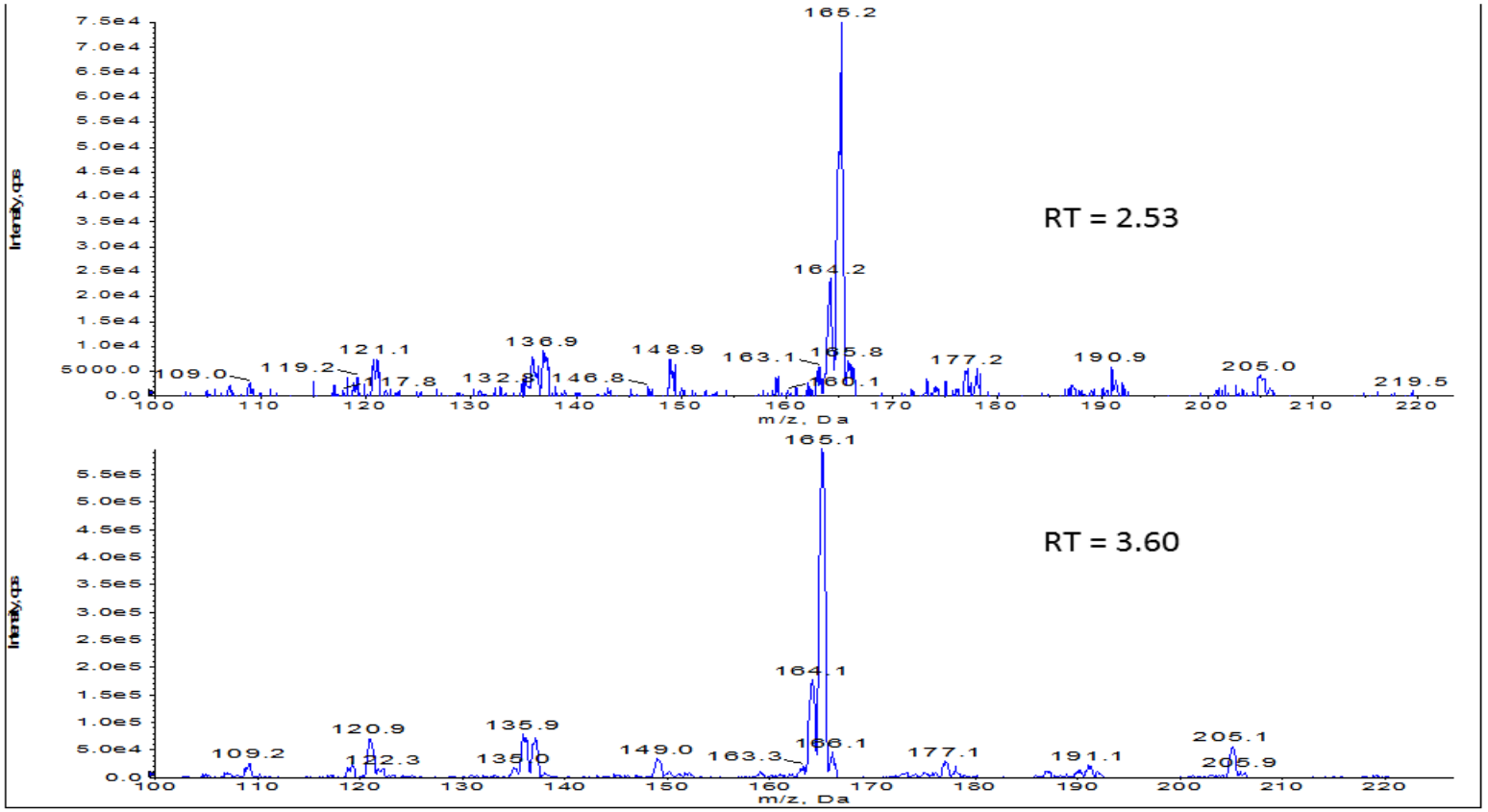


Figure 2.4. MS/MS spectra of unknown peak (retention time, RT=2.53) and alpha tocopherol (RT= 3.69), showing identical fragmentation behaviour.

To investigate why two peaks for alpha tocopherol were observed at different retention times, additional experiments were conducted. At first, it was hypothesized that homogenization that is used for the size reduction of liposomes may be the cause for this unusual behavior of alpha tocopherol. To test this hypothesis, liposomes were prepared without size reduction. However, peak X at retention time (RT) of 2.5 min was still observed. Further, with the assumption that hydration time of 3 hours during liposomal preparation was responsible for this behavior, the test was carried out by preparing liposomes after hydrating for only 1 min. However, peak X was still present. On the contrary, peak X was not observed in liposomal QCs, in which analytes are spiked in pre-formed liposomes.

Based on the above experiments, it can be concluded that the entrapment of alpha tocopherol in the liposomal bilayer of the phosphatidylcholine is responsible for the appearance of peak X (an extra peak of alpha tocopherol). To further confirm this conclusion, alpha tocopherol was processed mimicking the liposomal preparation process but without the addition of the phosphatidylcholine, the building block of the liposomes. As expected, no extra peak for alpha tocopherol was observed (data not shown). This finding implies that phosphatidylcholine in its ordered liposomal structure is responsible for the formation of peak X.

Synthetic alpha tocopherol, which was used for the formulation, is a mixture of 8 stereoisomers (as it has 3 chiral centers). It is well established in the literature that liposomal phosphatidylcholine has the ability for chiral recognition [28, 29]. Oscar *et. al*. showed using ^{1}H NMR that micelles and vesicles formed by phosphatidylcholine has the ability for chiral recognition of enantiomers of ditryptophan [28]. Similarly, L-DPPC liposomes showed chiral recognition towards L-amino acids after 48 hours incubation [29]. Therefore, we speculate that liposomes containing mixtures of stereoisomers of alpha tocopherol were separated during the analytical run due to the chiral

selectivity of liposomal phosphatidylcholine. It is still unclear, however, why other tocopherols (i.e., gamma and delta) did not show similar behavior. In any case, for the purpose of quantitative analysis, the two peaks of alpha tocopherol were integrated in order to determine its total concentration in tested liposomal samples.

Table 2.2 shows the quantity of PS and tocopherols entrapped in liposomes, accounting for over 90% of the added bioactives. We recently demonstrated the development of novel formulations intended for food application showing high entrapment efficiency of three different formulation strategies [19]. However, after running numerous samples, we noticed the lack of reproducibility at the LLOQ and LQC (i.e., not meeting regulatory guidelines), so we optimized the method employing quadratic relationship as discussed, for the first time, in the section below. In fact, sensitivity was also enhanced with the optimization of the quadratic relationship. In addition, there was no details about method development, validation and the presence of peak X in our past work that focused on formulation development [19]. A novel LC-MS/MS validated method to quantify liposomes containing PS and tocopherols as principal components is discussed in detail, in this manuscript for the first time.

Table 2.2. Quantity of phytosterols and tocopherols in entrapped in liposomal formulations.

Liposomal bioactives	**Quantity (mg/ml of liposomes)**
Brassicasterol	0.118 ± 0.010
Campesterol	0.162 ± 0.006
Beta-sitosterol	0.185 ± 0.018
Alpha tocopherol	0.0450 ± 0.0001
Gamma tocopherol	0.0480 ± 0.0005
Delta tocopherol	0.059 ± 0.002

2.4.3. Method application in quantifying PS and tocopherols present in CODD

The quantity of PS and tocopherols present in CODD is shown in **Table 2.3.** CODD showed the presence of four PS- brassicasterol, campesterol, stigamsterol and β-sitosterol. Among these PS, β-sitosterol's content was the highest (as shown in **Table 2.3**) whereas stigmasterol was present below the limit of quantitation, thus was not quantified. Among tocopherols, gamma tocopherols showed the highest abundance (**Table 2.3**). In the same way as stigmasterol, delta tocopherol could not be quantified.

Table 2.3. Quantity of phytosterols and tocopherols in canola oil deodorizer distillate (CODD).

Analytes in CODD	**Amount (in grams)**
Brasicasterol	0.11±0.0005
Campesterol	0.14±0.0015
β-sitosterol	0.19±0.0005
Gamma tocopherol	0.06±0.0000
Alpha tocopherol	0.04±0.0005
Total tocopherols	0.09±0.0000
Total PS	0.45±0.0010

2.4.4. Method validation

ICH guidance for bioanalytical method validation were used as a guideline to validate the LC-MS/MS method (http://www.gmp-compliance.org/guidemgr/files/Q2(R1).PDF).

2.4.4.1. Calibration range and sensitivity

Calibration curves for all PS and tocopherols are shown in **Appendix I, Figure S2.3**. PS showed linearity at a concentration range of 0.05 - 10 µg/mL (**Table 2.1**). For tocopherols, initial linear ranges were 0.25 – 10 µg/mL (for alpha and gamma) and 0.5-10 µg/mL (for delta tocopherol). Unfortunately, gamma and delta tocopherols failed to attain reproducibility at the LLOQs and LQCs under these conditions. To address this, linear ranges were narrowed to 0.5-10 µg/mL for gamma and 1-10 µg/mL for delta; however, reproducibility was still not obtained. Therefore, a quadratic calibration curve was adapted for gamma and delta tocopherols (**Table 2.1**). The use of quadratic calibration curve resulted in enhanced sensitivity for delta tocopherols with calibration range of 0.25-10 µg/mL. The use of quadratic calibration curves during method development and

validation is commonly used and has been reported in the literature [30-32]. In conclusion, all PS and alpha tocopherols showed linear relationship whereas gamma and delta tocopherols showed quadratic relationship.

The correlation coefficient (R) was 0.999 and 0.999 for tocopherols and PS, respectively. The achieved concentration range of both group of analytes were excellent for the intended application to quantify a wide range of concentrations of the target analytes incorporated in liposomal formulations.

LODs for all the analytes were in the range of 0.005-0.05 µg/mL while the LLOQ for all PS were 0.05 µg/mL and 0.25 µg/mL for tocopherols (**Table 2.1**).

2.4.4.2. Inter- and Intraday Accuracy and Precision

Accuracy and precision were evaluated on a single day (intraday) and three different days (interday) at four QC levels (**Table 2.4**). In case of intraday, 68 % of the QCs showed accuracy within standard deviation of ± 10 % and the remaining 32% of QCs were within the acceptable standard deviation of ± 15% from the nominal concentration. In the case of interday, 88% of QCs were within standard deviation of ± 10 % and 12 % of QCs were within deviation of ± 15 % from the nominal concentration, meeting the required validation criteria. Precision, which was measured, as coefficient of variation (CV), did not exceed 8.7 % at all QC levels

Table 2.4. Inter-day and intra-day accuracy and precision of phytosterols and tocopherols.

Compound	Quality controls	Concentration (µg/mL)	Intra-day (n=4)		Inter-day 1 (n=4)		Inter-day 2 (n=4)		Inter-day 3 (n=4)	
			Accuracy (%)	Precision (%RSD)	Accuracy (%)	Precision (%RSD)	Accuracy (%)	Precision (%RSD)	Accuracy (%)	Precision (%RSD)
δ-Tocopherol	LLOQC	0.25	110.03	6.37	100.46	2.54	102.48	8.15	113.22	3.56
	LQC	0.75	112.00	0.93	110.77	3.75	108.25	6.65	108.98	4.33
	MQC	5.5	91.19	2.27	110.64	3.63	100.82	6.94	100.15	5.65
	HQC	9	91.70	0.39	92.46	2.47	104.52	7.89	95.73	4.19
β/γ- Tocopherol	LLOQC	0.25	115.33	1.41	111.03	1.87	106.16	1.81	105.94	2.10
	LQC	0.75	113.27	2.04	111.01	3.79	113.79	6.41	110.39	3.40
	MQC	5.5	108.68	2.25	108.06	1.36	107.28	2.68	102.53	4.51
	HQC	9	111.04	1.03	104.45	3.44	104.02	3.12	102.37	4.17
α- Tocopherol	LLOQC	0.25	116.81	2.91	107.60	0.45	107.35	3.80	111.92	2.73
	LQC	0.75	113.88	0.47	109.83	3.37	110.93	4.68	105.00	2.98
	MQC	5.5	110.16	1.74	111.63	2.12	103.91	3.84	100.10	5.35
	HQC	9	113.99	1.46	100.96	4.67	107.66	4.25	101.60	5.42
Brassicasterol	LLOQC	0.05	100.88	4.75	102.20	6.63	106.94	2.12	100.37	6.28
	LQC	0.15	112.00	3.48	96.17	2.80	105.16	5.67	99.62	3.73
	MQC	5.5	91.19	0.09	105.93	7.38	103.70	3.71	94.92	2.47
	HQC	9	91.70	0.34	104.64	7.21	102.68	4.96	95.35	3.81
Campesterol	LLOQC	0.05	105.51	2.23	103.74	2.90	105.04	3.24	102.26	2.54
	LQC	0.15	105.65	4.52	101.36	2.39	101.48	2.75	98.64	3.75
	MQC	5.5	95.67	3.37	106.64	6.14	100.17	4.19	98.20	5.62
	HQC	9	104.85	5.21	104.40	4.72	103.07	4.83	98.20	3.74
Stigmasterol	LLOQC	0.05	99.87	9.72	99.12	4.00	113.92	2.84	105.37	6.28
	LQC	0.15	104.10	5.71	94.17	0.83	107.19	4.86	101.40	4.54
	MQC	5.5	93.66	0.10	103.06	6.67	99.67	3.83	97.41	3.79
	HQC	9	105.16	0.98	101.93	6.51	101.86	4.70	97.77	2.32
β-Sitosterol	LLOQC	0.05	110.88	0.02	97.54	5.35	109.63	5.88	102.43	6.66
	LQC	0.15	111.76	6.56	97.38	4.67	106.29	3.68	102.20	2.38
	MQC	5.5	96.40	0.98	109.65	6.30	103.61	2.57	97.67	2.72
	HQC	9	108.70	0.94	104.38	8.69	104.58	5.32	98.02	1.84

2.4.4.3. Dilution integrity

Accuracy and precision of the diluted sample was the basis for determining dilution integrity**.** **Appendix I, Table S2.1** shows that samples after dilution at 1.7 and 5 times lied within the acceptable limits for accuracy and precision, which is within ± 15 standard deviation for accuracy and CV of ± 15 % for precision. These results demonstrate that the dilution of samples within the expected range does not affect the analytical results during the analysis of liposomes containing the bio-actives.

2.4.4.4. Selectivity and Matrix effects

Selectivity assessment using blank liposomal matrix is shown in **Appendix I**, **Figure S2.4.** The chromatogram demonstrates that blank liposomes did not contain any interferences in the monitored analytes and internal standards MRM transitions. Thus, there is no interferences from component of the liposomal matrix during the analysis.

The matrix effects resulted in minor ion suppression at the various QC levels for all analytes, ranging between 86-92 % (**Table 2.5**).

Table 2.5. Matrix effect of phytosterols and tocopherols in liposomes at various QC concentrations (LQC, MQC. HQC).

Analytes	QCs µg/ml	Matrix effect (%)	Mean Matrix effect (%)
Delta	0.75(LQC) 5.5(MQC) 9(HQC)	95.11 89.45 90.81	91.79 ± 2.21
Alpha	0.75(LQC) 5.5(MQC) 9(HQC)	91.70 95.16 95.85	94.24 ± 2.22
Gamma	0.75(LQC) 5.5(MQC) 9(HQC)	91.83 90.43 91.74	91.33 ± 0.78
Brassicasterol	0.15(LQC) 5.5(MQC) 9(HQC)	88.29 93.94 95.87	92.7 ± 3.22
Campesterol	0.15(LQC) 5.5(MQC) 9(HQC)	90.63 101.05 95.30	95.66 ± 5.22
Stigmasterol	0.15(LQC) 5.5(MQC) 9(HQC)	87.34 100.95 95.73	94.67 ± 6.87
β-sitosterol	0.15(LQC) 5.5(MQC) 9(HQC)	90.31 94.52 94.64	93.16 ± 2.47

2.4.4.5. Stability evaluation

All prepared liposomal QCs undergoing three cycles of freeze-thaw met the accuracy limit as specified in the ICH guideline **Appendix I, Table S2.2 A.** This implies that repeated freezing and thawing does not degrade analytes within liposomal matrix. The QCs (LQC, MQC and HQC) for all the analytes prepared from reference stock solution and stored at -80 °C were stable for 4 weeks as shown in **Appendix I, Table S2.2 B**. This implies that liposomal PS and tocopherols can be

stored at -80 °C for a month. Further, all autosampler and benchtop QCs passed the required accuracy and precision, **Appendix I**, **Table S2.2 C and Table S2.2 D.** This shows that analytes were stable during sample preparation and data acquisition.

2.5. Conclusion

LC-MS/MS method was developed and validated as per the ICH guideline for the quantification of a mixture of PS and tocopherols entrapped in liposomal formulations. Further, we applied this method in quantifying target analyte in agricultural product (CODD). The method has a short run time of 7 min compared to currently existing methods. An isocratic elution was employed that simplifies the analytical procedure when compared to gradient elution. Better baseline separation in comparison to reported methods was achieved for PS making the method superior to current methods that are employed for the simultaneous determination of PS and tocopherols. Quantifying liposomal entrapped bioactives is required during formulation development for efficient therapeutic approach. A unique extra peak, however, was observed for alpha tocopherol which is assumed to be stereoisomers of alpha tocopherol (based on evidence obtained from several experimental trials) that got separated due to chiral selective phosphatidylcholine. Further studies such as nuclear magnetic resonance (NMR) will be conducted in the near future to validate this assumption. In sum, we developed and validated LC-MS/MS method with wide application.

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2.6. References

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CHAPTER 3

Development and characterization of liposomal formulations containing phytosterols extracted from canola oil deodorizer distillate along with tocopherols as food additives

Asmita Poudel, George Gachumi, Kishor M. Wasan, Zafer Dallal Bashi, Anas El-Aneed * and Ildiko Badea *

Drug Design and Discovery Group, College of Pharmacy and Nutrition, University of Saskatchewan, 107 Wiggins Road, Saskatoon, SK S7N 5E5, Canada

*Correspondence: anas.el-aneed@usask.ca ; ildiko.badea@usask.ca

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Transitioning rationale

The aim of this chapter is to investigate different preparation methods in developing liposomal phytosterols and tocopherols. Optimal method is selected based on the required physicochemical characteristics, such as size, zeta potential and entrapment efficiency. The entrapment efficiency was assessed using a robust analytical platform described in the previous chapter.

Contribution statement:

Asmita Poudel contributed to this paper by conducting experimental design, conducting experiment, data analysis, and writing the manuscript

3.1. Abstract

Phytosterols (PS) are plant sterols recommended as adjuvant therapy for hypercholesterolemia and tocopherols are well-established antioxidants. However, thermo-sensitivity, lipophilicity and formulation-dependent efficiency bring challenges in the development of food products enriched with PS and tocopherols. To address this, we developed liposomes containing brassicasterol, campesterol and β-sitosterol obtained from canola oil deodorizer distillate, along with alpha, gamma and delta tocopherol. Three approaches; thin film hydration-homogenization, thin film hydration-ultrasonication and Mozafari method were used for formulation. Validated liquid chromatographic tandem mass spectrometry (LC-MS/MS) was utilized to determine the entrapment efficiency of bioactives. Stability studies of liposomal formulations were conducted before and after pasteurization using high temperature short time (HTST) technique for a month. Vesicle size after homogenization and ultrasonication (<200 nm) was significantly lower than by Mozafari method (> 200 nm). However, zeta potential (−9 mV to −14 mV) was comparable which was adequate for colloidal stability. Entrapment efficiencies were greater than 89% for all the PS and tocopherols formulated by all three methods. Liposomes with optimum particle size and zeta potential were incorporated in model orange juice, showing adequate stability after pasteurization (72 °C for 15 s) for a month. Liposomes containing PS obtained from canola waste along with tocopherols were developed and successfully applied as a food additive using model orange juice.

Keywords: PS; tocopherols; liposomes; canola oil deodorizer distillate; model orange juice

3.2. Introduction

Nutraceuticals are increasing rapidly due to growing consumer preferences towards natural bioactives rather than synthetic drugs for disease prevention and treatment [1]. Phytosterols (PS) and tocopherols are such bioactives (plant metabolites) that have numerous health claims [2, 3].The primary health benefit of PS is to lower low-density lipoprotein (LDL) cholesterol levels in plasma [2, 4, 5]. PS compete with cholesterol for their solubilization in bile salt micelles, hindering the absorption of cholesterol in blood [6, 7]. Tocopherols, on the other hand, are free radical scavengers and natural anti-oxidants [8, 9]. Due to their anti-oxidant properties, tocopherols are used in the treatment of age related macular degeneration [10], Alzheimer's disease [11], glaucoma [3] and heart diseases [12].

Sources of PS and tocopherols include oil seeds such as canola and sesame, as well as nuts [13-15]. Among these, canola is a major source for edible vegetable oils, and the most abundant oilseed crop in Canada [16]. It is a rich source of four PS, namely beta-sitosterol, campesterol, stigmasterol and brassicasterol, and four tocopherols (alpha, beta, gamma and delta) [15, 17]. Canola oil loses some of its valuable components during the refining process [15]. Significant amount of PS and tocopherols are transferred to the waste stream, termed canola oil deodorizer distillate (CODD)[15] which offers an ideal source of these components.

However, formulation of these bioactives in food product has always been challenging due to their lipophilicity and light sensitivity [18]. In particular, degradation products of PS, phytosterols oxidation products (POPs), are known to have some negative impact on human health [19, 20]. Thus, the selection of suitable formulation approach is crucial during the development of enriched food that contain these bioactives. Encapsulation techniques, such as spray drying, fluidized bed coating, microemulsification and liposomal entrapment are emerging in the food industry to

address lipophilicity related challenges [21, 22]. Unfortunately, most of these techniques have shortcomings such as usage of high temperature (can possibly degrade PS and tocopherols) and the requirement of large quantities of emulsifiers and surfactants, which are deleterious to human health [22, 23]. All of these shortcomings can be addressed by employing liposomal formulations that require low heat and low quantities of surfactants or emulsifiers [24].

PS in both the free and esterified forms have been used in the food industry [2, 4]. Solubilization of esterified PS in fat containing foods, like margarine[25, 26], salad dressing[27] and yogurt[28] is prevalent in the food industry. However, this approach is not favorable to people who are on low fat diet. To overcome this, various low fat or non-fat food matrices such as low fat milk[29, 30], granola bars [31], orange juice [32] and non-fat beverages[33-35] are emerging as food products. However, for these type of food products, lipophilic PS should be well formulated prior to their addition into food products. In addition to the choice of the food matrix, the biological efficacy should also be carefully considered.

Various clinical trials have shown that the efficacy of PS depends on different parameters, such as solubility in the food matrix and the formulation [2, 36]. Esterified PS solubilized in fat/oil are driven favorably towards the bile salt micelles in the guts than the crystalline or the insolubilized forms [37, 38]. PS ester containing food products such as milk, spread and yogurt have showed reduction in LDL-cholesterol by 7-12% at daily dose of 1.6-2 g relative to control research participants [39-41]. In contrast, some failed clinical trials are also prevalent [42, 43]. For example, Ottestad et al. showed that PS ester in the capsular formulation revealed no significant reduction of LDL cholesterol [42]. Similarly, Denke et al. showed no significance in cholesterol reduction by sitostanol capsule relative to control[43]. Unlike PS capsule-based trials, lecithin-based free PS formulations have shown to impart efficacy as high as 14.3% at a daily dose of only 1.9g relative

to control [34]. In sum, literature reports show that the efficiency of PS depends greatly on the formulation approach, which provides insights regarding the possibility of further enhancing their efficiency by well formulating in suitable delivery systems.

The work of Shin at al. [33] and Spilburg at al. [34] provides a strong evidence that lecithin (phosphatidylcholine) can be effective carrier of PS to increase cholesterol-lowering efficiency. Both of these studies used lecithin micelles to formulate sterol/stanol which have shown promising cholesterol-lowering efficiency [33, 34]. Liposomes, which have same building blocks as micelles that is lecithin (i.e., phosphatidylcholine) but different architecture are another formulation strategy in which lecithin can be utilized, thus have potential of further enhancing its cholesterol-lowering efficiency. In addition, liposomes can prevent oxidation of thermo-sensitive bioactives and are biocompatible and biodegradable[44]. Further, co-formulation of tocopherols along with PS can enhance oxidative stability of PS [45].

Thus, in this work, with the aim of enhancing PS' oxidative stability and increasing its therapeutic efficiency, we formulated PS (obtained from CODD) and commercially available tocopherols into liposomes employing three different approaches, namely thin film hydration homogenization, thin film hydration ultra-sonication and Mozafari method. The liposomal formulation showing the highest entrapment efficiency, adequate size and zeta potential was incorporated into model orange juice (acidified solution). Thus, liposomal PS and tocopherols was developed, and its stability was assessed.

3.3. Materials and Methods

3.3.1. Chemicals and reagents

PS were extracted from CODD obtained from LMD foods (Yorkton, SK, Canada) Briefly, 5 g of CODD was saponified with 1 M potassium hydroxide in 95% ethanol for 1 h at 65 °C after which

water was added and the mixture was chilled at 9.5 °C for 1 h. After the crystallization of PS, vacuum filtration was performed, and the residue was washed before being dried under high vacuum. Tocopherols, chloroform, ethyl acetate and potassium hydroxide were purchased from Sigma Aldrich (Oakville, ON, Canada), and phosphatidylcholine (PC) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Purified water was obtained from Millipore (Bedford, MA, USA).

3.3.2. Formulation of liposomes

Three different formulation techniques namely thin film hydration homogenization; thin film hydration ultrasonication and Mozafari method were used for formulation in order to evaluate the formulation technique that can produce liposomes with optimum physicochemical properties for both oral delivery and industrial scale up.

3.3.2.1. Method I. Thin film hydration – homogenization

This method was adopted from Chung et al. [46] with some modifications. In brief, tocopherols (alpha, gamma and delta tocopherol), PS mixture (brassicasterol, campesterol and beta-sitosterol) and PC were dissolved in 5 mL ethyl acetate (food grade) in 0.1:0.9:2, 0.1:0.9:3, 0.1:0.9:4 and 0.1:0.9:5 ratio of tocopherol: PS: PC. Ethylacetate was evaporated using rotary evaporator at pressure of 90 mmHg. The thin lipid film containing bioactives and PC formed on the wall of the flask was lyophilized for 10 h to remove traces of ethylacetate and was hydrated with 20 mL of purified water for 3 h at 55°C with occasional vortexing in the presence of glass beads. The lipid dispersion was homogenized using recirculating high fluid pressure homogenizer (Microfluidics Corporation, Westwood, MA) at 60 psi for 20 min. The prepared liposomes were left overnight at 4 °C prior to size analysis.

3.3.2.2. Method II: Thin film hydration ultrasonication

This method was adopted from Akbarzadeh et al. [47]. Similarly to thin film hydration homogenization, tocopherols (alpha, gamma and delta tocopherol) and PS mixture (brassicasterol, campesterol and beta-sitosterol) were dissolved, along with PC in 5 mL ethyl acetate, in 0.1:0.9:2 ratio of tocopherols: PS: PC. Ethylacetate was evaporated using rotary evaporator at pressure of 90 mmHg. Thin lipid film containing bioactives and PC was formed at the bottom of the flask. Lipid film was lyophilized for 10 h to remove traces of ethylacetate and was hydrated with 20 mL of purified water maintained at 55°C. Lipid dispersion was ultrasonicated using bath sonicator (ELMA Corp., Singen, Germany) for 30 min maintained at 55 °C then was allowed to cool at room temperature. The prepared liposomes were left overnight at 4 °C prior to size analysis.

3.3.2.3. Method III: Mozafari Method

This method was adopted from Colas et al. [48]. 50 mg of PC was hydrated with 20 mL of purified water for 1 h and was heated to 55 °C. Nine mg of the PS mixture and 1 mg of the tocopherol mixture were heated with 3% *v*/*v* glycerol at 110 °C and 55 °C temperature, respectively for 15 min on a hot plate stirrer at approximately 1000 RPM (Corning Corporation, Midland, ON, Canada) and then was cooled down to 55 °C. PC dispersion, PS and tocopherols were mixed together with stirring on a hot plate for 30 min at approximately 1000 RPM. The formed liposomes were cooled down to room temperature and kept overnight at 4 °C prior to size analysis.

3.3.3. Characterization of particle Size, size distribution and zeta potential

Particle size and zeta potential measurement of the liposomes were performed using Zeta sizer, Nano ZS instrument, Malvern instruments Ltd (Worcestershire, England). All measurements were conducted in triplicates at 25 °C and reported as mean ± SD.

3.3.4. Transmission electron microscopy (TEM) analysis

TEM analysis was performed by negative staining. Briefly, a drop of liposomal sample was placed on copper- formvar coated TEM grid and was allowed to settle on grid surface for 1 min. Excess of the liquid was removed using absorbent tissue. Staining of grid was done using 0.5% phosphotungstic acid for 30 s and excess of stain is removed. Imaging was done using a HT 7700 TEM (Hitachi, Japan) at 80 kV.

3.3.5. LC-MS/MS method development and validation

LC-MS/MS method was developed and was validated as per International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) guidance for bioanalytical method validation guideline[49]. Briefly, chromatographic separation of the analytes was carried out on an Agilent Acquity UPLC (Agilent Technologies, Mississauga, ON, Canada) with an Agilent Poroshell C18 column (2.1 mm × 150 mm, 5µm) protected by a guard column (2.1 mm × 4.7 mm, 2.7 µm) of the same packing material. The column temperature was set at 30 °C and the injection volume was 2.5 µL. An isocratic elution consisting of acetonitrile: methanol (99:1 *v*/*v*) with 0.1% acetic acid was used at a flow rate of 0.8 mL/min. The detection and quantification were performed using an API 6500 QTRAP® quadruple-linear ion trap (QqQ-LIT) mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source obtained from AB Sciex (Mississauga, ON, Canada). The instrument was operated in the positive

ion mode and tandem mass spectrometric analysis (MS/MS) was employed using the following interface parameters: source temperature 380 °C, curtain gas 30 psi (gas), nebulizer current 2.5 µA, declustering potential 30 V and an ion source gas1 30 psi (gas) [50].

The parameters, selectivity, accuracy, precision, reproducibility, sensitivity, matrix effects, dilution integrity, stability were assessed [50]

3.3.6. Entrapment efficiency (EE)

In order to determine entrapment efficiency, free and entrapped bioactives were separated using ultracentrifugation. Ultracentrifuge (Beckman coulter, Inc., Indianapolis, IN, USA) with rotor SW 60Ti was used for ultracentrifugation. Briefly, 5 mL of liposomes was ultracentrifuged at 30, 60, 90 and 120 min at constant RPM 32,000 (G-force of 138000). The sediment at each time were analyzed using a validated LC-MS/MS method to optimize ultracentrifugation parameters. The liposomes (present in sediment) separated by ultracentrifugation were lyophilized using freeze dryer for 24 h. Similar lyophilization process was employed with 5 mL of unseparated liposomes for 24 h. Dried unseparated and separated liposomes were dissolved in 2 mL of chloroform separately. Aliquot of each were spiked with internal standard and diluted with acetonitrile. Samples were injected in LC-MS along with freshly prepared calibration and quality control standards, as described [50].The entrapment efficiency was calculated by measuring the ratio of entrapped bioactives in the formulation to the total bioactives present in the formulation and was determined using following equation:

$$\%\text{Entrapment efficiency} = \frac{\text{E bioactives}}{\text{T bioactives}} \text{X}100, \qquad (3.1)$$

Where, E bioactives = Entrapped bioactives in liposomes (present in sediment of separated liposomes); T bioactives = Total bioactives in liposomes (present in unseparated liposomes).

3.3.7. Development of orange juice containing liposomal phytosterols and tocopherols.

In order to preserve particle size during freeze drying, sucrose was added to liposomes as a cryoprotectant by adopting the procedure of Shaikh et al. [51]. Briefly, 5% *w*/*v* of sucrose was added to liposomes of well-defined size and was vortexed. Lyophilization then was employed for 24 h.

Freeze dried liposomes were re-suspended in model orange juice which is an orange juice mimic at 3.2 pH. A mimic was used instead of real orange juice to enable particle size analysis without the interference of particulate components existing in the orange juice. In fact, the acidified solution is considered a model juice and was prepared by using acetic acid as per the protocol of Marsansco et al. [52]. This protocol can be applied for fruit juice with a pH less than 5.0, such as orange juice and pineapple juice. Finally, liposomes with optimum entrapment efficiency in a dried form were incorporated into the model orange juice by vortexing for 5 min.

3.3.8. Pasteurization

High temperature short time (HTST) pasteurization technique was employed as described [53]. HTST is a commonly used strategy for the pasteurization of juice [54]. The liposomes containing model orange juice was pasteurized at 72 °C for 15 s. Unpasteurized formulation was used as a control. Both pasteurized and unpasteurized model juice were stored at 4 °C for stability evaluation.

3.3.9. Chemical stability studies

Both pasteurized and non-pasteurized model juice containing liposomal bioactives were analyzed using LC-MS/MS to assess the degradation of bioactive upon exposure to pasteurization temperature. Briefly, 5 mL each of pasteurized and non-pasteurized liposomal model juice were

lyophilized. Dried samples were dissolved in chloroform and were diluted with acetonitrile for LC-MS/MS analysis. The LC-MS/MS response was compared to obtain relative quantification data.

3.3.10. Physical stability studies

Physical stability evaluation was conducted at the interval of 7 days for a month. Particle size of pasteurized and non-pasteurized liposomes incorporated both in model orange juice were analyzed.

3.3.11. Statistical analysis

The statistical analysis of the samples was conducted with SPS statistical software version 24 (SPS Inc, Chicago, Ill, USA) using student *t*-test, and *p*-values < 0.05 were considered statistically significant. All data are reported as means ± standard deviations.

3.4. Results and Discussions

3.4.1. Physicochemical characterization

Size is an important parameter to assess the stability, the biological fate and the efficacy of formulated bioactives [55, 56]. Optimization of bioactive to lipid weight ratio (B/L ratio) was performed at ratio of 1:5, 1:4. 1:3, 1:2, 1:1 using liposomes prepared by thin film hydration-homogenization approach. An increase in particle size was observed at high B/L ratio (1:1) as shown in **Figure 3.1**. At 1:5 B/L ratio, the particle size was 149.53 nm; however, when B/L ratio increased to 1:1, the particle size increased to 258.31 nm (Figure 1). This observation is reported previously [57, 58], in which incremental vesicle size was observed when increasing the cholesterol (a sterol) concentration. While 1:1 ratio is preferable from a commercial point of view (less PC required for formulation), smaller particle size (less than 200 nm) attained at 1:2 ratio, is

optimum for liposomal stability. This optimum vesicle size (less than 200 nm) is consistent with several food-based liposomes [59-61]. Thus, 1:2 B/L ratio was selected for follow-up experiments. Same optimum B/L ratio was selected for thin-layer ultrasonication approach. However, in case of the Mozafari method, B/L higher than 1:5 led to the appearance of visible white precipitate. Loading techniques along with the preparation procedures are found to influence drug/lipid ratio of liposomes [62]. In both thin film hydration homogenization and thin film hydration ultrasonication, hydrated bioactives-PC film is subjected to cavitation and shearing forces unlike the Mozafari method where less intense magnetic stirring is used during the loading process. This might have led to differences in the B/L ratio of the Mozafari method in comparison with ultrasonication and homogenization methods. In this way, 1:5 B/L as optimum ratio was selected for formulations prepared by the Mozafari method.

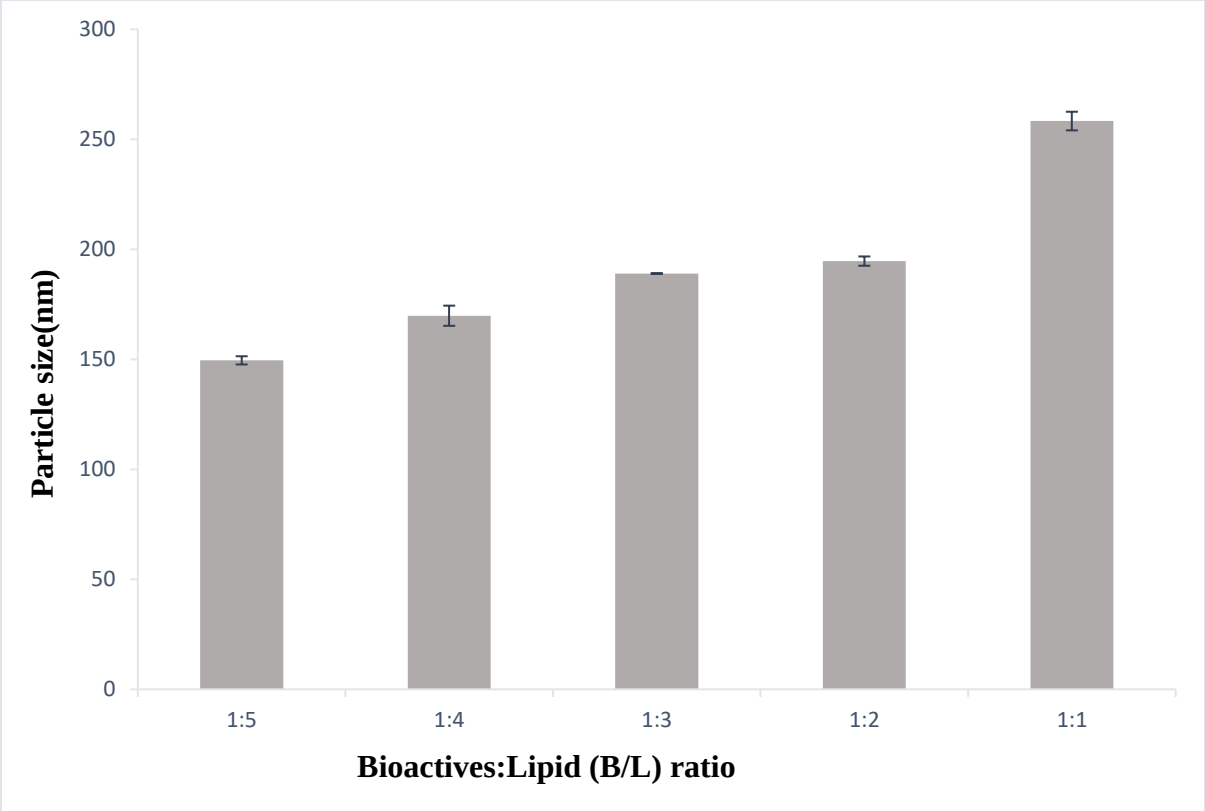

Figure 3.1. Particle size of liposomes prepared at different B/L ratio by homogenization method expressed as mean ± standard deviation.

The comparison of the vesicle size using the different formulation strategies is presented in **Table 3.1.** Thin film hydration homogenization and thin film hydration ultrasonication were comparable, showing sizes at 186.33 ± 4.38 nm and 196.2 ± 16.1 nm, respectively. On the other hand, the size was significantly larger in the case of the Mozafari method (260 ± 22.98 nm). It is possible that the high shear force and cavitation involved in size reduction during the homogenization and ultrasonication methods is the reason for the obtained smaller vesicles [63]. The Mozafari method uses a less intense magnetic stirring [64], probably yielding larger particles. Polydispersity index (PDI) shown in **Table 3.1** was found to be in the range from 0.29 to 0.37, which shows the desirable narrow size distribution for all formulations.

Table 3.1. Average particle size (nm), polydispersity index and zeta potential (mv) of liposomes prepared by thin film hydration homogenization, thin film hydration ultrasonication and Mozafari method expressed as mean ± standard deviation where * represents statistical significance (*$p < 0.05$) in average particle size of Mozafari method in comparison with homogenization and ultrasonication method.

Formulation Techniques	**Average Particle Size (nm)**	**Polydispersity Index (PDI)**	**Zeta Potential (mV)**
Thin film hydration Homogenization	186.3 ± 4.4	0.370 ± 0.001	−13.0 ± 5.0
Thin film hydration ultra-sonication	196.2 ± 16.1	0.294 ± 0.084	−14.0 ± 3.4
Mozafari method	260.0 ± 23.0*	0.348 ± 0.087	−9.8 ± 0.3

Zeta potential (surface charge) is another important parameter that determines the stability of liposomal dispersions[65]. All the liposomes**,** demonstrated similar zeta potential values (**Table 3.1)**, that is in the range of -9 mV to -14 mV, indicating relatively stable systems [66]. Thus, based on particle size and zeta potential, the developed liposomal formulations are theoretically stable that was confirmed experimentally by conducting the stability studies.

Finally, TEM analysis of liposomes shows spherical shaped particles with a single lipid bilayer (**Figure 3.2**), representing the expected morphology of unilamellar liposomal vesicles (ULV) [67, 68]. The size of approximately 200 nm is consistent with the size range measured by dynamic light scattering (DLS) (**Table 3.1**). Some aggregated particles were observed in ultra-sonication and Mozafari method as shown in **Figure 3.2.**

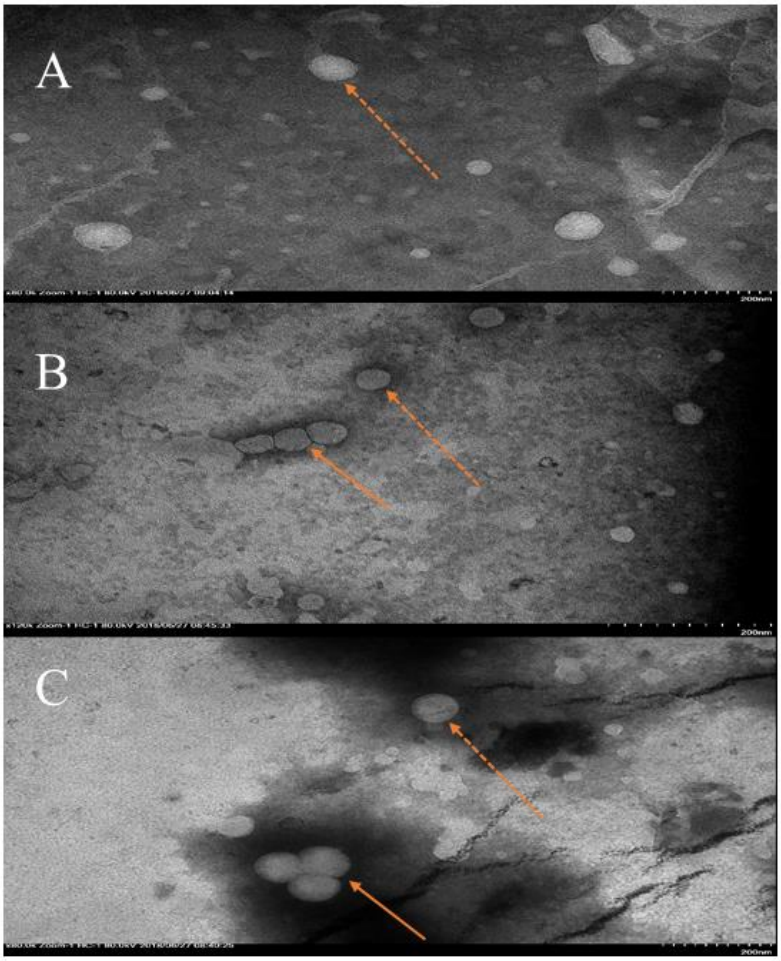

Figure 3.2. Transmission electron microscopy analysis of liposomes prepared by; **A**. homogenization method, **B**. ultrasonication and **C.** Mozafari method. Sample of unilamellar vesicles are shown with a dotted arrow while aggregates are indicated by solid arrows. Scale bar in the figure A, B and C indicates 200nm.

3.4.2. Entrapment efficiency (%EE)

The developed LC-MS/MS method (representative chromatogram shown in **Figure 3.3**) was able to separate and quantify four PS (brassicasterol, campesterol, stigmasterol and β-sitosterol) and three tocopherols (alpha, gamma and delta). Both ultracentrifugation parameters and entrapment efficiencies were determined by analyzing bioactive using LC-MS/MS. The separation of the

liposomes during ultracentrifugation was time-dependent. Relatively low amounts of liposomes sedimented after 30 min (around 80% for all bioactives) of ultracentrifugation, whereas high sedimentation of liposomes was observed at 60, 90 and 120 min. There was no significant difference in sedimentation at 60, 90 and 120 min of ultracentrifugation. This supports the notion that after 60 min of ultracentrifugation at RPM 32,000 (G-force of 138000), a significant amount of liposomes was sedimented, leaving free bioactives in the supernatant.

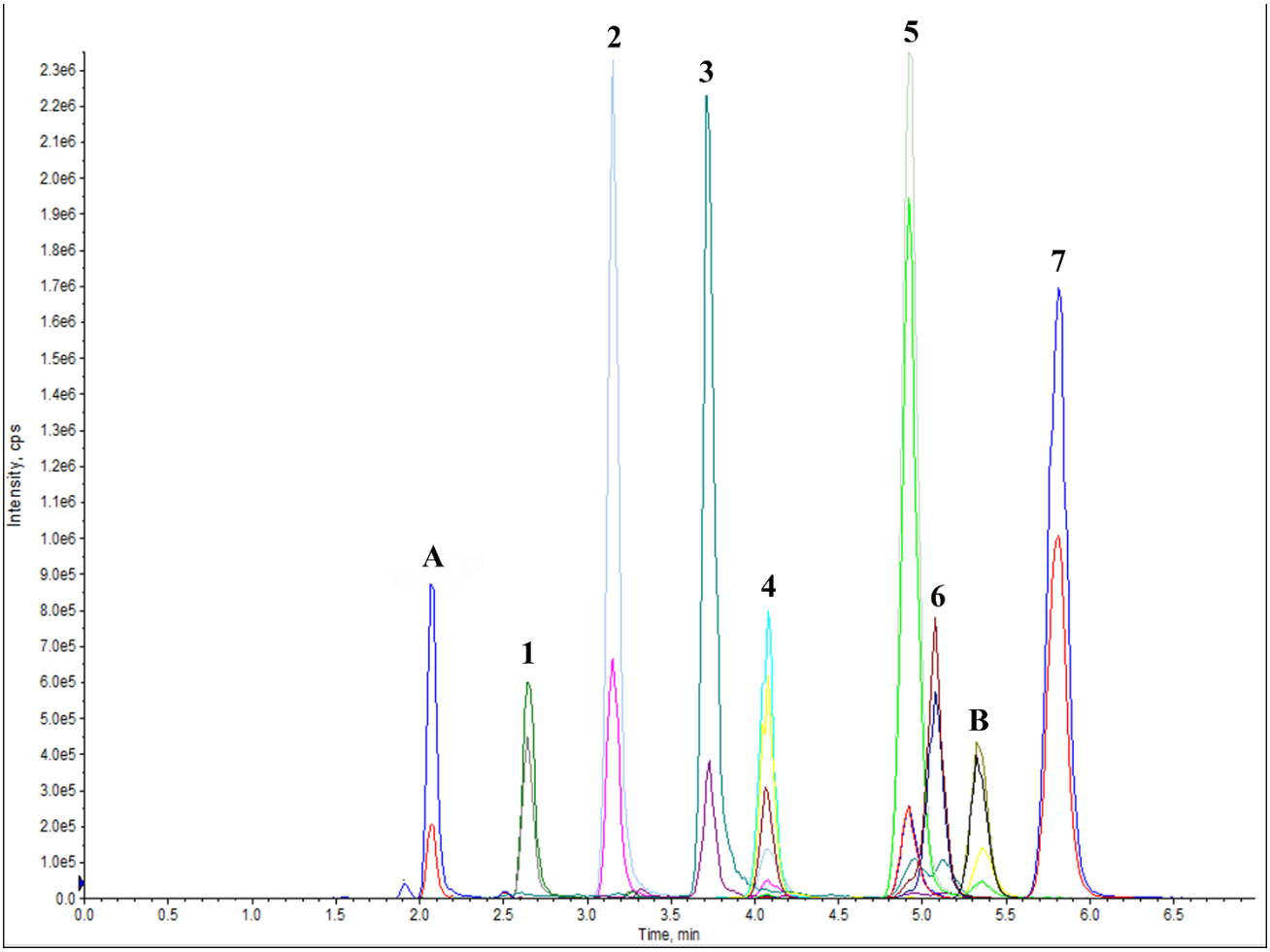

Figure 3.3. LC-MS/MS chromatogram of tocopherols: 1-δ tocopherol, 2-γ tocopherol, 3-α tocopherol; and phytosterols: 4-brassicasterol, 5-campesterol, 6-stigmasterol and 7-β-sitosterol. A-Rac tocol and B-cholestanol are internal standards.

The optimum entrapment efficiencies of PS and tocopherols into the liposomes obtained by the thin film hydration homogenization, thin film hydration ultra-sonication and Mozafari method is shown in **Table 3.2.** The results demonstrate that all three methods resulted in entrapment efficiency > 89% for PS and tocopherols. **Table 3.2** does not show any specific pattern in entrapment efficiency for bioactives. For example, in case of thin film hydration homogenization method, brassicasterol showed the highest entrapment efficiency among all PS; however, in the case of the Mozafari method, brassicasterol has the lowest entrapment efficiency. Similarly, the Mozafari method showed the highest entrapment efficiency for gamma tocopherols among all tocopherols. On the other hand, thin film hydration ultrasonication showed the lowest entrapment efficiency for gamma tocopherol. Thus, no concrete conclusion was obtained regarding entrapment differences between these bioactives. The entrapment efficiency of some of lipophilic compounds were reported to be almost close to 100% [69, 70]. However, **Table 3.2** shows EE in the range of 89–97% for various bioactives, evaluated in our work. It is possible that some of the liposomes were too small and failed to sediment during ultracentrifugation. This will lead to decreased EE (the amount of bioactives in the sediment were taken as a basis to calculate EE). Nevertheless, the obtained EE (shown in **Table 3.2**) is consistent with entrapment efficiency of nutraceuticals such as vitamin E, resveratrol and retinol specified in the literature [52, 60, 71]. High entrapment efficiency, that is, greater than 85% is considered economical for industrial application because it eliminates the cost of separating free and entrapped bioactives that will be required in case of low entrapment efficiency

Table 3.2. Entrapment efficiency of bioactives (PS and tocopherols) into liposomes prepared by the thin film hydration homogenization, thin film hydration ultra-sonication and Mozafari method expressed as mean ± standard deviation.

Methods	**Entrapment Efficiency (EE %)**					
	Brassicasterol	**Campesterol**	**β-Sitosterol**	**Alpha Tocopherol**	**Gamma Tocopherol**	**Delta Tocopherol**
Thin film hydration-Homogenization	95.9 ± 1.7	94.0 ± 2.2	94.8 ± 3.0	91.6 ± 2.4	90.5 ± 2.9	91.6 ± 3.6
Thin film hydration-Ultrasonication	91.5 ± 2.4	92.3 ± 3.4	90.1 ± 1.9	91.2 ± 2.1	89.8 ± 3.1	90.1 ± 2.3
Mozafari method	89.4 ± 2.8	93.7 ± 6.0	93.1 ± 6.0	92.3 ± 7.5	97.4 ± 1.9	95.3 ± 1.4

3.4.3. Effect of lyophilization on the physicochemical properties

Freeze-drying of liposomes resulted in the increase in particle size, reaching up to 500 nm (**Figure 3.4**). Various food compatible cryoprotectants such as sucrose, mannitol and lactose can be used to address this issue [72]. Thus, food compatible sucrose was tested as a cryoprotectant [51]. The addition of sucrose maintained the desired particle size (**Figure 3.4**). The lyophilized liposomes were then incorporated into model orange juice. Lyophilization is one of the crucial steps used for stabilization of liposomes [73]. It extends the shelf life of liposomes and can prevent thermosensitive bioactives from degradation [73].

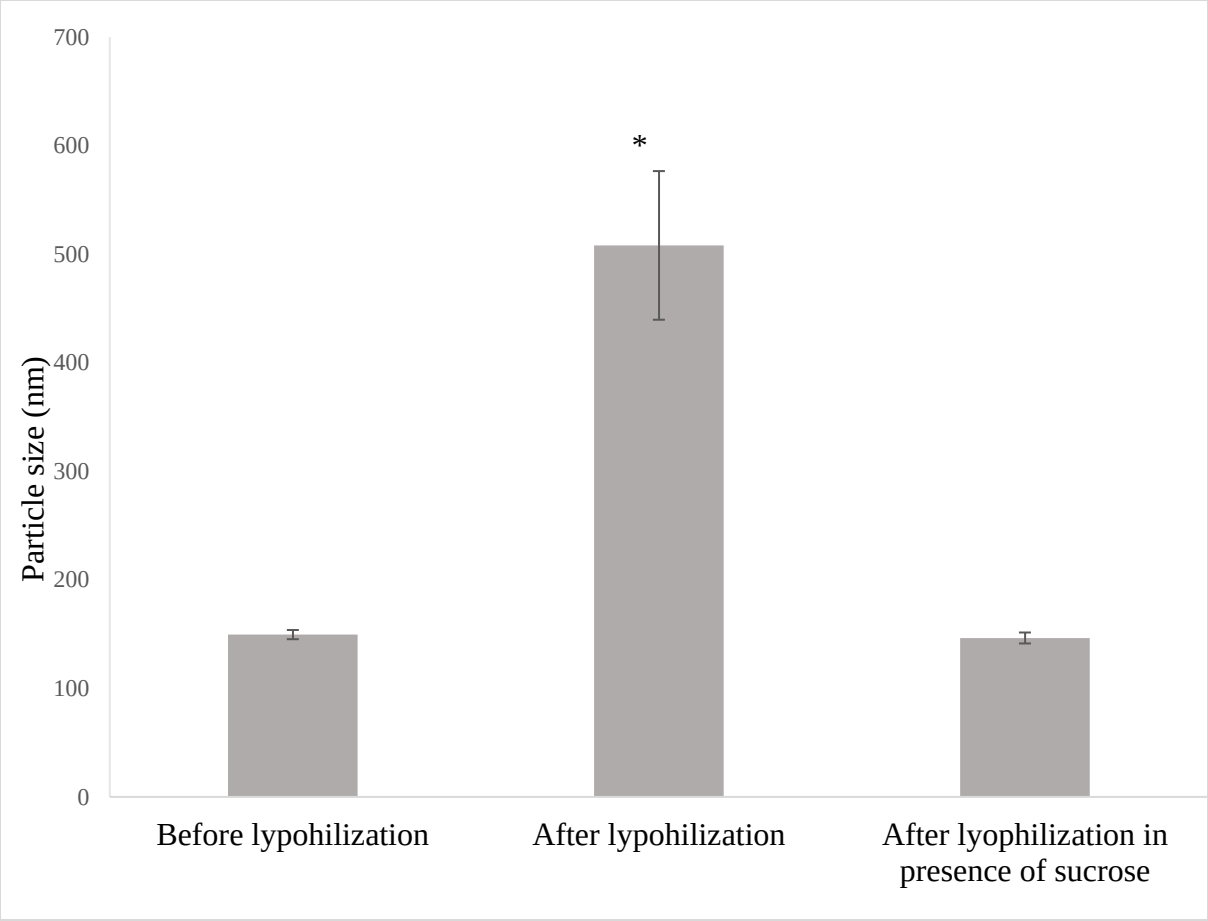

Figure 3.4. Effect of addition of sucrose as a cryo-protectant on particle size of liposomes before and after lyophilization expressed as mean ± standard deviation where * represents statistical significance (*$p < 0.05$) in particle size after lyophilization in comparison to before lyophilization and after lyophilization in presence of sucrose.

3.4.4. Chemical stability studies

Pasteurization technique did not compromise the stability of bioactives as shown in **Table 3.3**. There was no significant change in the LC-MS/MS response for pasteurized and non-pasteurized formulations, ranging from 0.5 to 2.59% (**Table 3.3**). This shows that exposure to temperature of 72 °C for short time of 15 s does not degrade bioactives entrapped within liposomes in the model juice

Table 3.3. Relative change in the concentration (represented by area under curve, AUC) of PS and tocopherol before and after pasteurization.

Bioactives	**AUC of Non-Pasteurized Bioactives**	**AUC of Pasteurized Bioactives**	**Percentage Relative Change in AUC (%) of Pasteurized and Non-Pasteurized**
Brassicasterol	5.63×10^{6}	5.60×10^{6}	0.53
Campesterol	2.32×10^{7}	2.26×10^{7}	2.59
β-sitosterol	3.40×10^{6}	3.37×10^{6}	0.88
α-tocopherol	2.76×10^{7}	2.72×10^{7}	1.45
γ-tocopherol	4.94×10^{6}	4.89×10^{6}	1.01
δ-tocopherol	4.84×10^{6}	4.73×10^{6}	2.27

3.4.5. Physical stability studies

Both pasteurized and non-pasteurized liposomes in model orange juice showed similar trend in particle size (**Figure 3.5**). This implies that high temperature in HTST pasteurization process did not compromise the stability of the liposomes. Further, particle size of vesicle did not change significantly during the one-month storage at 4 °C (**Figure 3.5**). This shows that liposomal orange juice can be stored in 4 °C for a month with adequate stability. Regarding zeta potential, unlike liposomes in purified water, liposomal model juice was found to have positive zeta potential in the range of 5.6–8.9 mV. Even though this zeta potential value is generally considered an indicator of instability to the colloidal system [66], liposomal model orange juice showed adequate storage stability. Probably, the optimized smaller vesicular size maintained the stability of particles preventing its aggregation.

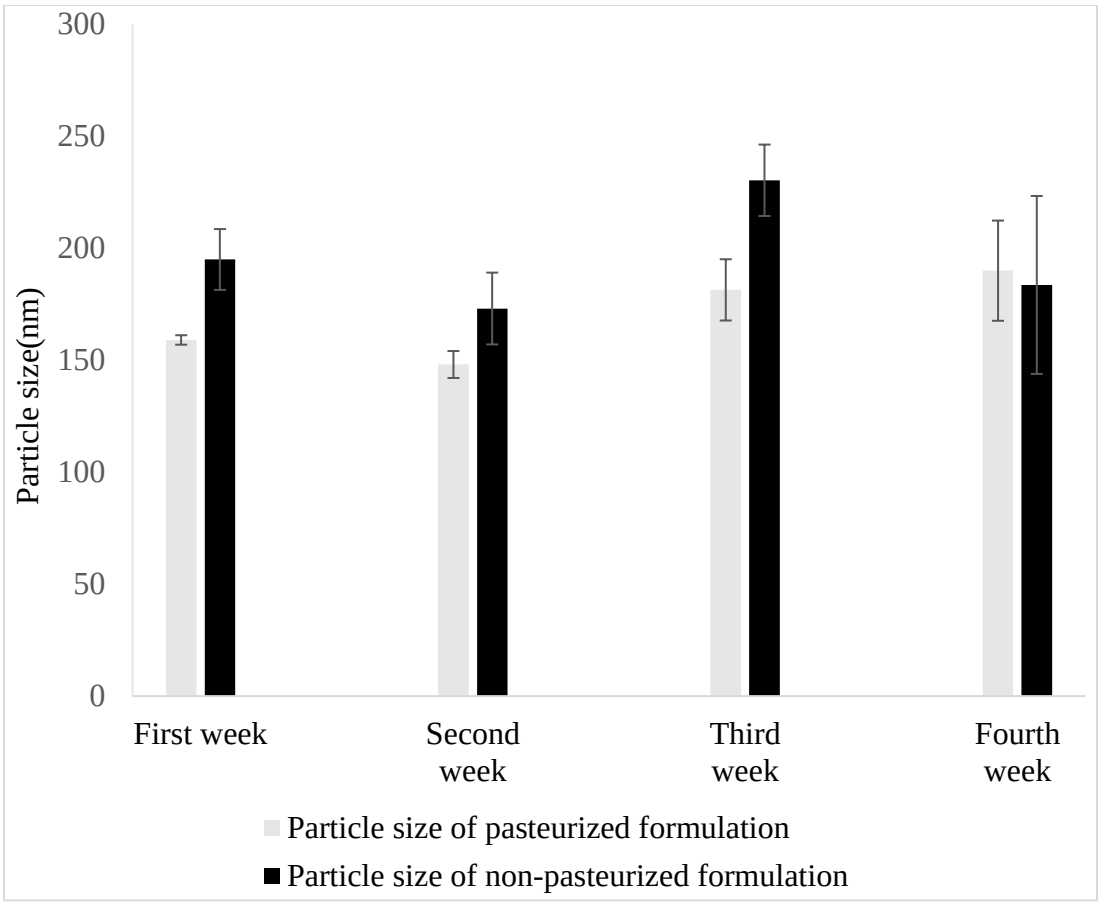

Figure 3.5. Particle size of pasteurized and non-pasteurized liposomes incorporated in model orange juice during storage period of one month at 4 °C expressed as mean ± standard deviation.

3.5. Conclusion

To address the lipophilicity, heat and light sensitivity challenges, unilamellar liposomes containing PS obtained from CODD and tocopherols were formulated and were fortified into a juice. Three different formulation approaches were employed and were compared for their suitability in formulating PS and tocopherols. All three methods showed optimum physicochemical properties and excellent entrapment efficiencies that were greater than 89%. Mozafari method was found to be simple and quick for formulating liposomes; however, the use of high temperature can possibly degrade thermosensitive bioactives. In addition, its low B/L ratio (not economical for scaling up) makes the Mozafari method less suitable method for PS in comparison to thin film hydration ultrasonication and thin film hydration homogenization method. Both ultrasonication and homogenization seemed to be equally suitable at a laboratory scale. At an industrial scale, however, the homogenization method is more feasible due to the availability of homogenizers of large capacity. Thus, thin film hydration-homogenization seems to be the best method for scaling-up the liposomal formulation containing PS and tocopherols. The pasteurization technique did not affect the chemical stability of tested bioactives. Moreover, model orange juice containing liposomes maintained an adequate physical stability during a period of one-month storage at 4 °C. In the future, liposomes containing PS will be tested for cholesterol-lowering efficiency by conducting animal and human trials.

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CHAPTER 4

Determination of phytosterols oxidation products in pharmaceutical liposomal formulations and plant vegetable oil extracts using novel fast liquid chromatography - tandem mass spectrometric methods

Asmita Poudel [1], George Gachumi [1], Randy Purves [1, 2], Ildiko Badea [1], and Anas El-Aneed [1]

[1] Drug Discovery and Development Research Group, College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, SK, Canada

[2] Canadian Food Inspection Agency, Saskatoon, SK, Canada

* to whom correspondence should be addressed: Dr. Anas El-Aneed, Phone: +1-306-966-2013, email: anas.el-aneed@usask.ca.

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Transitioning rationale

In the previous chapter, we developed a liposomal formulation containing phytosterols and tocopherols. However, it is crucial to assess whether the liposomes and antioxidant tocopherols can maintain oxidative stability of phytosterols, i.e., to prevent the generation of phytosterols oxidation products (POPs). The oxidized species of phytosterols, in particular, polar derivatives, could have negative health implications. Thus, the type and amount in the formulation needs to be determined to ensure the safety of the final product. This chapter (IV) aims to develop a robust LC-MS/MS in order to determine POPs in the liposomal formulations.

Contribution statement:

Asmita Poudel contributed to this paper by conducting experimental design, conducting experiment, data acquisition, data analysis and writing the manuscript.

4.1. Abstract

Phytosterols oxidation products (POPs) formed by the auto-oxidation of phytosterols (PS) can lead to negative health consequences. New liquid chromatography-tandem mass spectrometry (LC-MS/MS) quantitative and qualitative approaches were developed. For quantification, sixteen PS oxidation products (POPs) in liposomal formulations; namely 7-keto, 7-hydroxy, 5,6-epoxy, and 5,6-dihydroxy derivatives of brassicasterol, campesterol, stigmasterol, and β-sitosterol were quantified. The method has a short run time of five minutes, achieved on a Poroshell C18 column, using isocratic elution. To the best of our knowledge, this is the shortest run time among reported methods for the quantitative analysis of POPs. Atmospheric pressure chemical ionization (APCI) was used, and the mobile phase was composed of acetonitrile/methanol (99:1 v/v). The quantitative method was validated as per the FDA guidelines for linearity, accuracy, precision, selectivity, sensitivity, matrix effect, dilution integrity, and stability. The method was applied for the quantification of POPs in liposomal PS formulations prepared with and without tocopherols, as antioxidants. The formulation process had little impact on the formation of POPs as only 7-ketobrassicasterol was quantified in tested samples. The quantified value of POPs in liposomal samples was insignificant to impart any toxicological effects. Other degradation products such as 7-hydroxy, 5,6-epoxy and 5,6-dihydroxy derivatives of brassicasterol, campesterol and β-sitosterol were below the lower limit of quantification. PS-containing formulations were then assessed for their oxidative stability after microwave exposure for 5 minutes. The incorporation of tocopherols

significantly increased the stability of PS in the liposomal formulations. Finally, LC-MS/MS qualitative identification of PS obtained from extra virgin olive oil was performed. New POPs, namely 7-ketoavenasterol, and 7-ketomethylenecycloartenol were putatively identified, illustrating the applicability of the method to identify POPs with varying structures present in various PS sources. In fact, it is the first time that 7-ketomethylenecycloartenol is reported as a POP.

4.2. Introduction

Phytosterols (PS) are secondary plant metabolites, structurally analogous to cholesterol (**Figure 4.1**). They can be obtained from vegetable oil seeds, such as canola, soybean, olive, and sunflower [1, 2]. They are also abundant in the oil waste stream, termed the deodorizer distillate, which is obtained during the refining process of vegetable oils [1]. PS have been widely used as nutraceuticals due to their ability to reduce the levels of low-density lipoprotein cholesterol (LDL-C) in serum in the range of 8-10% [3-5]. Health Canada and the United States Food and Drug Administration (FDA) have approved PS as cholesterol-lowering agents at a daily oral dose of two grams [6, 7]. This has led to the emergence of numerous PS containing marketed products in the form of enriched foods and supplements. Some of the current food examples in the Canadian market include PS- enriched Minute Maid® orange juice, Yoplait® healthy heart yogurt, and Benecol® margarine.

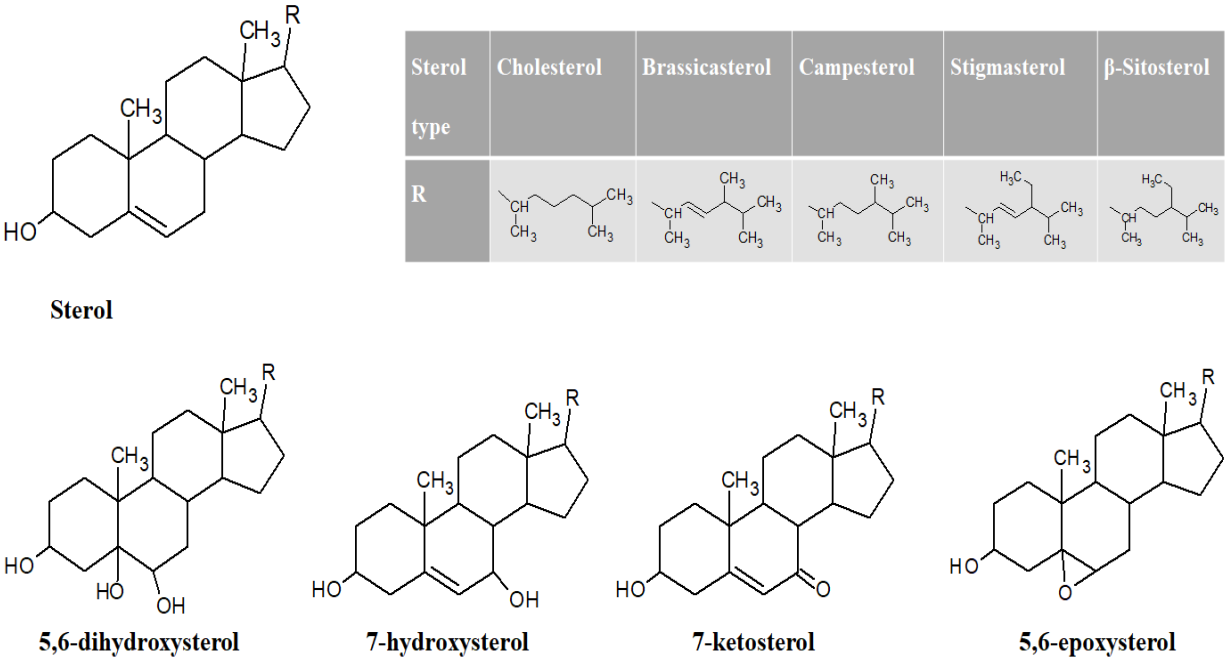

Figure 4.1. General structure of sterols and oxidized derivatives. The table shows common PS structures along with cholesterol. Polar sterol oxidation products are shown; 5,6-dihydroxysterol, 7-hydroxysterol, 7-ketosterol, and 5,6-epoxysterol.

Unfortunately, PS tend to undergo auto-oxidation in the presence of heat and light, leading to the generation of various phytosterols oxidation products (POPs) of varying polarities [8, 9]. The most abundant POPs generated from PS are polar, such as 7-keto, 7-hydroxy, 5,6-epoxy, and 5,6-dihydroxy sterol (**Figure 4.1**) [10-12]. Non-polar derivatives such as 3,5-dienes and 4,5-diene-3-one [9] are also formed, albeit at a lower extent. The above-mentioned POPs are formed primarily by oxidation of PS at their steroid ring. The occurrence of POPs in PS-enriched food has been widely reported [13-17]. For instance, 3.4 mg of POPs per 100 grams of food were present in PS-enriched margarine [18]. Similarly, PS-enriched orange juice contained 0.8 mg of POPs per 100 grams [17]. POPs in food products can cause negative health consequences due to possibly their

pro-atherogenic and pro-inflammatory effects, evident from animal studies [19-21]. Screening and quantifying POPs is required to ensure the safety of the final food or supplement products, especially polar POPs due to their high prevalence and their higher proatherogenic properties in comparison to non-polar POPs, as illustrated in preclinical studies [12, 22].

Gas chromatography (GC) coupled with mass spectrometer (MS) or flame ionization detector (FID) has been routinely utilized for the analysis of POPs in food [11, 23, 24]. For instance, 5,6-epoxy, 7-hydroxy, and 7-ketosterol in PS enriched margarine were quantified using GC-FID [23]. Similarly, GC-MS was used to quantify 5,6-epoxy, 7-hydroxy, 7-keto, and 5,6-dihydroxy derivatives of campesterol, stigmasterol, and β-sitosterol present in cooked and baked food products [24]. A comprehensive two-dimensional GCXGC-Time of Flight (TOF)-MS with enhanced separation power and sensitivity was developed in order to quantify 7-hydroxy and 7-keto PS present in human serum [11]. Despite their wide utility, GC-based analytical approaches remain tedious and time-consuming due to the required derivatization step.

Liquid chromatography (LC), on the other hand, does not require derivatization. Despite this, there are few reported LC methods that have been used for the quantification of POPs [9, 25, 26]. Saynajoki et al. [25] analyzed stigmasterol oxidation products using normal-phase LC combined with ultraviolet (UV) and fluorescence detectors. The drawback of such detectors is the lack of selectivity for co-eluting POPs; in addition, sterol oxidation products do not absorb UV light adequately [27, 28]. Thus, the use of MS can enhance selectively for LC-based POP analysis. LC-MS method using normal phase chromatography was developed for quantifying POPs of diverse polarities with a diol column that have intermediate polarity [9]. In addition, normal phase LC-MS was used to quantify stigmasterol oxidation products using unmodified silica column [29]. However, the run time of both methods was long, requiring 30 minutes of analysis [9, 29].

To shorten the run time, reverse phase chromatography LC-MS was used to analyze stigmasterol oxidation products with a run time of 11 minutes [26]. However, the method utilized electrospray ionization (ESI), which usually shows low ionization efficiency for hydrophobic compounds, such as POPs [30]. Alternatively, atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI) are better suited options for ionizing PS and their oxidation products [9, 30, 31].To the best of our knowledge, no study showed the utilization of reverse phase LC in conjunction with APPI or APCI.

In this work, a novel reverse-phase LC-APCI-MS/MS method was developed and validated to quantify 16 POPs obtained from brassicasterol, campesterol, stigmasterol, and β-sitosterol. The method had a run time of five minutes and was applied to quantify POPs in liposomal formulations consisting of PS and tocopherols (vitamin E) [32] under normal conditions or after microwave heat-mediated oxidation. Finally, the novelty of the method was demonstrated by identifying other POPs such as 7-ketomethylenecycolartenol and 7-ketoavenasterol in the extract of extra virgin olive oil. To the best of our knowledge, this is the first report identifying (putatively) 7-ketomethylenecycolartenol as a POP.

4.3. Materials and Methods

4.3.1. Reagents and Chemicals

7-keto cholesterol, 5α, 6α -epoxycholesterol, and 5α,6β-dihydroxycholesterol were obtained from Steraloids Inc. (Newport, RI, USA). 7α -hydroxy cholesterol and phosphatidylcholine (PC) was obtained from Avanti polar lipids (Alabaster, AL, USA). Deuterated (d7)-7-keto cholesterol was obtained from CDN isotopes (Pointe-claire, Quebec, Canada). β-sitosterol, campesterol, stigmasterol, and brassicasterol at 98% purity were purchased from Toronto Research Chemicals

(North York, Ontario, Canada). Tocopherols (liquid form), chloroform, acetonitrile (LC/MS grade), methanol (LC/MS grade), and ethyl acetate were purchased from Sigma Aldrich (Oakville, Canada). PS oxidation products were obtained by heating PS standards in an oil bath at 180°C for 180 minutes in a glass vial [12]. Canola oil deodorizer distillate (CODD) was a gift from LDM foods (Yorkton, Saskatchewan, Canada) while olive oil (Organic Extra Virgin, Terra Delyssa®) was purchased from a local supermarket.

4.3.2. Extraction of PS from CODD and unsaponifiable fraction containing PS from extra virgin olive oil (EVOO)

PS were extracted from CODD using previously described method [32]. For the extraction of unsaponifiable fraction from EVOO, 5 g of oil was dissolved in 1M potassium hydroxide, prepared in 95% ethanol. The mixture was refluxed for 1 hour at 75 °C. It was then cooled to room temperature and 50 mL of distilled water was added. Unsaponifiables were extracted three times with 50 mL heptane. The combined organic phase was washed with 10% ethanol until the washings were neutral to phenolphthalein. The organic phase containing the unsaponifiables was dried using anhydrous sodium sulfate followed by evaporation of heptane in a rotary evaporator (Buchi corp, DE, USA). The final residue was further dried under high vacuum using Trivac vacuum D4A (Leybold vacuum products Inc., PA, USA).

PS obtained from CODD were quantified using the previously developed LC-MS/MS method in our lab [30]. The identification of PS in EVOO was conducted using its established tandem mass spectrometric fingerprint detailed in our previously published work [31]. Finally, the CODD PS extract was utilized for the formulation of liposomes whereas EVOO phytosterols were further subjected to forced oxidation (detailed in section 4.3.7.3).

4.3.3. Preparation of liposomes

The preparation of liposomes was described previously [32]**.** Briefly, PS obtained from CODD, namely brassicasterol, campesterol, and β-sitosterol, and tocopherols (α, γ, and δ)) were solubilized together with phosphatidylcholine in ethyl acetate. The solvent was subsequently evaporated using rotary evaporator, which led to the formation of thin lipid film. Hydration of the lipid film was performed using milliQ water at 55 °C. The resulting pro-liposomes were homogenized in high-pressure homogenizer to obtain unilamillar liposomes [32]. A control set of the liposomal formulations was prepared without tocopherols. Empty liposomes were also prepared without PS and tocopherols in order to obtain the liposomal matrix required to prepare calibration and quality control standards. All three sets of liposomes were lyophilized to obtain a dried residue, which was re-constituted prior to use.

4.3.4. Liquid chromatography-mass spectrometry conditions

Agilent Acquity UPLC (Agilent Technologies, Mississauga, ON, Canada) system with an Agilent Poroshell C18 column (2.1 mm × 150 mm, 5 µm) protected by a guard column (2.1 mm × 4.7 mm, 2.7 µm) of the same packing material was utilized for chromatographic separation. The column temperature was set at 20 °C, and the injection volume was 2.5 µL. An isocratic elution of acetonitrile: methanol (99:1 v/v) was used at a flow rate of 0.8 mL/min. The detection and quantification were performed using an AB Sciex 6500 QTRAP® quadruple-linear ion trap (QqQ-LIT) mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source (AB Sciex, Concord, ON, Canada). APCI tandem mass spectrometric analysis (MS/MS) was employed in the positive ion mode. Interface parameters were set as follows: source temperature 380°C, curtain gas 35 psi, and nebulizer current 2.5 µA.

Cholesterol oxidation products were directly infused to optimize the ion source and compound dependent parameters for peak intensity and signal stability. The collision energy to induce the fragmentation of the analytes and the internal standards ranged from 24-34 V (**Table 4.1**)

Table 4.1. Precursor ions and product ions of 5, 6-dihydroxy, 7-hydroxy, 7-keto and 5, 6-epoxy derivative of cholesterol, brassicasterol, campesterol, stigmasterol, β-sitosterol and the internal standard (d7-7-ketocholesterol). Two isomers of the 5, 6 epoxy derivative, namely 5α, 6α and 5β, 6β, were found to elute at two different retention times in the optimized chromatographic conditions (detailed in section 3.1).

Compounds	**Oxidation Products**	**Retention Time (min)**	**Precursor ion (*m/z*)**	**Monitored fragment ions MRM (*m/z*)**	**CE (V)**
Cholesterol	5,6-dihydroxycholesterol	1.44	**385.3**	**367.3** 255.3	24 24
	7-hydroxycholesterol	1.72	**385.3**	**367.3** 255.3	24 24
	7-ketocholesterol	1.73	**401.3**	**383.3** 365.3	34 34
	5,6-epoxycholesterol	2.35 and 2.62	**385.3**	**367.3** 255.3	24 24
Brassicasterol	5,6-dihydroxybrassicasterol	1.22	**397.3**	**379.3** 255.3	30 28
	7-hydroxybrassicasterol	1.72	**397.3**	**379.3** 255.3	30 28
	7-ketobrassicasterol	1.69	**413.3**	**395.3** 377.3	33 32
	5,6-epoxybrassicasterol	2.29 and 2.54	**397.3**	**379.3** 255.3	30 28

Campesterol	5,6-dihydroxycampesterol	1.63	**399.3**	**381.3** 255.3	31 30
	7-hydroxycampesterol	2.02	**399.3**	**381.3** 255.3	31 30
	7-ketocampesterol	1.99	**415.3**	**397.3** 379.3	28 29
	5,6-epoxycampesterol	2.73 and 3.07	399.3	**381.3** 255.3	31 30
Stigmasterol	5,6-dihydroxystigmasterol	1.42	411.3	**393.3** 255.3	28 30
	7-hydroxystigmasterol	2.05	411.3	**393.3** 255.3	28 30
	7-ketostigmasterol	2.04	427.3	**409.3** 391.3	32 30
	5,6-epoxystigmasterol	3.83 and 3.89	411.3	**393.3** 255.3	28 30
β-sitosterol	5,6-dihydroxysitosterol	1.70	413.3	**395.3** 255.3	30 32
	7-hydroxysitosterol	2.34	413.3	**395.3** 255.3	30 32
	7-ketositosterol	2.31	429.4	**411.4** 393.4	31 31
	5,6-epoxysitosterol	3.18 and 3.58	413.3	**395.3** 255.3	30 32
Internal standard	d7-7-ketocholesterol	1.69	408.3	**390.3** 372.3	31 31

4.3.5. Calibration standard preparation

Due to the unavailability of commercial POP standards, cholesterol oxidation products (COPs) (**Figure 4.1**) were used as reference standards to prepare the calibration curves. This approach has been successfully used in the past due to the difficulty in obtaining authentic standards of POPs [23, 24, 33]. The individual stock solution of COPs (5,6-dihydroxysterol, 7-hydroxycholesterol, 7-ketocholesterol, 5, 6-epoxycholesterol) and the internal standard (d7-7-ketocholesterol) were prepared at a concentration of 1 mg mL^{-1} in chloroform. The working solution comprising all COPs were then prepared at a concentration of 50 µg mL^{-1} by diluting the stock solution of COPs with the required volume of acetonitrile. Internal standard (IS) working solution was prepared at a concentration of 100 µg mL^{-1}. Calibration standards (0.01 – 5 µg mL^{-1}) were prepared in liposomal matrix (empty liposomes) by spiking the required volume of working solution of COPs and internal standard (50 µL) and diluting it with acetonitrile. The final concentration of internal standard in calibrant was 2.5 µg mL^{-1}.

4.3.6. Method validation

The developed LC-MS/MS method was validated as per FDA guidance for bioanalytical method validation [34]. Validation parameters, namely linearity, selectivity, precision, repeatability, sensitivity, dilution integrity, matrix effect, and stability were assessed.

4.3.6.1. Linearity and Sensitivity

Linearity was assessed by plotting the calibration curve of COPs ranging from 0.05 to 5 µg mL^{-1}. The regression equations of the calibration curve were obtained using a least square regression model with a weighting factor of 1/x. For the calibration curve to be accepted, all the points in the

curve should lie within ±15% standard deviation of their nominal value except for the lower limit of quantitation (LLOQ) which can lie within ±20% standard deviation from its nominal value.

Limit of detection (LOD) and LLOQ were assessed to determine sensitivity. LOD was assessed at the concentration range of 0.001-0.05 µg mL^{-1}. The lowest detectable concentration with a signal to noise (S/N) ratio ≥ 3 was selected as LOD. On the other hand, LLOQ was selected as the lowest concentration with accuracy within a standard deviation of ±20% from nominal value.

4.3.6.2. Interday and Intraday accuracy and precision

Interday and intraday accuracy and precision were determined using four different concentrations of quality controls (QCs); LLOQ (0.05 µg mL^{-1}), low quality control (LQC: 0.15 µg mL^{-1}), middle quality control (MQC: 2.5 µg mL^{-1}), and high-quality control (HQC: 4 µg mL^{-1}). For intraday measurements, QC replicates (n=6) were analyzed in a single analytical run, whereas for interday day, QC replicates (n=6) were run on three consecutive days. The accuracy of LQC, MQC and HQC should lie within a standard deviation of ±15% from nominal value while LLOQ can be within ±20%. On the other hand, the precision of all QCs must be within a relative standard deviation of ±15% except for LLOQ, which can be within ±20%.

4.3.6.3. Dilution integrity

To determine dilution integrity, mixed stock of all COPs was prepared at a concentration of 50 µg mL^{-1}. This was followed by preparing 12 µg mL^{-1} concentration from the mixed stock. A test concentration of 2.5 µg mL^{-1} (5-fold dilution) and 1.5 µg mL^{-1} (8-fold dilution) in liposomal matrix was prepared from the 12 µg mL^{-1} solution by pipetting the required volume of analytes and diluting it with acetonitrile in the presence of internal standard. The dilution of 5 and 8 folds was carried out to mimic the expected dilution of the experimental sample.

4.3.6.4. Selectivity and matrix effect

A blank liposomal matrix was analyzed without the addition of the analytes of interest to assess if a blank sample interferes in the analysis of target analytes. Matrix effect was evaluated using LQC, MQC and HQC prepared with and without liposomal matrix, in the absence of the internal standard. The comparison of analytes response in both sets of QCs was made to evaluate matrix effect. Eq. (1) was utilized in the calculation of matrix effect:

$$Matrix\ Effect = \left(\frac{Analytes\ response\ in\ liposomal\ matrix}{Analytes\ response\ in\ neat\ solution}\right) * 100 \quad (1)$$

4.3.6.5. Stability studies

Unlike the conventional approach where stability studies are conducted using authentic standards or the target analyte, in this study, the application samples were utilized for stability assessment. Using COP standards may not represent the actual stability of the targeted POPs. Thus, an assessment using an application sample containing POPs can be conducted to truly evaluate the stability of the measured analyte. Such an assessment can also be beneficial in optimizing the storage conditions for liposomal formulations. Liposomal PS application samples were stored in -20 °C, 4° C, and benchtop for stability evaluation. After a month of storage, samples were analyzed using LC-MS/MS.

4.3.7. Method application

4.3.7.1 Quantification of POPs in liposomal formulation.

The validated method was applied for the quantification of POPs present in liposomal PS prepared with and without tocopherols. Freeze dried formulation were dissolved in chloroform, followed by diluting an aliquot with acetonitrile in the presence of the internal standard.

4.3.7.2. Quantification of POPs in microwaved liposomal PS (accelerated stability testing)

Liposomal PS prepared with and without tocopherols along with PS extract were subjected to microwave heating for 5 minutes at 1000 W [35]. Microwave heating is utilized as an accelerated stability testing [36]. The microwaved samples were then dissolved in chloroform, followed by diluting an aliquot with acetonitrile in the presence of internal standard and finally were analyzed using LC-MS/MS.

4.3.7.3. Identification of POPs obtained from extra virgin olive oil extract.

EVOO extract was screened for PS prior to identifying their oxidized derivatives. Our previously established MS/MS fingerprints and screening method of PS, was utilized [31]. Prior to the screening experiment, 5 mg of EVOO PS extract was dissolved in 1 ml chloroform, followed by further diluting 10 µL of the chloroform solution with acetonitrile to make a final volume of 1 mL. On the other hand, EVOO PS extract were heated in an oil bath at 180 °C for 180 min in a glass vial in order to generate POPs. Five milligrams of oxidized PS were dissolved in 1 mL of chloroform followed by diluting 100 µL of the solution with acetonitrile to a final volume of 1 mL.

Finally, both samples (PS extract of EVOO and its forced oxidized derivatives) were analyzed qualitatively using LC-MS/MS. Full scan and product ion scan MS was performed for PS and POPs using a SCIEX 6500 QTRAP® using MS parameters described in section 2.4. Diagnostic ions of PS and POPs were utilized for their profiling, as detailed in section 4.4.3.3. For exact mass measurement to confirm the identity of new POP isolated from olive oil, Thermo Fisher Q-ExactiveTM® Quadrupole – Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) was utilized. The mass resolution for the full scan was 140,000 at m/z 200. For the

MS/MS experiment, the instrument was set in parallel reaction mode (PRM) at mass resolution of 17,500 using the precursor ion at m/z 455.3889 and a normalized collision energy (NCE) of 30 V to produce the product ions.

4.4. Results and Discussions

4.4.1. Method development

Selection of reference and internal standards

The challenge in method development for POPs is the lack of commercial reference standards. Most reported methods have utilized cholesterol oxidation products (COPs) as reference standards [23, 24, 33] with only few studies using synthesized in-house reference POPs [37, 38]. In-house synthesis of POPs is time-consuming and non-economical. It is documented that the response factor (the ratio of analyte's signal to its concentration) of COPs is similar to that of POPs [39]; thus, they are suitable reference standards for the quantification of POPs [24, 39, 40]. In addition, COPs are commercially available. Therefore, COPs were selected as reference standards in this study. Internal standards, such as deuterated 7-hydroxycholesterol and, deuterated 7-ketocholesterol have been widely used for the analysis of sterol oxidation products [11, 24, 39, 41]. Accordingly, a deuterated derivative of 7-ketocholesterol, d7-7-ketocholesterol, was utilized as an IS for both COPs and POPs.

MS detection

ESI has low ionization efficiency for hydrophobic compounds; thus, it is not ideal for the analysis of POPs and COPs [31]. On the other hand, APCI can be successfully utilized for the analysis of non-polar compounds, such as PS and their oxidation products [31]. Therefore, APCI was selected

as the ideal ionization source. Commercial COPs and POPs, obtained via forced oxidation of PS, were utilized for MS optimization.

Two multiple reaction monitoring (MRM) transitions were used for the analysis of target analytes (Table 1). The in-source loss of one water molecule was observed during the ionization of 7-hydroxy and 5,6-epoxysterol, as shown in **Figure 4.2** and **Appendix II, Table S4.1**. Both 7-hydroxy and 5,6 epoxy derivatives were ionized by forming $[M + H - H_2O]^+$ ion whereas, 5,6-dihydroxy (triol) derivative showed a loss of two water molecules, $[M + H - 2H_2O]^+$ as shown in **Appendix II, Table S4.1**. The precursor ions of these derivatives lost an additional water molecule during CID-MS/MS analysis, forming $[M + H - 2H_2O]^+$ for 7-hydroxy and 5,6-epoxy whereas $[M + H - 3H_2O]^+$ was formed for 5,6-dihyrodroxy derivative as an abundant product ion, which were used as quantifier ions. The second most abundant product ion that was monitored as a qualifier ion was formed by the loss of the side chain from the steroid ring at carbon 17 as shown in **Appendix II, Table S4.1**, which can be considered diagnostic elimination for the tested molecules.

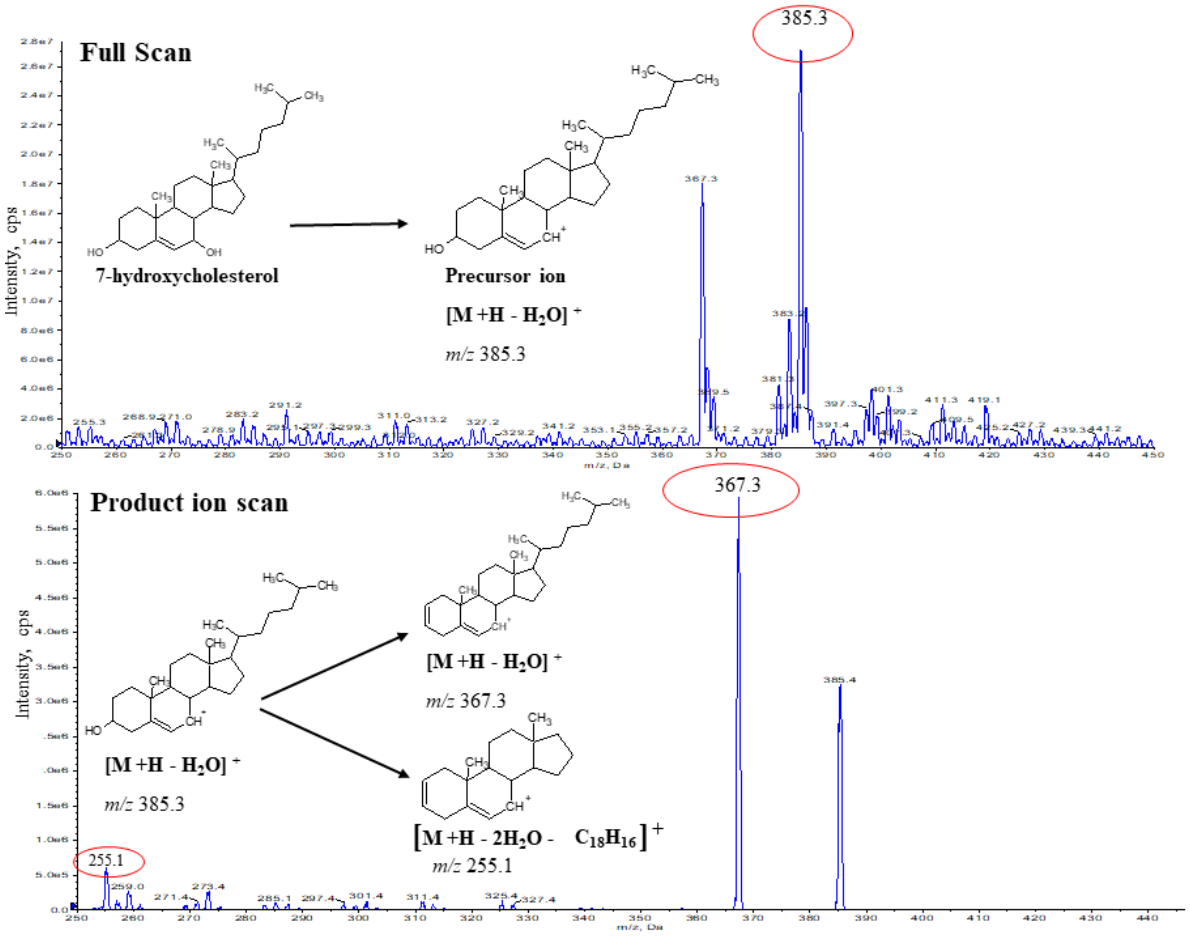

Figure 4.2. (A) Full scan MS analysis of 7-hydroxycholesterol (as a representative structure of cholesterol oxidation products) showing the formation of $[M+H-H_2O]^+$ ion and (B) the product ion scan showing the selected quantifier and qualifier ion (circled).

Unlike the above three COPs, the ionization of the 7-keto derivative, proceeded by formation of a protonated ion $[M + H]^+$ without the loss of water molecule (**Appendix II, Table S4.1)**. This suggests that 7-keto derivatives are more stable than 7-hydroxy, 5,6-epoxy and 5,6-dihydroxy derivatives. The designated $[M + H]^+$ ion of 7-ketosterol loses one or two water molecules, yielding the ions designated as $[M +H - H_2O]^+$ and $[M + H - 2H_2O]^+$ during collision induced dissociation (CID)-MS/MS. The former ion was utilized as the quantifier ion while the latter was

used as the qualifier ion **(Appendix II, Table S4.1)**. Similar ionization and MS/MS fragmentation behavior of COP and POPs have been reported [9, 42-44].

The MRM transitions of both POPs and COPs is shown in **Table 4.1**. 7-ketosterol showed unique MRM transitions, whereas; 5, 6-dihydroxysterol, 7-hydroxysterol, and 5, 6-epoxysterols showed the same MRM transitions (**Table 4.1**). However, they were all chromatographically separated, and the retention times were used for identification.

Chromatographic separation

Reverse-phase chromatography with a C18 column was utilized for the separation of COPs and POPs. Acetonitrile/methanol 99:1(v/v) was used as the mobile phase. The optimization of the mobile phase was done with and without 0.1% acetic acid as an additive. The presence of the acid did not show any significant effect on the ionization of POPs (data not shown). Thus, mobile phase solvent without an acid was utilized. Chromatographic separation of target analytes is shown in **Figure 4.3 I**. A co-elution of 7-keto and 7-hydroxycholesterol at a retention time of 1.72 min is observed. However, the co-eluting compounds have different MRM transitions, allowing for their proper identification and quantification.

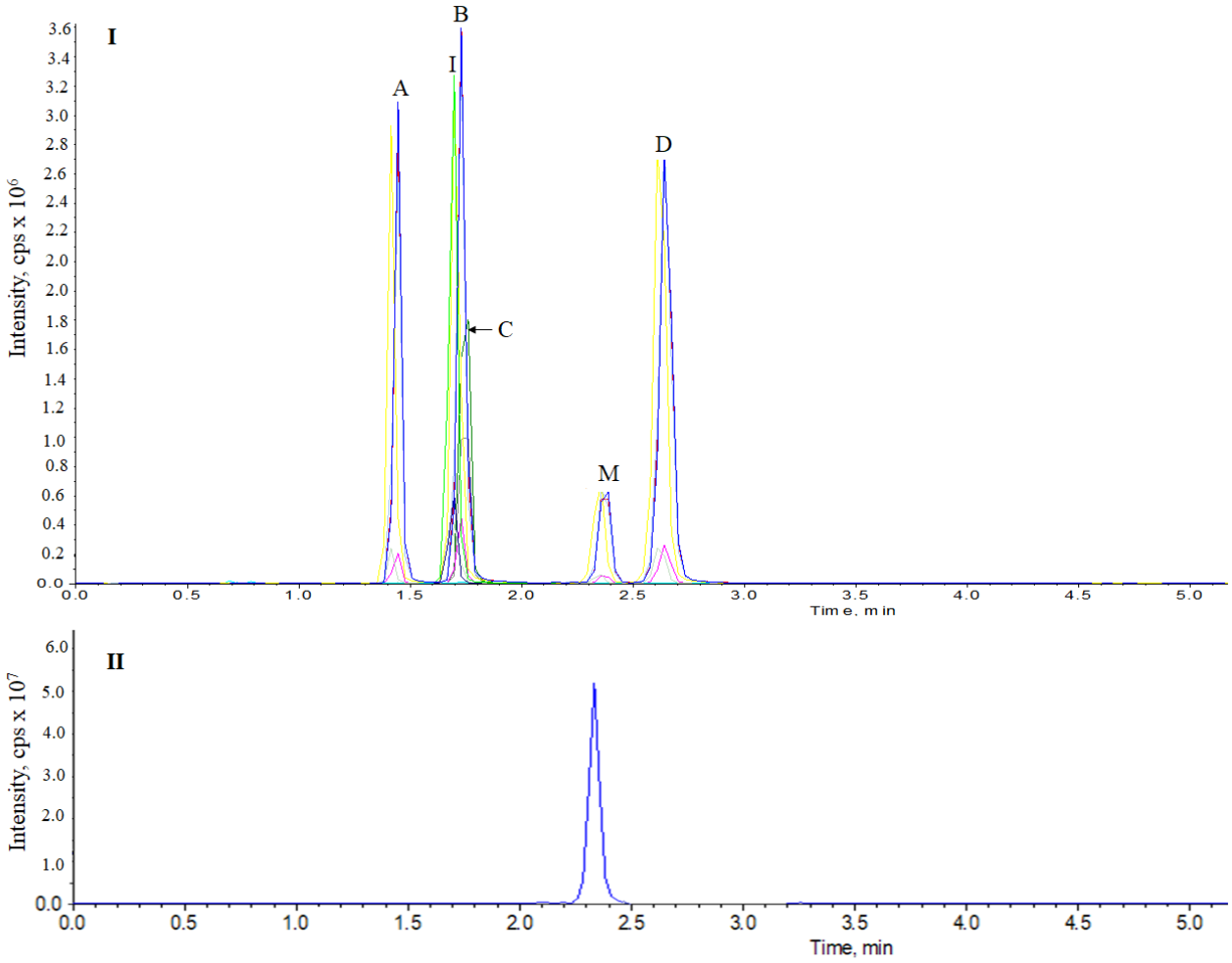

Figure 4.3. (I) Chromatographic separation of cholesterol oxidation products; 5,6-dihydroxycholesterol (A), 7-hydroxycholesterol (B), 7-ketocholesterol (C) and 5,6-epoxychoelsterol (D) along with internal standard d7-7-ketocholesterol (I). M represents the minor peak of 5,6-epoxycholesterol. (II) Chromatographic separation of 5β, 6β-epoxycholesterol eluting at the same time as that of peak M, confirming that the peak M is β derivative of epoxy cholesterol.

The analytes, 5,6-dihydroxycholesterol, 7-hydroxycholesterol, and 5, 6 epoxy cholesterol with the similar MRM transitions were well-resolved from each other at a retention time of 1.45 min (peak A), 1.73 min (peak B), and 2.64 min (peak D), respectively (**Figure 4.3 I**). On the other hand, two different peaks corresponding to the quantifier ion 385.3→367.3 and qualifier ion 385.5→255.3 of 5, 6-epoxycholesterol were observed at the retention time of 2.40 min (M peak) and 2.60 min (D peak) (**Figure 4.3 I**). Both peaks showed the same MS/MS fragmentation pattern (data not shown). Also, the two peaks were present when 5, 6-epoxycholesterol was injected alone.

The epoxy derivative of cholesterol exists in two stereoisomeric form; 5α,6α-epoxycholesterol and 5β,6β-epoxycholesterol (**Appendix II, Figure S4.1)** [45]. The presence of one isomer as an impurity in the reference standard of another isomeric form may be the reason for the appearance of two distinct peaks. To confirm this hypothesis, 5β,6β-epoxycholesterol reference standard was purchased. As expected 5β,6β-epoxycholesterol eluted at 2.4 min (**Figure 4.3 II**), confirming the identity of the observed minor peak. Thus, the peak (M) most likely represents 5β,6β-epoxycholesterol.

POPs showed similar chromatography pattern for their oxidation products to that of cholesterol (**Figure 4.3**). For example, extracted ion chromatogram of brassicasterol oxidation products is shown in **Figure 4.4,** and that of campesterol, stigmasterol, and β-sitosterol oxidation products are shown in **Appendix II, Figures S4.2- S4.4**. As expected, the elution order of POPs is the same as that of COPs. However, two major abundant peaks of 5β,6β-epoxy PS, and 5α,6α-epoxy PS for POPs were observed as shown in **Figure 4.4**, unlike COP which contained a minor peak of 5β,6β-epoxycholesterol**.** This is expected since POPs obtained by forced oxidation of PS can contain both isomers in similar quantities, unlike pure standard of COP where one isomer was present as an impurity in the standard of another isomer. To accurately represent the total amount of 5, 6-epoxy

derivatives present in the sample, both peaks were integrated during its quantification. The retention time (RT) of individual POPs and COPs are shown in **Table 4.1.**

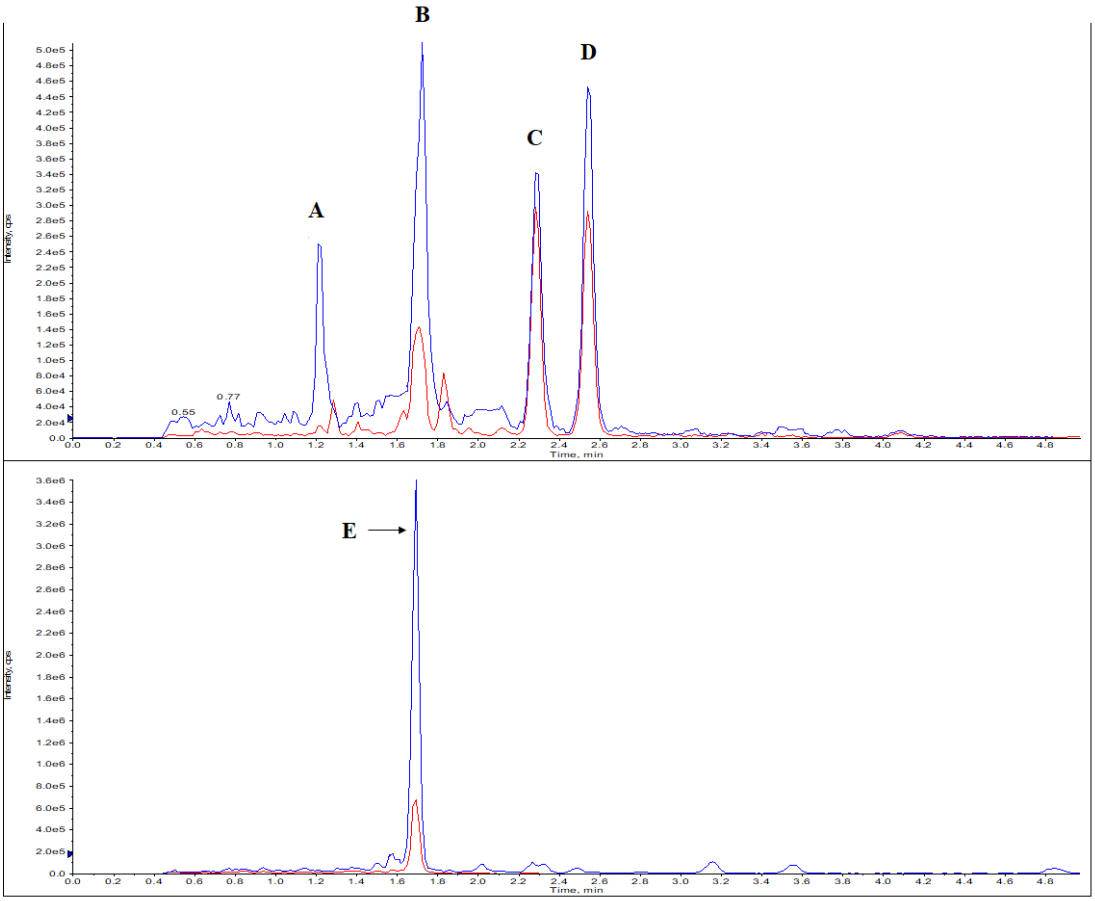

Figure 4.4. Extracted ion chromatogram of brassicasterol oxidation products; 5,6-dihydroxybrassicasterol (A), 7-hydroxybrassicasterol (B), 5,6-epoxybrassicasterol (C is 5β,6β-epoxy and D is 5α,6α-epoxy), and 7-ketobrassicasterol (E). A, B, C and D shares the same MRM transitions so they appear together in an extracted ion chromatogram. It is only E that have unique MRM transitions.

4.4.2. Method validation

4.4.2.1. Linearity and sensitivity

All COPs showed excellent linearity at the concentration range from 0.05 to 5 µg mL^{-1}, with correlation coefficient (R) for all the analytes > 0.99 (**Table 4.2**). LODs for 7-hydroxycholesterol and 7-ketocholesterol were 0.005 µg mL^{-1} while the LODs for 5,6-dihydroxycholesterol and 5,6-epoxycholesterol were 0.01 µg mL^{-1}. LLOQ for all analytes was 0.05 µg mL^{-1}.

Table 4.2. Linear regression parameters of cholesterol oxidation products along with the limit of detection and lower limit of quantification.

Cholesterol oxidation products	**Concentration range (µg mL^{-1})**	**Regression equation**	**Correlation coefficient (R)**	**LOD (µg mL^{-1})**	**LLOQ (µg mL^{-1})**
5,6-dihydroxycholesterol	0.05 - 5	y = 0.857x + 0.000136	0.99	0.01	0.05
7-hydroxycholesterol	0.05 - 5	y = 1.17x + 0.0123	0.99	0.005	0.05
7-ketocholesterol	0.05 - 5	y = 0.821x + 0.0122	0.99	0.005	0.05
5,6-epoxycholesterol	0.05 - 5	y = 1.52x + 0.00695	0.99	0.01	0.05

4.4.2.2. Repeatability: interday and intraday accuracy and precision

Interday and intraday accuracy and precision were evaluated by analyzing QCs at four concentrations, LLOQ, LQC, MQC, and HQC, as shown in **Appendix II ,Table S4.2.** Overall, all samples met the USFDA guidelines for precision and accuracy [34]. In the case of intraday, about 93% of QCs showed accuracy within ±10% standard deviation from the nominal value, whereas the remaining 7% were within ±15% standard deviation. The accuracy of 81% of interday QCs lie

within ±10% standard deviation while the remaining 19% were within ±15% standard deviation. Precision (coefficient of variation) of all the intraday and interday QCs was below 7.2%.

4.4.2.3. Dilution integrity

Dilution integrity was evaluated by determining the accuracy and precision of the diluted samples. The test sample after five- and eight-fold dilution showed accuracy within a standard deviation of ±6%, whereas precision lied within ±10% (**Appendix II, Table S4.3**). This demonstrates that diluting samples during POP analysis does not affect the accuracy of the quantitative results.

4.4.2.4. Selectivity and matrix effects

Selectivity evaluation using blank liposomal matrix did not show any interference of liposomal components with the MRM transitions of the analytes of interest, as demonstrated in chromatogram in **Appendix II, Figure S4.5**. This depicts that the liposomal matrix cannot overestimate the quantitative results. Matrix effects were evaluated at various levels of QCs (LQC, MQC, and HQC) showing a minor ion suppression. The mean matrix effect ranged between 92-94 %, as shown in **Appendix II, Table S4.4.**

4.4.2.5. Stability studies

The stability assessment using COP standards does not represent accurately the stability of the measured POPs. Therefore, in this method, stability was evaluated using an application sample containing POPs. Liposomal PS stored at -20 °C, 4 °C, and benchtop were analyzed after a month. **Appendix II, Table S4.5** shows the POP before and after 30 days of storage at various temperatures. As expected, POPs such as 7-ketocampesterol and 7-ketositosterol, which were initially found to be below the LLOQ, were quantifiable after a month of storage. However, there

was no significant increase in the amount of 7-ketobrassicasterol before and after the storage (**Appendix II, Table S4.5**). On the other hand, 7-hydroxy, 5,6-epoxy, and 5,6-dihydroxy derivatives of brassicasterol, campesterol, and β-sitosterol were below the LLOQ during the formulation stability assessment. Interestingly, there was no significant variation in the amount of POPs stored at various temperatures (20 °C, 4 °C, and benchtop). This could possibly be due to the short duration of storage. In fact, the quantity of POPs present in these samples is considered too insignificant to impart any possible toxicological effect (as detailed in section 3.3.1) [14, 46].

4.4.3. Method application

4.4.3.1. Quantification of POPs in liposomal formulation.

The LC-MS/MS method was applied to quantify PS oxidation products present in liposomal formulations. Even though the developed method can quantify oxidation products obtained from four different PS, only the oxidized derivatives of brassicasterol, campesterol, and β-sitosterol are expected to be in the test samples. This is because PS extraced from CODD abundantly contains brassicasterol, campesterol, and β-sitosterol, whereas stigmasterol is a minor constituent [47].

Liposomes prepared by thin-film hydration homogenization involves the utilization of heat (55° C) and pressure (60 psi). Both factors may theoretically generate reactive oxygen species that can possibly oxidize PS. Further, there may already be a presence of POPs in the purified PS extract prior to incorporation in the formulation. In this work, it is hypothesized that the optimum heating temperature and the pressure applied during the homogenization process will not generate a toxic level of POPs, as established in preclinical studies [14, 46]. It is also hypothesized that the presence of tocopherols will enhance oxidative stability of the PS in liposomal formulation (due to their antioxidant activities), thus further preventing the generation of POPs. In order to test these hypotheses, three different samples were analyzed: 1) PS extract, 2) liposomes containing PS, and

3) liposomes containing PS and tocopherols. 5, 6-dihydroxybrassicasterol, 5, 6-dihydroxycampesterol, and 5, 6-dihydroxysitosterol were not detected in any of these PS containing samples. We speculate two possible reasons for such finding-i) lower rate of formation of 5,6-dihydroxy derivative in comparison to other oxidation products, and ii) instability of 5,6-dihydroxy derivative. Other oxidation products, namely 7-hydroxybrassicasterol, 5,6-epoxybrassicasterol, 7-hydroxycampesterol, 7-ketocampesterol, 5,6-epoxycampesterol, 7-hydroxysitosterol, 7-ketositosterol, and 5,6-epoxysitosterol were all detected; however, they were present below the LLOQ. Only 7-ketobrassicasterol was quantified showing concentration in the range 10-12 µg per 10 mg of PS for all tested samples. Previously published work estimated the toxicological limit or no observed effect limit (NOEL) of POPs as 128 mg/kg body weight /day [14, 46]. In our work, POPs quantified in liposomes only show 0.03 mg/kg body weight/day (estimated at the daily PS dose of 2 grams) which is around 4200 times lesser than the NOEL of POPs. Thus, the developed liposomal PS is considered safe for human consumption.

It is noteworthy that the descending order abundance of PS extracted from the CODD is β-sitosterol > brassicasterol ≥ campesterol [47]. Therefore, the higher oxidation products can be expected from β-sitosterol in comparison to brassicasterol and campesterol. Surprisingly, our findings only quantified POP from brassicasterol. A possible explanation is that brassicasterol might have lower oxidative stability in comparison to β-sitosterol. The amount of 7-ketobrassicasterol present in PS extract, liposomal PS, and liposomal PS containing tocopherols were similar, indicating that the formulation process did not contribute to its generation. In fact, the finding obtained with 7-ketobrassicasterol can be theoretically extrapolated to other POPs detected below the LLOQ. The change in the quantity of 7-ketobrassicasterol during storage was statistically insignificant in all the samples, highlighting that the optimum liposomal formulation

parameters were able to maintain the oxidative stability of PS. However, the contribution of tocopherols in preventing the generation of POPs remained unknown. Its role in POP generation was investigated in the accelerated stability study (using microwave heating) of liposomal PS as described below.

4.4.3.2 Quantification of POPs in microwaved liposomal PS

To evaluate if liposomes can prevent the oxidation of PS, accelerated stability study was conducted using microwave heating [35]. It is noteworthy that phytosterols heating by both microwave and conventional oven showed a similar POP profile [48]. Also, the role of tocopherols in preventing the formation of POPs was assessed. Liposomal PS, liposomal PS containing tocopherols, and crude PS were comparatively evaluated for POP content upon microwave treatment. As expected, order of the POPs concentration in these samples from the lowest to the highest were liposomal PS containing tocopherol < liposomal PS < crude PS (**Table 4.3**). The finding suggests that the entrapment of PS in liposomes, even without antioxidants, can enhance the oxidative stability of PS. A possible explanation could be due to the prevention of direct exposure to reactive free radical upon liposomal entrapment [49]. In fact, similar protective effects of liposomes have been reported in the literature for thermo-sensitive natural compounds [50, 51]. However, best oxidative stability is obtained via the dual action of liposomes and the antioxidant activity of tocopherols (**Table 4.3)**. In sum, the formulation of PS in liposomes along with tocopherols prevents the generation of POPs and thus can enhance PS' oxidative stability. It should be noted that quantification in crude oil sample constitutes semi-quantification (i.e., fit-for-purpose) since the method was only validated for the formulation matrix that will be used clinically in the future. However, the substantial presence of POPs in the crude oil samples clearly shows the protective ability of liposomes and tocopherols.

Table 4.3. Phytosterol oxidation products (POPs) present in various phytosterols samples after microwave heating for five minutes expressed as mean ± standard deviation where. Different letters in each row donates statistical significance at $P < 0.05$.

Phytosterols oxidation products	**Crude PS (µg per 10mg of PS)**	**Liposomal PS (µg per 10mg of PS)**	**Liposomal PS and tocopherols (µg per 10mg of PS)**
5,6-epoxybrassicaterol	0.73±0.02[a]	0.930±0.0006[b]	<LLOQ
7-ketobrassicaterol	23.77±0.69[a]	17.71±0.13[b]	17.33±0.24[b]
7-hydroxybrassicaterol	6.39±0.08[a]	4.79±0.02[b]	0.06±0.018[c]
5,6-dihydroxybrassicasterol	2.20±0.02[a]	0.80±0.03[b]	<LLOQ
5,6-epoxycampesterol	2.46±0.03[a]	2.800±0.005[b]	<LLOQ
7-ketocampesterol	6.79±0.18[a]	4.06±0.12[b]	2.06±0.02[c]
7-hydroxycampesterol	6.00±0.11[a]	9.79±0.18[b]	1.27±0.02[a]
5,6-dihydroxycampesterol	3.80±0.06[a]	1.33±0.01[b]	<LLOQ
5,6-epoxysitosterol	3.90±0.04[a]	4.26±0.07[b]	<LLOQ
7-ketositosterol	7.86±0.28[a]	4.60±0.11[b]	2.73±0.04[c]
7-hydroxysitosterol	3.59±0.09[a]	0.600±0.007[b]	0.66±0.01[b]
5,6-dihydroxysitosterol	8.99±0.15[a]	7.46±0.04[b]	7.0±0.13[c]
Total	76.48±1.75[a]	59.13±0.72[b]	31.11±0.48[c]

4.4.3.3 Identification of POPs obtained from extra virgin olive oil extract

In order to assess the wide applicability of the developed method, it was also applied qualitatively, for identifying POPs in oxidized PS (i.e., forced oxidized) obtained from EVOO. Prior to such analysis, it is crucial to identify the PS profile of EVOO so that its oxidized derivatives can be predicted allowing for the possibility of identifying POPs that may exist in EVOO. The previously established MS/MS fingerprint of PS in our lab [31] was utilized for profiling PS in EVOO extract. In addition, reported PS profile in EVOO [52, 53] was used in order to obtain additional confirmation during the identification process of POPs. Theoretical MS/MS product ions of PS that can possibly be present in EVOO are shown in **Appendix II, Table S4.6**. The analysis revealed the possible presence of three major sterols, namely campesterol, stigmasterol, and β-sitosterol along with avenasterol and 24-methylenecycloartenol as shown in the LC-MRM-MS/MS chromatogram **(Appendix II, Figure S4.6).** Briefly, campesterol, stigmasterol, and β-sitosterol were putitavely identified using the following MRM transitions, i) 383.4 → 161.1 and 147.1, ii) 395.4 → 83.2 and 297.4, and iii) 397.4 → 135.1 and 255.2 respectively (**Appendix II, Table S4.6)**. Avenasterol, on the other hand, shows the same *m/z* values as that of stigmasterol, as both of these sterols structurally differ only in the position of the double bond at the side chain. Nevertheless, they elute at different retention times, as shown recently [31]. Thus, retention time along with *m/z* values was utilized in distinguishing between avenasterol and stigmasterol (**Appendix II, Figure S4.6)**. Methylenecycloartenol, on the other hand, was identified using the theoretical *m/z* 423.3 of the precursor ion along with its diagnostic product ions at *m/z* 169, 147, 161, and 215 [31]. Similar PS profiles in EVOO was reported in the literature [52].

After extraction, PS of EVOO were subjected to forced oxidation and screened for the presence of POPs. Theoretical *m/z* values for the precursor ions and diagnostic product ions were utilized

in identifying the PS and their oxidized derivatives. The oxidation products of campesterol, stigmasterol, and β-sitosterol were detected in the same way as that of CODD extract using its diagnostic product ion (2 MRM transitions were utilized as shown in section 3.1). In addition, new POPs, namely 7-ketoavenasterol, and 7-ketomethylenecycloartenol were recognized as shown in **Figure 4.5**. 7-ketoavenasterol shares the same MRM transitions (427.3 → 409.3 and 427.3 → 391.3) with 7-ketositgmasterol, thus retention times (1.72 min) was utilized to confirm its identity. Regarding 7-ketomethylenecycloartenol, it is represented with theoretical *m/z* 455, and its diagnostic product ions at *m/z* 437, 419 and 409 which was obtained by the loss of one and two water molecules , along with side chain (propyl group) respectively **Figure 4.5**. As seen in **Figure 4.5 II**, only the MRM 455 → 409 was observed whereas the second transition 455 → 437 has low abundance. However, MS/MS anlayis (**Figure 4.5 III)** confirmed that the eluted ion is indeed the POP designated as 7-ketomethylenecycloartenol. To confirm the idenity of the new POP, accurate mass measurement using quadrupole-orbitrap instrument was conducted to verify the presence of 7-ketomethylenecycloartenol. LC-MS full scan analysis showed the presence of the 7-ketomethylenecycloartenol at *m/z* 455.3886 (theoretical *m/z* 455.3884), with mass accuracy of 0.44 ppm (Figure 6). Product ion scan was then conducted (**Figure 4.6**) where product ion with theoretical *m/z* 437. 3778 was identified (mass error < 10 ppm). Mass tolerance of 5 ppm is advisable to identify analytes in full scan mode whereas in the case of product ion scan mass tolerance can be up to 20 ppm [54].

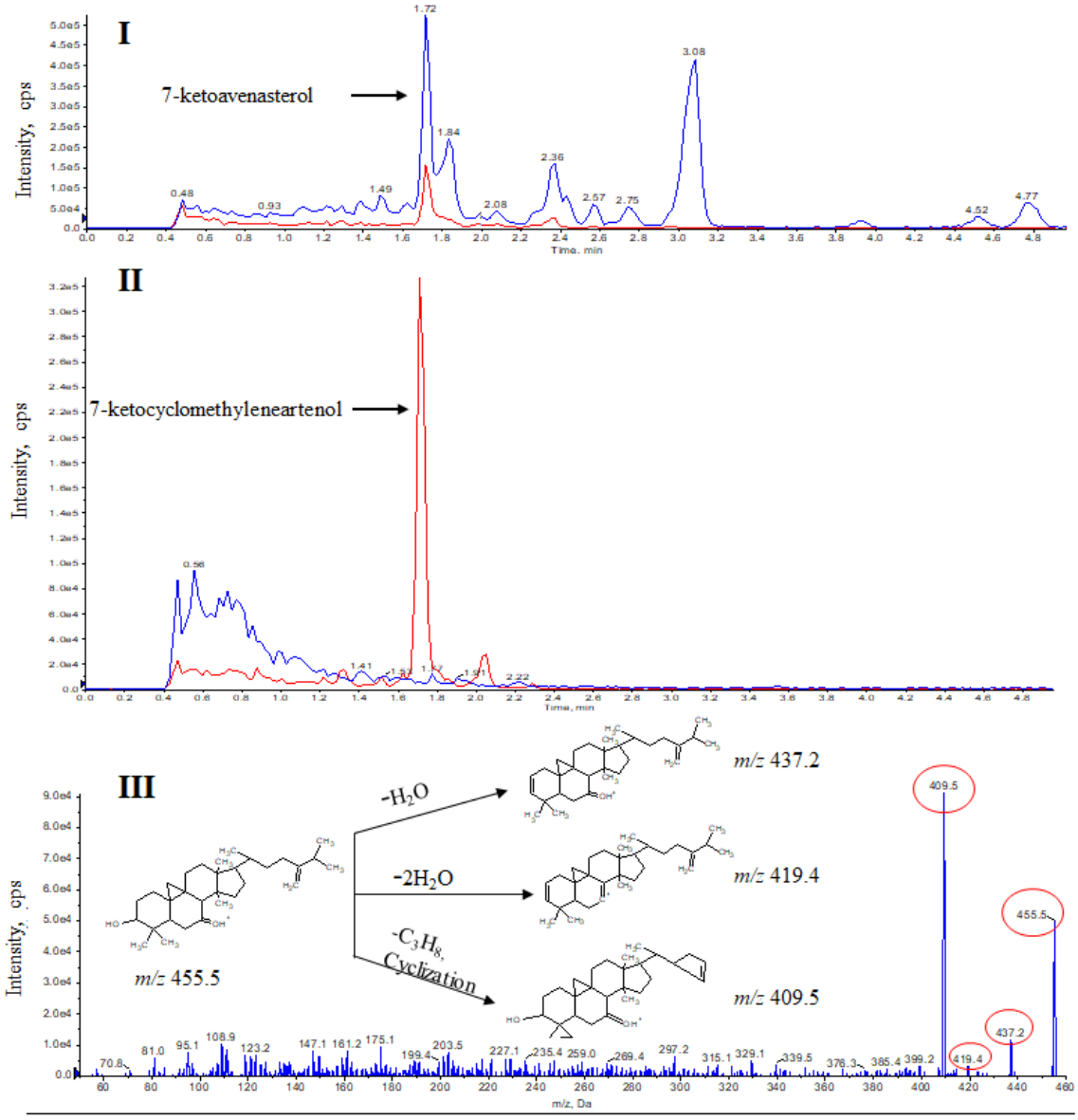

Figure 4.5. (**I-II)** LC-MRM chromatogram showing POPs of extra virgin olive oil; 7-ketoavenasterol [2 MRMs, *m/z* 427.3 → *m/z* 409.3 (blue) and *m/z* 427.3 → *m/z* 391.3 (red)], and 7-ketocyclomethyleneartenol [2MRMs, *m/z* 455.3 → *m/z* 409.5 (red) and *m/z* 455.3 → *m/z* 437.2 (blue)] identified in PS extract of virgin olive oil. The PS extract was underwent forced oxidation. (**III**) Product ion scan of 7-ketocyclomethyleneartenol showing diagnostic product ions.

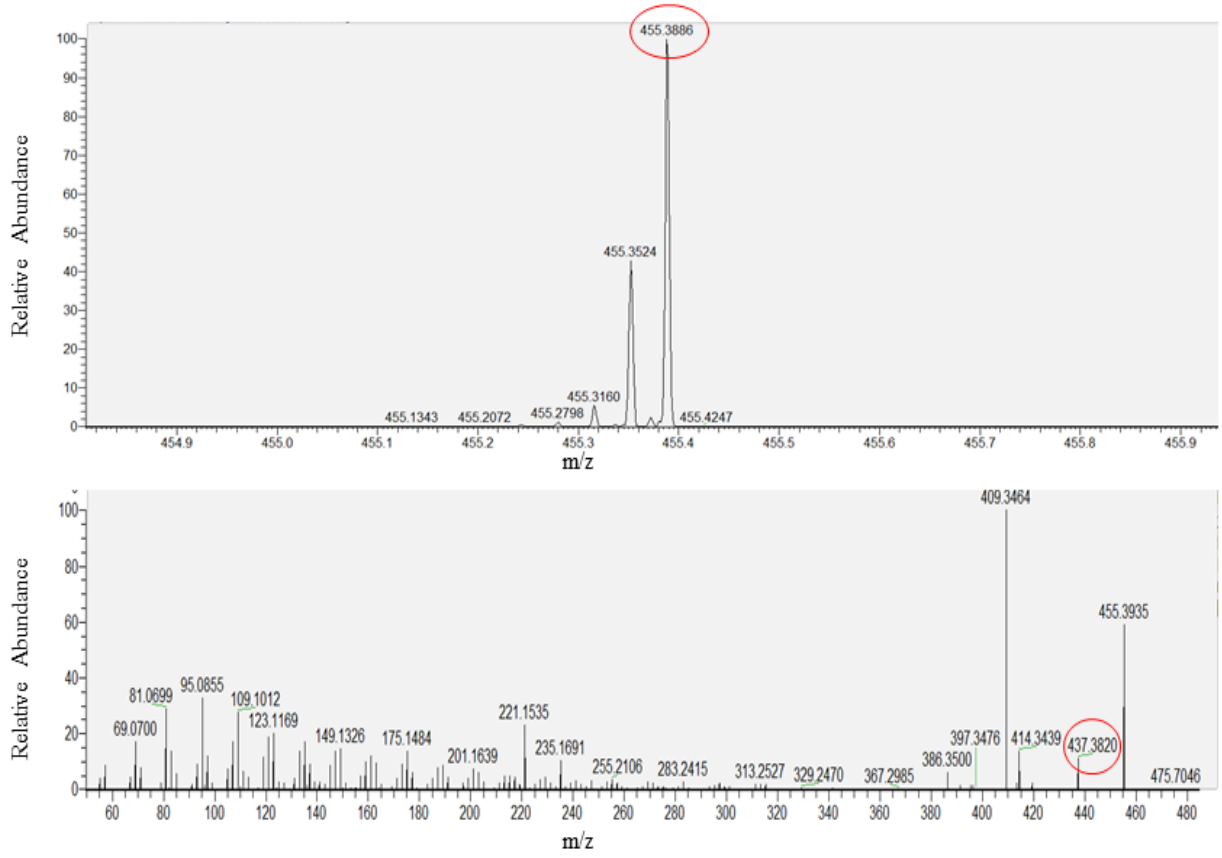

Figure 4.6. Exact mass measurement using quadrupole-orbitrap instrument confirmed the presence of 7-ketocyclomethyleneartenol at 455.3886 (top panel). Similarly, MS/MS analysis (bottom panel) was similar to the data obtained with the quadrupole linear ion trap, including the presence of the ion observed at 437.3778.

To the best of our knowledge, only one investigation reported the presence of 7-ketoavenasterol in olive oil [55]. On the other hand, it is the first time that 7-ketomethylenecycloartenol is putatively identified in extra virgin olive oil. The above example illustrates that the developed method can be applied for the analysis of an array of PS oxidation products from various sources.

4.5. Conclusion

LC-APCI-MS/MS method was developed and validated for the quantification of sixteen POPs using reversed-phase chromatography. The developed method requires five minutes run time making it the shortest reported methods for POP analysis. The existing shortcomings of POP quantitative methods such as longer run time due to NPLC, and tedious derivatization, in case of GC-MS, can be addressed using the newly developed reversed-phase LC-APCI-MS/MS method.

The method was successfully applied for the quantification of POPs present in liposomal PS under storage and accelerated stability studies. The wide utility of the method was also demonstrated for the qualitative identification of POPs present in extra virgin olive oil.

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4.6. References

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CHAPTER 5

Liposomal phytosterols as LDL-cholesterol lowering agents in diet-induced hyperlipidemia

Asmita Poudel [1], George Gachumi [1], Phyllis G. Paterson [1], Anas El-Aneed [1], Ildiko Badea* [1]

[1] College of Pharmacy and Nutrition, University of Saskatchewan, Saskatoon, SK, Canada

* to whom correspondence should be addressed: Dr. Ildiko Badea, Phone: +1-306-966-6349, email: ildiko.badea@usask.ca.

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Transitioning rationale

We developed and characterized a novel liposomal formulation containing phytosterols and tocopherols in the previous chapters. In addition, oxidative stability of the phytosterols was maintained in the formulation during the accelerated stability study. In this chapter (V), we aim to assess the LDL-cholesterol lowering ability of the liposomal phytosterols using a hamster model and compare it with the marketed phytosterols containing product and the control.

Contribution statement:

Asmita Poudel contributed to this paper by designing and conducting experiments, data analysis and writing the manuscript.

5.1. Abstract

High blood levels of low-density lipoprotein cholesterol (LDL-C) is a primary risk factor for cardiovascular disease. Plant sterols, known as phytosterols (PS), can reduce LDL-C in a range of 8-14%. The extent of LDL-C reduction depends on its formulation. Liposomes are one formulation strategy that can enhance PS efficiency. The PS, campesterol, stigmasterol, and β-sitosterol, have repeatedly been assessed alone or in combination for their LDL-C lowering ability. However, one naturally abundant PS, brassicasterol, has not yet been tested for its efficacy. We have previously developed novel liposomal formulations containing the PS mixture present naturally in canola that is composed of brassicasterol, campesterol, and β-sitosterol. In addition, tocopherols were added to the formulation to reduce oxidation. In this work, the efficacy of our novel liposomal PS formulation that includes brassicasterol was assessed. The formulation was delivered in two different vehicles, orange juice and water. Hamsters were selected as an animal model to assess PS efficacy. Animals were divided into five groups which received: i) liposomal PS in orange juice, ii) liposomal PS in water, iii) marketed PS in orange juice, iv) control orange juice, and v) control water. A high fat, cholesterol-supplemented (0.5%) diet was fed to hamsters to induce hypercholesterolemia. Animals were orally fed with the treatment and the control formulations for four weeks. Fasting blood samples were collected at baseline, week 2 and week 4. The extent of the reduction of total cholesterol, LDL-C, high density lipoprotein cholesterol (HDL-C), and triglycerides was compared among the groups. Liposomal PS in both orange juice and water significantly reduced LDL-C compared to their controls. Furthermore, liposomal PS was as effective as a marketed PS-containing product in reducing LDL-C. Both liposomal PS orange juice and liposomal PS water showed similar efficacy in LDL-C reduction, highlighting that these vehicles or food matrices do not affect the efficacy of PS. This is the first time that liposomal

formulation of a natural PS mixture extracted from canola oil with brassicasterol constituent, as a major component, has shown a significant effect in LDL-C reduction.

5.2. Introduction

Hyperlipidemia is one of the major risk factors for cardiovascular disease (CVD) [1, 2]. Increased serum low-density lipoprotein cholesterol (LDL-C) promotes the progression of atherosclerosis, which ultimately contributes to CVD [1]. Statin therapy is highly effective in reducing LDL-C up to 50% in hypercholesterolemic individuals [3]. Along with medication, dietary phytosterols (PS) have gained popularity as adjuvant therapy due to their well-established LDL-C lowering properties [4-6]. PS at a daily oral dose of two grams can reduce serum LDL-C in the range of 8-14% [7-11]. PS inhibits the intestinal absorption of cholesterol by competing with cholesterol for solubilization in bile salt micelles [12]. Numerous clinical studies have demonstrated that the extent of LDL-C reduction by PS is dependent on the way that it is formulated. For instance, PS formulated in a capsule (comprised of an oil suspension of phytosterols) did not reduce LDL-C significantly. On the other hand, micellar PS exerted a clinically significant reduction in LDL-C concentration by 14.3% in mildly hypercholesterolemic individuals [11, 13, 14]. In addition, a water-dispersible PS formulation composed of polysorbate as emulsifier and fatty acids enhanced the efficacy of PS by reducing LDL-C around 12% [15]. Similarly, a micellar PS formulation composed of phospholipids, mostly present as phosphatidylcholine (PC) showed a reduction of total cholesterol around 32-38% and LDL-C around 14.7% [11, 13]. Simiarly to micelles, liposomes composed of PC can potentially enhance the therapeutic efficacy of PS, due to their ability to accelerate micellar solubilization of bile salts, which is the mechanism of action of PS [16, 17].

It is important to note that PS can undergo autoxidation generating PS oxidation products (POPs) that may impart a pro-atherogenic effect [18, 19]. Thus, an optimum formulation should prevent the autooxidation of PS and render PS safe for consumption. Our recent work showed that liposomal entrapment maintained the oxidation stability of PS, preventing the generation of POPs [20].

In addition to the formulation approach, the source of PS can have an impact on their therapeutic effects. PS, namely campesterol, stigmasterol, and β-sitosterol, obtained from soybean and palm oils, are abundant in nature and have been tested for their efficacy in humans [9, 21]. The FDA and Health Canada have approved their use as cholesterol-lowering agents [22, 23]. However, brassicasterol, an abundant PS present in canola have not been tested for its therapeutic efficacy. A major source of canola PS is the canola oil deodorizer distillate (CODD), a by-product obtained during the refinement process of canola oil [24]. Thus, there are unexplored health and economic gains of extracting PS from the CODD, with its unique composition of brassicasterol, campesterol, and β-sitosterol [24], and developing it into a health supplement.

We previously developed and characterized liposomal formulation composed of phospholipids to enhance the efficacy of PS extracted from the CODD while at the same time preventing their autooxidation by incorporating alpha, gamma and delta tocopherols as antioxidants [20, 25, 26]. The liposomes were able to entrap PS and tocopherols at an efficiency greater than 90% while maintaining oxidative stability [20, 25]. Since the liposomal formulation had optimal physicochemical properties and adequate stability, we proceeded to assess the LDL-cholesterol lowering ability of the PS in comparison with a marketed PS containing product.

For preclinical testing of the therapeutic efficacy of the new PS formulation, hamsters were chosen as the animal model because of similarities in cholesterol and bile acid metabolism to that of

humans [27, 28]. Similarly to humans, hamsters express cholesteryl ester transport protein, a protein that transports cholesterol between lipoproteins [29]. Thus, findings from hamster studies are more clinically translatable to humans than those from some other laboratory rodents [30]. Several studies have previously utilized hamsters to assess plant sterols and other LDL-cholesterol-lowering compounds [31-33].

Thus, the objective of the study was to evaluate liposomal PS extracted from the CODD for their LDL-C lowering ability in a hamster model of diet-induced hypercholesteremia.

5.3. Materials and Methods

5.3.1. Chemicals

Canola oil deodorizer distillate (CODD) was gifted from LDM foods (Yorkton, SK, Canada). PC (95% purity with lyso PC as a minor constitunet) was purchased from Avanti Polar Lipids (Alabaster, AL, USA). Tocopherols (a combination of alpha, gamma and delta tocopherols), ethyl acetate, and potassium hydroxide were obtained from Sigma Aldrich (Oakville, ON, Canada).

5.3.2. Extraction of PS and PS formulation

PS were extracted from CODD. Briefly, the CODD was saponified with potassium hydroxide in the presence of 95% ethanol, and this was followed by the addition of water. The resulting mixture was chilled for 1 hour. During the chilling process, PS crystallized and was collected by vacuum filtration. The PS was composed of brassicasterol, campesterol, and β-sitosterol (**Table 5.1**), and the purity of the extract was >90 % as determined by our previously developed LC-MS/MS method [24]. The development of the liposomal PS was reported in our published work that showed 90% entrapment efficacy [25, 26]. Briefly, PS was formulated into liposomes composed of PC and tocopherols. The formulation was a ratio of 1:9:20 ratio of tocopherol: PS: PC. This ratio led to

the formation of liposomes of desirable physicochemical properties [34]. Fresh formulations were prepared weekly and were characterized for size, zeta potential, and entrapment efficiency **(Appendix III, Table S5.1)**. Finally, the liposomal formulation was incorporated into orange juice or sterile purified water to deliver in a 1 mL volume a dosage of 0.03 g/kg body weight/day, as recommended by Health Canada and the FDA [22, 35]. The efficacy of the formulated PS and the influence of the vehicles (orange juice or water) on its efficacy was tested.

Table 5.1. Percentage composition of phytosterols in the extract obtained from canola oil deodorizer distillate.

Phytosterols	**Composition in phytosterol extract**
Brassicasterol	24.3 %
Campesterol	32.8 %
β-sitosterol	43.1%

5.3.3. Preparation of commercial PS product

Orange juice (Minute Maid® Premium Heart Wise®) containing approximately one gram of PS per 250 mL as per the package labeling was obtained from a local supermarket. The product was within expiry and kept at the same storage conditions as in the supermarket. The commercial product PS is composed of campesterol, stigmasterol, and β-sitosterol [21]. The marketed PS containing orange juice was lyophilized using a FreeZone Plus 6 freezer dryer (Labconco; Kansas City, MO, USA). This was followed by reconstitution with sterile purified water to obtain the desired dosage (0.03 g/kg body weight/day in 1 mL solution, water or orange juice) as recommended by Health Canada and the FDA [22, 35].

5.3.4. Animal Studies

Animals and Diet

Six-week-old male Golden Syrian hamsters were purchased from Charles River (St. Constant, Quebec, Canada). Animal experiments were conducted in two cohorts, each containing 30 hamsters. Hamsters were group-housed with 2-3 animals per cage in a temperature-controlled room with a 12h-light/dark cycle. All animals had free access to water. They were acclimatized to handling for two weeks prior to the experiment, during which all hamsters were fed with regular rodent chow.

To test the PS formulations versus the vehicle control solutions, hamsters were then randomly assigned to 1 of 5 different treatment groups (n=12) for 4 weeks (see details below). During this experimental period, all animals had free access to a purified diet to induce hyperlipidemia. The diet purchased from Research Diets (Catalog # D0605050; New Brunswick, NJ, USA), had a high fat content (44 % of energy) with added cholesterol (0.5% by weight), which mimics a Western diet. This diet has been widely utilized in the study of cholesterol lowering compounds as it significantly increases serum LDL-C compared to a normal diet with added cholesterol [31]. In addition, the presence of hydrogenated coconut oil and sucrose in diet will impart further atherogenic effect in animals. Diet composition is shown in **Table 5.2**.

The animal study was approved by the University of Saskatchewan's Animal Research Ethics Board (AUP 20200094), and the experiment adhered to the Canadian Council on Animal Care guidelines for humane animal use.

Table 5.2. Composition of the purified high fat hamster diet with 0.5% added cholesterol.

Ingredients	**Composition in gm**	**Composition in kcal**
Casein, 80 Mesh	250	1000
L-Methionine	5	20
MaltoDextrin 10	125	500
Corn Starch	156	624
Sucrose	95	380
Cellulose, BW200	150	0
Coconut Oil 101 Hydrogenated	200	1800
Soybean Oil	20	180
Mineral Mix S10001	35	0
Vitamin Mix V10001	10	40
Choline Bitartrate	2	0
Cholesterol, USP	**5.3**	**0**
Red Dye, FD&C #40	0.05	0
Total	**1053.35**	**4544**
	Composition in gm%	**Composition in kcal%**
Protein	24	22
Carbohydrate	37	34
Fat	**21**	**44**
Total		**100**
Kcal/gm	**4.31**	

Experimental Groups

Thirty hamsters in each of the two cohorts were randomly assigned to one of five different experimental groups, each containing six animals (**Table 5.3**). The daily dose of PS in the first three treatment groups was 0.03 g/kg body weight. On each day, for four weeks, hamsters were fed 1 mL of the PS formulation or control solution by oral gavage (flexible gavage needle code: FTP-18-75, Instech labs, Plymouth Meeting, PA, USA) while under light anesthesia with isoflurane. Body weight and food intake per cage were recorded on a weekly basis.

Table 5.3. Study and control groups in the experimental trial.

Treatment Group	**Description**
Group I: Liposomal phytosterols in orange juice (L-PS-OJ)	Treatment group Liposomal PS and tocopherols (size: 150- 200 nm) Formulation incorporated in pulp-free orange juice
Group II: Liposomal phytosterols in water (L-PS-W)	Treatment group Liposomal PS and tocopherols (size: 150-200 nm) Formulation incorporated in sterile water
Group III: Marketed phytosterols in orange juice (M-PS-OJ)	Treatment group Minute Maid® Premium Heart Wise® orange juice containing PS.
Group IV: Orange juice (OJ)	Control group for PS formulation in OJ Pulp-free orange juice
Group V: Water (W)	Control group for PS formulation in sterile water.

Blood collection and processing

All blood samples were collected after an 8-hour fasting period. Fasting samples were collected after two weeks of acclimatization for baseline lipid concentrations, as well as at experimental

weeks 2 and 4. At weeks 0 and 2, blood was withdrawn from the hamster's gingival sinus while under isoflurane anesthesia. At the termination stage (i.e., end of week 4), animals were euthanized under anesthesia followed by a cardiac puncture.

Blood samples were allowed to clot at room temperature for 30 minutes and centrifuged at 2320 x g for 20 minutes. The separated serum layer was collected in a tube and was stored at -80 °C until further analysis.

Serum triglyceride analysis

Serum triglycerides were analyzed colorimetrically based on an enzymatic assay [36, 37] by utilizing a triglyceride colorimetric assay kit obtained from Cayman Chemical (Ann Arbor, MI, USA). The assay was conducted as described by the manufacturer. The principle of triglyceride enzymatic assay involves the hydrolysis of triglyceride into glycerol and fatty acids, catalyzed by lipoprotein lipase [38]. This is followed by phosphorylation of glycerol to glycerol-3-phosphate by glycerol kinase in the presence of ATP. Glycerol 3-phosphate is then oxidized, generating dihydroxyacetone phosphate and hydrogen peroxide. The obtained hydrogen peroxide undergoes redox coupled reaction with 4-aminoantipyrine and N-ethyl-N-(3-sulfopropyl)-m-anisidine producing a purple color for colorimetric analysis [38].

The calibration curve was plotted using triglyceride standard in the concentration range of 3.125-200 mg/dL. For the baseline (week 0), 10 µL of serum was diluted with 40 µL of assay diluent, whereas for the week 2 and week 4 experimental samples, 10 µL of serum was diluted with 300 µL of assay diluent. The diluted samples were added to a 96 well-plate along with a reagent mixture (lipoprotein lipase, ATP, glycerol kinase, glycerol phosphate oxidase, peroxidase, 4-aminoantipyine, N-ethyl-N-(3-sulfopropyl)-m-anisidine) and were incubated at 37 °C for 30 min.

Finally, they were analyzed using a BioTek Synergy® HT Multi-Mode Microplate Reader (VT, USA) at an absorbance wavelength of 530 nm. All the samples were analyzed in triplicates.

In addition, free glycerol in the serum was determined using a glycerol colorimetric kit obtained from Cayman Chemical (Ann Arbor, MI, USA). The week 0 serum was diluted with assay diluent in the ratio of 1:1, whereas serum from week 2 and week 4 were diluted in the ratio of 1:10. The assay was conducted in the same way as described above for triglyceride determination, except that the enzyme mixture did not contain lipoprotein lipase; this prevented the hydrolysis of serum triglycerides into glycerol so that only free glycerol was determined. Final triglyceride concentrations were obtained by subtracting free glycerol concentration from the total glycerol concentration obtained above in the triglyceride colorimetric assay.

Serum cholesterol analysis

Analysis of serum total cholesterol (T-C) and high density lipoprotein-cholesterol (HDL-C) were conducted using a fluorometry-based enzymatic assay [39] using a kit purchased from Cell, Biolabs, Inc. (San Diego, CA, USA). The assay was conducted as described by manufacturer's protocol. The principle of the enzymatic cholesterol assay involves the hydrolysis of cholesteryl esterase to free cholesterol, which is further oxidized by cholesterol oxidase into ketone-cholest-4-en-3-one along with hydrogen peroxide [39]. Finally, the hydrogen peroxide is detected with a fluorescence probe in a reaction catalyzed by horseradish peroxidase.

A calibration curve was plotted using a cholesterol standard in the concentration range of 1µM to 18 µM. For the determination of total cholesterol at week 0, serum was diluted with assay diluent where a dilution factor of serum to diluent was 1:195. For the week 2 and week 4, the dilution factor of 1:6231 serum to diluent were employed.

In order to determine HDL-C, 10 µL of serum were mixed with 20 µL of precipitating reagent. The mixture was allowed to precipitate by incubating it for 10 minutes at room temperature. After precipitation, the mixture was centrifuged at 11,250 x g for 10 minutes. A pellet containing LDL-C/very low-density lipoprotein cholesterol (VLDL-C) fraction was formed after centrifugation along with clear supernatant containing HDL. The supernatant HDL fraction was transferred to another tube and was further diluted in the same way as total cholesterol to achieve the desired concentration. Fifty microliters of each diluted sample were added to 96-well microplates in triplicates along with an enzyme mixture containing fluorescence probe, horseradish peroxidase, and cholesterol esterase (at the ratio of 5:5:5:1) in order to compare with a known standard. The plates were then incubated for 45 min at 37 °C and were analyzed in a BioTek Synergy® HT Multi-Mode Microplate Reader (VT, USA) at an excitation and emission wavelength of 530 nm and 620 nm, respectively.

VLDL-C was calculated by dividing the triglyceride value with an adjustable factor provided in Martin's formula for the cholesterol measurement [40]. The adjustable factor was obtained based on the non-HDL and triglyceride concentration in the serum [40].

Finally, LDL-C was determined using Martin's formula as shown below:

$$LDL\ cholesterol = Total\ cholesterol - HDL\ cholesterol - VLDL\ cholesterol$$

5.3.5 Statistical analyses

The effect of the two independent variables, treatment and time (fixed factors), on the dependent variables, serum T-C, HDL-C, LDL-C, VLDL-C, and triglycerides, body weight, and food intake was assessed. Dividing the experiment into two cohorts can lead to the presence of an additional random factor, which might affect the results obtained for the dependent variables within groups

[41]. Thus, to assess the effect of cohort on the primary endpoint, serum LDL-C concentration, a mixed model ANOVA/ split-plot ANOVA was conducted [42]. Since the effect of cohort was found to be significant (see Results section), the body weights of the 2 cohorts at week 0 was compared by Student's *t* test to determine if differences in body weight might contribute to the cohort effect. All data are presented as the mean ± the pooled average of the standard error of the mean (SEM) for the two cohorts. All data were tested for a normal distribution using the Shapiro Wilk test, and equality of variance between the experimental groups of each cohort by Levene test.

Since outliers were identified for T-C, triglycerides, VLDL-C and LDL-C concentrations in four different serum samples, and the serum turbidity was considered as a potential contributor. Therefore, turbidity of outlier samples was compared with that of the non-outlier samples by measuring the absorbance of visible light at 530 nm. The turbidity assessment is independent from the cholesterol and triglyceride enzymatic assays where these lipids need to be oxidized and hydrolyzed, respectively to determine their concentration. Thus, turbidity measurement was used to investigate whether the lipid concentration was overestimated due to the components of the serum other than the target lipid. Apart from this assessment, the value of the outlier was investigated if lies than Q3 + (3 X IQR) or lower than Q1 – (3 X 1QR) where Q3, Q1 and IQR are 3^{rd} quartile, 1^{st} quartile and interquartile range respectively.

Statistical analysis was performed using a two-way ANOVA to assess the effect of formulation and time (independent variables) on serum lipid concentrations and body weight. One-way ANOVA was conducted to evaluate the effect of formulation on food intake. Post hoc testing was conducted using the Bonferroni test when significant differences and interactions were observed. Bonferroni test was opted in order to minimize type I error. Five different groups were compared in post hoc analysis namely i) L-PS-OJ versus OJ, ii) L-PS-W versus W, iii) M-PS-OJ versus OJ,

iv) L-PS-OJ versus M-PS-OJ, and v) L-PS-OJ versus L-PS. All the data were expressed as mean ± pooled SEM. IBM SPS 28, GraphPad Prism, and MS excel were utilized for statistical analyses. Differences were considered significant if $P < 0.05$.

5.4. Results and Discussions

The purpose of the study was to determine the ability of lowering LDL-C of PS obtained from CODD, encapsulated into liposomal formulation. CODD-derived PS contains a unique combination of brassicasterol, campesterol, and β-sitosterol. Liposomal PS was tested and compared to a marketed PS-containing product. Apart from different formulations, the products have different PS compositions. Liposomal PS contained brassicasterol, campesterol, and β -sitosterol while marketed PS product contained campesterol, stigmasterol, and β-sitosterol. Furthermore, this study compared the effect of liposomal PS in orange juice relative to the liposomal PS in water to evaluate the influence of food vehicles/matrices on the efficacy of PS.

The assessment of the cohort effect on primary endpoint, (LDL-C concentration) using mixed model ANOVA/split-plot ANOVA showed a significant impact of a cohort on LDL-C concentration within a group ($P < 0.001$). The source of the cohort effect could be due to the differences in the body weight of the hamsters between cohorts at week 0 (discussed below in 5.4.1).

5.4.1. Food intake and body weight

Body weight and food intake of the animals were monitored on a weekly basis. Due to group housing, it was not possible to monitor the food intake of an individual animal. Thus, we measured the overall food intake in each group. There was no significant difference in the weekly food intake (average of four weeks) among the five groups (**Appendix III, Figure S5.1**). Previously published

work, where animals were fed with a high fat cholesterol containing diet along with PS, also demonstrated similar results [43, 44].

Student's *t* test showed the significant difference in the body weight (at week 0) of hamsters between the cohorts ($P<0.001$). This highlights that the possible source of random variable or cohort effect could be the difference in the initial body weight of hamsters between the cohorts. Two-way ANOVA showed a significant effect of formulation on hamster's body weight ($P= 0.0482$), on the other hand, time did not have a significant impact on their body weight. Furthermore, no significant interaction was observed between formulation and time. In week 0, hamster body weight ranged from 113.0 ± 9.3 grams to 122.0 ± 14.2 grams. Similar range (112.7 ± 21.7 to 136.5 ± 13.4 grams) was observed in week 4 (**Figure 5.1**). Regarding, multiple comparison between groups, we did not see any significant differences in treatment groups compared to their respective control. This highlights that treatment formulation does not seem to influence the body weight of the animals. Similarly to our animal trial, no difference in body weight were observed in hamsters fed with soybean oil PS compared with the control during a two month trial [45].

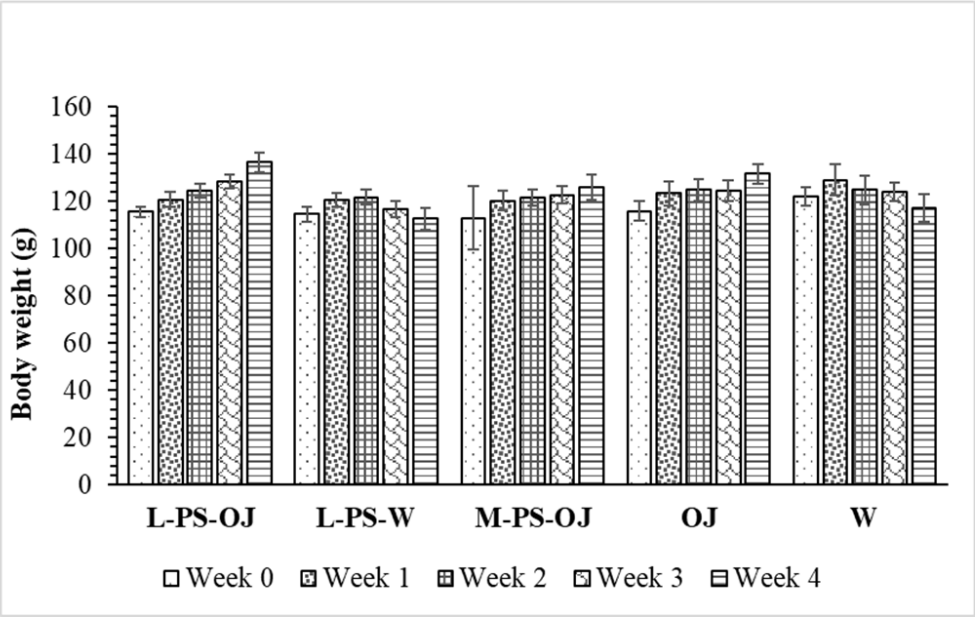

Figure 5.1. Body weight of hamsters of treatment and control groups at a weekly interval during a month of experimental trial after consuming a high-fat cholesterol-containing diet. The data are presented as the. mean ± the pooled average of standard error of mean (SEM) for the two cohorts. Abbreviations: L= Liposomal, PS = PS, M = Marketed, OJ = Orange juice, and W = Water. N values: i) week 0; L-PS-OJ (N=12), L-PS-W (N=12), M-PS-OJ (N=12), OJ (N=12), and W (N=12); ii) week 1; L-PS-OJ (N=12), L-PS-W (N=12), M-PS-OJ (N=11), OJ (N=12), and W (N=12); iii) week 2; L-PS-OJ (N=12), L-PS-W (N=12), M-PS-OJ (N=11), OJ (N=12), and W (N=12); iv) week 3; L-PS-OJ (N=11), L-PS-W (N=11), M-PS-OJ (N=11), OJ (N=11), and W (N=10); v) week 4; L-PS-OJ (N=11), L-PS-W (N=11), M-PS-OJ (N=11), OJ (N=11), and W (N=9). The decrease in N value in week 2, week 3, and week 4 is due to death of animals. "*" above the dotted line represents the statistical significance.

It is noteworthy that the body weight of five hamsters, two from the L-PS-W group and 1 each from M-PS-OJ, orange juice, and water group were reduced significantly in week 4 due to diarrhea. A total of seven hamsters died prior to the final week of the experimental trial. One in each L-PS-OJ, L-PS-W, M-PS-OJ, and control orange juice whereas 3 in control water; 4 of which died due to diarrhea, and 3 due to anesthetic overdose during the blood withdrawal procedure. To limit the use of animals in this proof-of-concept study, the control group fed with matched control diet (i.e.,

not high in fat and cholesterol) was not included. Thus, we were not able to directly ascertain whether this diarrhea was due to the presence of a high-fat diet or not. Nevertheless, it is well-documented in the literature that exposure to a high-fat diet for as short as two weeks can cause a shift in the gut microbiota in hamsters, rats, and mice [46-48]. This could lead to the diarrhea of hamsters, as observed in this study. Reduction in the body weight of rodents can be related with less amount of food intake, which will influence their hyperlipidemic state needed for this experimental trial. Thus, hamsters with more than 20% weight reduction were excluded from the statistical analysis in order to limit confounders.

5.4.2. Serum lipid analysis

Serum T-C, HDL-C, and triglycerides were directly assessed with the enzymatic assay. VLDL-C was obtained from triglyceride values using Martin's equation [40]. Finally, primary end point LDL-C was calculated from T-C, HDL-C, and VLDL-C.

It is important to note that the concentration of T-C, triglycerides, VLDL-C, and LDL-C in four different serum samples, one (M-PS-OJ) in week 2 and three (one in each L-PS-OJ, M-PS-OJ and W groups) in week 4 were identified as outliers. We hypothesized two different reasons for this anomaly: i) turbidity of sample and adsorption of the fluorescent substrate on the suspended particles (protein or cell debris) which led to a non-linear increase in fluorescence [49], overestimating the lipid concentration, and ii) presence of haem pigment in the serum due to haemolysed red blood cells [50]. In order to investigate the first hypothesis, absorption of samples in the normal range were compared with the outlier samples directly, without any enzymatic reaction. As expected, the outliers showed significantly higher absorbance at visible light of wavelength 530 nm compared to normal samples, corelated with higher calculated lipid values of the outliers (**Appendix III, Table S5.2**).

In addition, reported LDL-C concentration in hamsters (6-10 weeks old) fed with a high-fat cholesterol-containing diet (the same diet that we employed) for a time point of at least one month shows LDL-C in the range of 310- 650 mg/dL [51-53], whereas the LDL-C concentration of the outliers in our study was 2500-3000 mg/dL (**Appendix III, Table S5.3**). This suggests that the observed LDL-C concentration in the outlier sample is unusual for hamsters of the age groups 6-8 weeks fed with a high-fat cholesterol diet for a month. Thus, these outliers were excluded during the descriptive and statistical analysis.

HDL-C measurements did not identify these four samples as outliers. This is probably due to HDL-C extraction from the serum prior to its analysis (detailed in section 5.2. Material and methods), unlike T-C and triglycerides, which were directly analyzed with enzymatic assay without any extraction.

Determination of triglycerides

Serum triglycerides are an independent risk factor for CVD [54]. In this study, we assessed the effect of treatment on the concentration of serum triglycerides. Two-way ANOVA showed a significant effect of treatment ($P < 0.001$) and time ($P < 0.001$) on serum triglyceride concentration as well as a significant interaction between treatment and time ($P = 0.0137$).

Table 5.4 shows the serum triglyceride concentration in weeks 0, 2, and 4 of the experiment. Serum triglyceride concentration was in the range of 160.5 ± 12.8 mg/dL to 187.9 ± 15.3 mg/dL in week 0, whereas by week 2, after consuming the high-fat cholesterol-supplemented diet, the values increased significantly into the range of 1652.8 ± 191.8 mg/dL to 2662.2 ± 243.3 mg/dL in all the study and control groups ($P<0.001$). However, there was no significant change in triglyceride concentration between week 2 and week 4 in any experimental group. For the selected

treatment comparisons in week 4, none of the groups fed with a PS formulation, showed a significant change in serum triglycerides compared to their respective control group. These results are consistent with previously published work investigating the effect of PS-containing milk powder on hamsters, where PS did not contribute to reducing serum triglycerides during a four-week trial [31]. Another trial, conducted for six weeks, also showed a similar trend in hamsters [44]. To sum, this study showed the minimal impact of PS extracted from CODD, with its unique composition, on serum triglyceride concentration of hamsters during a month of an experimental trial.

Table 5.4. Serum triglyceride concentration in hamsters of treatment and control groups during a month of an experimental trial. The values are shown as the mean ± the pooled average of standard error of mean (SEM) for the two cohorts. Superscripts (a, b) were used to express statistical significance at $P<0.05$ in each column. The different letters in each column denotes the statistical significance at a different time point within a group. No significant differences were observed between treatments relative to their respective control at a given timepoint. Abbreviations: L= Liposomal, PS = PS, M = Marketed, OJ = Orange juice, and W = Water. N values: i) week 0; L-PS-OJ (N=12), L-PS-W (N=12), M-PS-OJ (N=12), OJ (N=12), and W (N=12); ii) week 2; L-PS-OJ (N=12), L-PS-W (N=12), M-PS-OJ (N=10), OJ (N=12), and W (N=12) and; iii) week 4; L-PS-OJ (N=11), L-PS-W (N=8), M-PS-OJ (N=9), OJ (N=9) and W (N=7), Abbreviations: L= Liposomal, PS = PS, M = Marketed, OJ = Orange juice, and W = Water. The decrease in N value in week 2 and week 4 are due to, i) presence of outlier, ii) death of animals, iii) exclusion of animals with diarrhea with significant weight reduction.

Week	Triglyceride concentration (mg/dL)				
	L-PS-OJ	**L-PS-W**	**M-PS-OJ**	**OJ**	**Water**
0	182.4 ± 10.8[a]	187.9 ± 15.3 [a]	172.9 ± 6.6[a]	173.6 ± 11.9 [a]	160.5 ± 12.8 [a]
2	1652.8 ± 191.8 [b]	2048.4 ± 335.3 [b]	2484.1 ± 421.8 [b]	1689.6 ± 291.2 [b]	2662.2 ± 243.3 [b]
4	1168.4 ± 153.0 [b]	1213.2 ± 148.2 [b]	2349.2 ± 247.6 [b]	1897.5 ± 223.3 [b]	1820.3 ± 342.8 [b]

Determination of total-C, LDL-C, and VLDL-C

Clinically, elevated serum levels of total-C, VLDL-C, and LDL-C are the primary risk factors of CVDs [55, 56]. Among these, LDL-C is the most potent contributor as evident from numerous randomized clinical trials and epidemiological studies [55, 56]. In this study, we evaluated the effect of the novel PS formulation on these lipoprotein cholesterols. **Figure 5.2** shows T-C, VLDL-C, and LDL-C at week 0, week 2, and week 4 of the experimental trial.

Two-way ANOVA showed a significant effect of formulation on serum T-C concentration with $P < 0.001$. Similarly, time had a significant effect ($P= 0.0053$) on T-C concentration. Regarding interaction between formulation and time, it did not reach a level of statistical significance ($P = 0.0534$). Similar behaviour as T-C was observed in the case of LDL-C and VLDL-C where formulation showed significant effect on the concentration of these lipids with $P < 0.001$. Furthermore, time significantly affected LDL-C and VLDL-C with $P= 0.0096$ and $P= 0.0045$, respectively. Unlike T-C, there was a significant interaction between time and formulation for both LDL-C and VLDL-C ($P = 0.0361$ and $P= 0.0246$, respectively).

A significant increase in the serum T-C, LDL-C, and VLDL-C ($P < 0.01$) was observed in all groups only at week 2 of the trial compared to the week 0 or baseline. Such observation is expected due to the nature of the diet, a high fat cholesterol containing diet inducing hyperlipidemia. At week 2 animals consuming liposomal PS in either orange juice or water (L-PS-OJ and L-PS-W groups) did not show any significant reduction of T-C, VLDL-C, and LDL-C compared to control orange juice (OJ group) and water (W group). Similarly, in pairwise comparison, marketed PS orange juice (M-PS-OJ group) showed no significant difference compared to control orange juice (OJ group) at week 2. This lack of statistical difference could be attributed to the short treatment period of only two weeks, which is a mid-interval of this experimental trial. In fact, clinical studies

indicated that at least four weeks of supplementation is needed for the PS to show significant efficacy [57]. It was also noted that the serum T-C concentration was lower in the hamsters receiving the L-PS-OJ group as compared to those administered the marketed M-PS-OJ group ($P < 0.001$). L-PS-OJ and L-PS-W groups on the other hand did not show significant difference at 2 weeks.

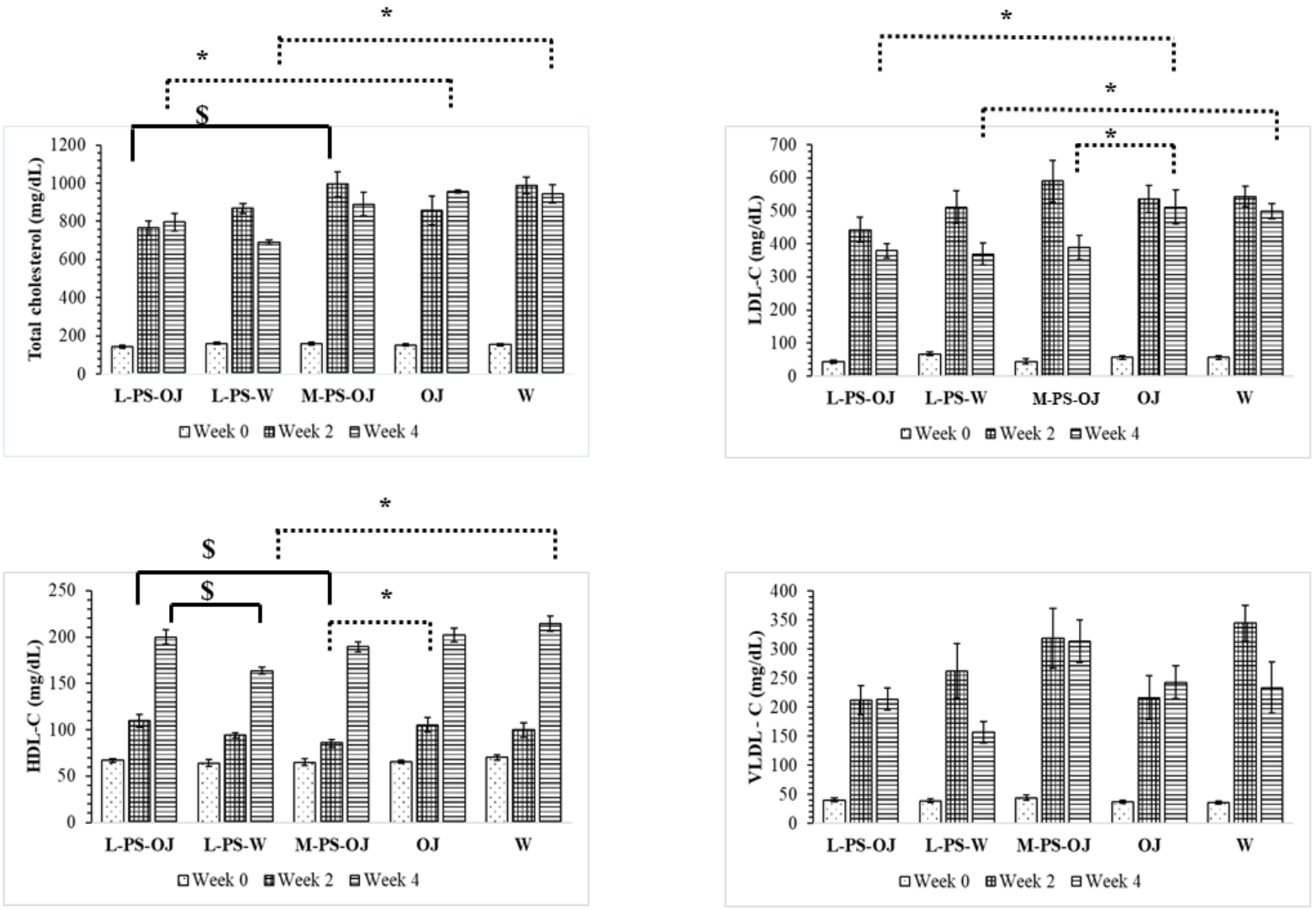

Figure 5.2. Serum TC, LDL-C, HDL-C, and VLDL-C concentration in hamsters of treatment and control groups during a month of an experimental trial. The values are shown as the mean ± the pooled average of standard error of mean (SEM) for the two cohorts. Symbols (*, $) show the presence of statistical significance at P <0.05. "*" above the dotted line represents the statistical significance of the

treatment groups with their respective control group at a constant time point. OJ group is control of treatment groups L-PS-OJ and M-PS-PJ, whereas W group is control of the treatment group L-PS-W. “$” above the solid line represents the statistical significance between treatment groups at a constant time point. N values: i) week 0; L-PS-OJ (N=12), L-PS-W (N=12), M-PS-OJ (N=12), OJ (N=12), and W (N=12); ii) week 2; L-PS-OJ (N=12), L-PS-W (N=12), M-PS-OJ (N=10), OJ (N=12), and W (N=12) and; iii) week 4; L-PS-OJ (N=11), L-PS-W (N=8), M-PS-OJ (N=9), OJ (N=9) and W (N=7), Abbreviations : L= Liposomal, PS = PS, M = Marketed, OJ = Orange juice, and W = Water. The decrease in N value in week 2 and week 4 are due to, i) presence of outlier, ii) death of animals, iii) exclusion of animals with diarrhea with significant weight reduction.

By week 4, liposomal PS formulated in orange juice and water (L-PS-OJ and L-PS-W groups) significantly reduced T-C in hamsters compared to the control orange juice ($P = 0.0425$) and water ($P < 0.001$) (**Figure 5.2**). In contrast, the marketed PS product (M-PS-OJ) did not reduce T-C concentration significantly relative to its control. The vehicle (orange juice or water) had no effect on T-C concentration since no significant difference was observed between liposomal PS in orange juice (L-PS-OJ) and liposomal PS in water (L-PS-W). Regarding VLDL-C by week 4, there was no significant differences in treatments (L-PS-OJ, L-PS-W, and M-PS-OJ) compared to their respective control. Furthermore, no differences were observed in the groups L-PS-OJ versus M-PS-OJ, and L-PS-OJ versus L-PS-W for serum VLDL-C concentrations.

Since serum LDL-C is a clinical contributor to CVD in humans [1, 2], studying the effect of treatment on LDL-C in animals is key for translation to humans. By week 4 liposomal PS in both orange juice and water (L-PS-OJ and L-PS-W groups) significantly reduced serum LDL-C at ($P < 0.001$) in hamsters compared to their respective control matrices (OJ and W, respectively) (**Figure 5.2**). Unlike, T-C where marketed PS orange juice (M-PS-OJ) did not show significant reduction relative to its control, in the case of LDL-C it exerted a significant effect in comparison to control orange juice ($P = 0.0393$). However, pairwise comparisons between L-PS-OJ versus M-PS-OJ, and L-PS-OJ versus L-PS-W did not show a significant difference in serum LDL-C concentration in week 4.

Overall, our findings demonstrate that a unique PS combination of brassicasterol, campesterol, and β-sitosterol extracted from the CODD and formulated in a novel liposomal formulation with tocopherols significantly reduces LDL-C and is comparable to a marketed product, commonly obtained from soybean oils (most abundant in of campesterol, stigmasterol, and β-sitosterol) [21]. This is the first report of therapeutic efficacy of PS mixture containing brassicasterol as major PS.

Furthermore, the vehicle did not affect LDL-C concentration, as both liposomal PS in orange juice and liposomal PS in water showed comparable effects. This outcome is consistent with previous studies, which highlighted that the food components or the vehicle do not influence the efficacy of PS [58]. PS formulations such as lecithin micelles [11], emulsion [59], capsules [60], and water-dispersible formulation [15] have been clinically tested for their LDL-C lowering efficacy. It is to be noted that in this proof-of-concept experiment we opted for only male hamsters due to time and resources. It is unknown whether these outcomes apply to females, therefore a future study should definitely be undertaken.

Reduction of the LDL-C around 10% is considered clinically significant in order to prevent CVD [61]. Lecithin PS, water-dispersible PS, and PS emulsion reduced LDL-C in the clinically significant range by 14.3% relative to control, while PS capsules only a reduction of 3% [11, 15, 59, 60]. In this study, a reduction of LDL-C higher than 20% was attained, indicating the feasibility of clinical translation to humans. It is to note that our liposomal formulation also comprises antioxidant mixtures (α, γ, and δ tocopherols). There is clinical evidence that tocopherols, at an optimum dose, decrease susceptibility to oxidation of LDL, preventing the generation of oxidized LDL (ox-LDL) [62], which are cytotoxic and accelerate atherosclerosis [63, 64]. Thus, in addition to reducing LDL-C, our liposomal formulation containing tocopherols can potentially prevent the oxidation of LDL. While we demonstrated that PS oxidation products were reduced in liposomal PS [20] , the effect on LDL can only be confirmed by conducting *in vitro* or *in vivo* studies.

Determination of HDL-C

HDL-cholesterol possesses anti-atherogenic properties; thus, its concentration is inversely associated with the risk of CVD [65]. In this study, the effect of liposomal PS on serum HDL-C concentration were evaluated. Two-way ANOVA showed a significant effect of both treatment

(P<0.001) and time (P= 0.0134) on HDL-C concentration. However, no significant interaction between treatment and time was observed. HDL-C concentration ranged from 64.2 ± 3.6 mg/dL to 70 ± 2.8 mg/dL in week 0 in all groups whereas in week 4 it ranged from 163.8 ± 3.7 mg/dL to 214.2 ± 8.3 mg/dL (**Figure 5.2**). There was a significant difference between the two liposomal formulations (L-PS-OJ versus L-PS-W) in week 4 (P=0.0093). Unexpectedly, the control water group showed significantly higher HDL-C concentration (of 214 ± 8.3 mg/dL) compared to L-PS-W (of 163.8 ± 3.7 mg/dL), P<0.001. The reason behind this observation is unknown. However, neither the liposomal PS (L-PS-OJ and L-PS-W) nor the marketed PS (M-PS-OJ) affected HDL-C concentration significantly compared to controls. In fact, a similar effect of PS on HDL-C was demonstrated previously where hamsters were fed with PS containing lactic acid fermented milk powder along with a high fat high cholesterol diet [31]. Furthermore, several meta-analyses of a randomized clinical trials demonstrated no effect of PS on HDL-C [9, 66].

5.5. Conclusion

This proof-of-concept study of a novel liposomal PS composed of phosphatidylcholine and tocopherols demonstrated a significant effect in reducing LDL-C. This new formulation can be a feasible alternative to existing commercial products in delivering PS as it showed similar efficacy as that of the marketed micellar PS. Additionally, the presence of tocopherols in the formulation prevents the formation of POPs. We demonstrated that the vehicle (juice or water) did not have effect on the therapeutic outcome, thus potentially expanding the commercial options to fortified water and other beverages. In addition, it is the first time that a canola PS with brassicasterol as major PS has shown evidence of LDL-C lowering which could be explored to amend health claims of PS filed with regulatory agencies. Furthermore, the unique PS combination obtained from CODD showed similar therapeutic efficacy to marketed soybean PS product, highlighting that

CODD can be an effective source for PS supplement/enriched food products. To fully elucidate the effect of CODD-derived PS and brassicasterol, long-term studies are needed in animal models and human clinical trials.

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5.6. References

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CHAPTER 6

General Discussions

6.1. General Discussions

Phytosterols (PS) are increasing in popularity due to their ability to reduce LDL-cholesterol in the range of 8-14% [1-3]. They are approved by the FDA and Health Canada as cholesterol lowering agents at a daily oral dose of 2 grams [4, 5]. PS are utilized as adjuvant therapy in hypercholesterolemic individuals [4, 5]. Furthermore, PS are a great therapeutic alternative in mild hypercholesterolemia where synthetic drugs cannot be prescribed. PS can be obtained from various natural sources such as vegetable oil (canola, palm, or soybean oil) and their by-product, the deodorizer distillate [6, 7]. Canola oil deodorizer distillate (CODD) is obtained after refining crude canola oil and is an excellent source for PS in the range of 21-25% [8]. Thus, there are economic gains from extracting PS from the CODD. However, the commercial potential of the extracts can only be realized by developing nutraceutical formulations. Significant limitations in the development of nutraceutical formulation containing PS, are caused by the lipophilic nature of PS, thus the need to be incorporated in food products, leading to formulation-dependent efficacy of PS. Moreover, PS can undergo oxidation when exposed to high temperature generating PS oxidation products (POPs). Encapsulation techniques such as spray drying [9, 10], spray chilling [11, 12], inclusion encapsulation [13, 14], rapid expansion of supercritical fluid (RESS) [15, 16], and micro-emulsification [17] can be utilized for addressing lipophilicity. Unfortunately, these techniques suffer from limitations such as usage of high heat, high cost for scale up procedures, and a usage of deleterious surfactants or emulsifiers. Lipid based liposomal formulations can be

utilized to address these limitations. Liposomes made of biodegradable phospholipid (phosphatidylcholine) are able to entrap lipophilic PS, thus addressing lipophilicity and preventing their direct exposure to reactive free radicals, which in turn can maintain their oxidative stability [18]. In fact, liposomes have already been widely utilized to enhance the oxidative stability of various sensitive nutraceuticals such as quercetin [19], curcumin [20], and nisin [21]. In addition, the entrapment of anti-oxidants tocopherols inside liposomes can further enhance the stability of PS [22]. Both natural as well as synthetic tocopherols namely alpha, beta, gamma, and delta have been widely utilized as antioxidants [23-25]. Regarding efficacy, previously published work has demonstrated that micellar PS can reduce low density lipoprotein cholesterol (LDL-C) around 14% [26, 27]. Liposomes are made up of the same building block as that of micelles. Thus, liposomes can possibly enhance LDL-C lowering ability of PS. In addition, liposomes are superior to micelles in terms of stability and entrapment efficiency [28, 29].

Our main goal was to develop a liposomal formulation containing PS extracted from the CODD along with commercially available tocopherols (alpha, gamma, and delta) to enhance PS' LDL-C lowering ability, impart aqueous solubility, and prevent the generation of POPs. To achieve such goal, analytical strategies were also developed to ensure efficient entrapment of PS within liposomes and evaluate their oxidative stability.

6.1.1. Development of liposomal formulations containing phytosterols and tocopherols, characterization of formulations, and determination of phytosterols, tocopherols and oxidation products using liquid chromatography tandem mass spectrometry (LC-MS/MS)

Liposomal formulations are developed using various preparation methods such as thin layer hydration homogenization [30], thin layer hydration ultrasonication [31], Mozafari method [32]. As such, we evaluated these techniques for their suitability in preparing liposomal PS and tocopherols. The criteria were: uniform particle distribution in nano-range, zeta potential around |20 mV|, low bioactive to lipid (B/L), and high entrapment efficiency. Particle size and zeta potential are important parameters that determine stability and bioavailability of the formulation [33, 34]. Thus, the optimum preparation method should produce liposomes with desirable particle size and zeta potential. In our study, thin layer hydration homogenization and ultrasonication methods produced liposomes of size 186.3 ± 4.4 nm and 196.2 ± 16.1 nm, respectively at the B/L ratio of 1:2, on the other hand, Mozafari method produced liposomes of size 260.0 ± 23.0 nm and required B/L ratio of 1:5. Unlike homogenization and ultra-sonication, the Mozafari method utilizes low shearing force (imparted by magnetic stirring) during liposomal preparation leading to larger particle size (**Table 3.1, Chapter 3**) [35]. Even though low shear force prevents the exposure of formulation to harsh condition, it does not seem to be adequate for obtaining smaller vesicles. Furthermore, transmission electron microscopy (TEM) analysis confirmed the size of liposomes and ULV morphology (**Figure 3.2, Chapter 3**). The obtained particle size is consistent with published work where liposomes were utilized for oral delivery [36, 37] . Zeta potential, which was comparable among all the preparation methods, ranged from -13.0 mV to -9.8 mV. Even though, the ideal zeta potential value as per DLVO theory is ± 20 to 30 mV, the obtained

zeta potential was adequate to maintain the liposomes' initial particle size, during the long term storage [38].

In order to determine the entrapment efficiency of PS and tocopherols in liposomes, a robust analytical method is needed. However, there are numerous analytical challenges in the quantification of these target analytes. For instance, GC coupled with detectors such as FID and MS has been utilized to determine PS and tocopherols [39, 40]; however, GC requires derivatization, thus it is tedious and time-consuming. LC-based method can be used to avoid derivatization. Detectors, such as DAD and ELSD can be coupled with an LC system for quantification; however, these detectors do not provide selectivity for co-eluting analytes [41, 42]. Fortunately, LC-MS can address all the above-mentioned challenges. We developed and validated a novel LC-MS/MS method to simultaneously determine PS and tocopherols entrapped in liposomal formulation. ESI is one of the commonly utilized ion sources for MS analysis [43, 44]. However, in case of lipophilic compounds such as PS, ESI showed lower ionization efficiency. Thus, we utilized APCI for ionizing these analytes. The optimized chromatographic condition using poroshell C18 column along with acetonitrile and methanol (99:1 V/V) imparted substantially better baseline separation for campesterol and stigmasterol compared to the reported methods. Furthermore, most of the published work utilizes complex gradient elution in order to separate target analytes; on the other hand, the developed method uses simpler isocratic elution. The method has a total run time of seven minutes which is the shortest run time among the reported methods for the simultaneous quantification of PS and tocopherols that utilizes MS with conventional chromatography [45-47]. Regarding tocopherols, positional isomers beta and gamma tocopherols co-eluted, thus were quantified collectively. The co-elution of beta and gamma tocopherols in C18 has been widely reported and in fact is one of the major challenges in the

determination of tocopherols [46, 48]. It is noteworthy, that interesting chromatographic phenomenon was observed while analyzing the formulation sample where unlike standards, the formulation sample showed the presence of unknown peak (termed peak X), which shared exactly the same MRM transitions of that alpha tocopherol (**Figure 2.3 and 2.4, Chapter 2**). After experimental investigation and literature search, peak X was concluded to be an stereoisomer of alpha tocopherols which got separated due to the chiral recognition ability of phosphatidylcholine (component of liposomes) [49]. This is the first time to report to such a dual separation of stereoisomers of alpha tocopherol using a C18 column. However, the reason why gamma and delta tocopherols did not show the same behavior is still unknown.

The fully validated LC-MS/MS method was successfully utilized to determine PS and tocopherols showed entrapment efficiency greater than 89% for the liposomes prepared with all three methods (**Table 3.2, Chapter 3**). Such excellent entrapment values prevent the need to remove free bioactive (additional preparatory step), thus making the preparatory process more economical. Even though all three methods showed optimum entrapment efficiency and physicochemical properties required for oral delivery, the Mozafari method may not be favorable for PS due to usage of heat (~110 °C). In fact, when comparing ultrasonication and homogenization, it was found that the latter is better suited for scale up procedures due to the availability of large size homogenizer. Thus, we concluded that thin layer hydration homogenization is the most suitable method in the developing liposomal PS and tocopherols.

In addition to investigating the suitability of the preparatory methods, we also evaluated the capability of liposomes along with tocopherols in preventing the generation of PS oxidation products (POPs). Such an assessment is crucial as there are some pre-clinical evidences that highlighted the negative health implications of POPs [50]. The determination of POPs in PS

formulation helps to ensure the safety of the final product, in this case, the developed liposomal formulation. Therefore, we also developed another LC-MS/MS method to quantify POPs in liposomal formulations.

One of the major challenges in POPs determination is the unavailability of authentic reference standards. Alternatively, we utilized cholesterol oxidation products (COPs) as they have similar response factor as that of POPs [51]. There are three major shortcomings in the POPs analysis, namely, i) longer run time, ii) requirement of tedious derivatization step, and iii) non-specific detectors (**detailed in Table 1.5, Chapter 1**). The newly developed method has the shortest run time, does not require derivatization of POPs, and imparts the desired selectivity [52]. The developed method has a total run time of five minutes and is able to quantify sixteen different POPs, namely, 7-hydroxy, 7-keto, 5,6 epoxy, 5,6 dihydroxy derivatives of brassicasterol, campesterol, stigmasterol, and β-sitosterol (**Figure 1.7, Chapter 1**).

We applied the method to quantify and compare POPs in three different samples; namely, liposomal PS, liposomal PS and tocopherol, and crude PS. Only one POP (7-ketobrassicasterol) was in the quantifiable range in all samples; however, the quantified value for this POP was too low to cause cytotoxicity. Also, there was no significant difference between the quantified value of POPs in liposomal PS (both with and without tocopherols) and the crude PS extract. This demonstrates that the liposomal preparation procedure did not contribute to the oxidation of PS. Furthermore, we evaluated the protective action of liposomes, alone or in combination with antioxidants for POP generation, and for this, three samples mentioned above were assessed by conducting accelerated stability study (by microwave heating). Liposomal PS produced significantly lower POPs than crude PS upon microwave heating. In fact, previous work demonstrated the ability of liposomes to enhance the stability of sensitive compounds such as

vitamin A and polyphenols (**Table 1.4, Chapter 1**), thus liposomes are an ideal carrier to deliver the oxidation prone compounds. In addition, our results demonstrated that liposomal PS containing tocopherols resulted in the formation of a significantly lower amount of POPs compared to liposomal PS. This was the expected outcomes due to the well-known potency of tocopherols and their derivatives in imparting antioxidant activity both *in vitro* and *in vivo* [53, 54]. The ascending order for the POPs in the microwaved samples are liposomal PS and tocopherols < liposomal PS < crude PS. This highlights that the best way to prevent the generation of POPs during long term storage is by developing PS with liposomes along with antioxidants. In addition to quantifying PS in liposomes, we expanded the method for wider applications: identification or profiling of possible POPs in extra virgin olive oil (EVOO). This led us to putatively identify 7-ketomethylenecycloartenol, a novel POP which has not been reported before. The identity of 7-ketomethylenecycloartenol, was confirmed by conducting accurate mass measurement using a quadrupole-orbitrap instrument.

Finally, optimized liposomes showing high entrapment efficiency, B/L, and oxidative stability were fortified in a model orange juice (acetic acid solution maintained at 3.2 pH) by vortexing for five minutes. Model orange juice was used instead of real orange juice in order to enable particle size determination without the interference from the particulate component present in real orange juice. Liposomes fortified model juice was then pasteurized at 72 °C for 15 sec, HTST method of pasteurization. Pasteurization is a crucial step in the development of beverages in the food industry to kill any microorganism that might be present in the final product to ensure the product is safe for humans. Pasteurized formulation was compared with the non-pasteurized formulation (control) for chemical stability by conducting LC-MS analysis. No significant differences in the quantity of PS and tocopherols between the two formulations, demonstrating that exposure to HTST

pasteurization does not degrade/oxidize PS or tocopherols. Furthermore, pasteurized liposomal formulations stored for a month at 4 °C were able to maintain their physical stability.

To sum, in this work, we developed and characterized an optimum liposomal formulation containing PS and tocopherols, which imparted adequate physicochemical properties, entrapment efficiency, and enhanced oxidative stability. In addition, liposomes fortified in model juice maintained physical and chemical stability. This way, nutraceutical formulation containing PS and tocopherols were successfully designed and were fortified in orange juice.

6.1.2. Assessment of LDL-C lowering ability of the developed PS formulation

The novel liposomal PS formulations were assessed for their ability in reducing LDL-C compared to control and marketed PS-containing product. Liposomal PS were fortified in two vehicles, namely, orange juice and water, and this was done in order to assess influence of the food vehicle on the therapeutic efficiency of PS. Minute maid® premium Heartwise® is the only commercial product where PS are enriched in orange juice. Thus, it was selected in this study. The PS formulation of this marketed product is unknown. Regarding composition, it consists of campesterol, stigmasterol, and β-sitosterol as demonstrated in a published clinical trial [55] . Based on this composition, the source of PS in Minute maid® premium Heartwise® is most likely soybean and/or corn oil [56]. Both FDA and Health Canada have approved three PS, campesterol, stigmasterol, and β-sitosterol, for oral administration at a daily dose of two grams as a cholesterol lowering agent [4, 5]. However, brassicasterol, an abundant PS present in canola, has not been included in these health claims as its efficacy is yet to be determined. Therefore, we tested, for the first time, unique PS composition containing brassicasterol along with campesterol, and β-sitosterol for their LDL-C lowering ability.

Hamsters were selected as animal model as they have similar cholesterol and bile acid metabolism as that of humans, thus results can be better translated to humans compared to some other lab rodents [57]. During a month of experimental trial, animals were fed with a high fat cholesterol containing diet to mimic western diet and induce hyperlipidemia. The negative control groups were orange juice and water, while the marketed PS-enforced orange juice was the positive control. The two investigational groups were liposomal PS in orange juice (L-PS-OJ) and liposomal PS in water (L-PS-W). As this study was a proof-of concept study, all animals were on the same high-fat diet. Every day for a month, hamsters were orally administered with treatment and control samples. Parameters such as total cholesterol, triglycerides, HDL-C were assessed in the serum obtained from fasting blood at baseline, week 2 and week 4 of experimental trial whereas the primary outcome, LDL-C, was determined using the Martin's equation [58] .

We observed a significant increment in total cholesterol (T-C), triglycerides, and LDL-C in all the animal groups just within 2^{nd} week compared to baseline. This observation was expected due to the nature of the diet, which induces hyperlipidemia in hamsters. However, no significant difference in these parameters was observed among groups in the 2^{nd} week. This demonstrates that a two-week period is relatively short for PS to contribute to cholesterol reduction. In fact, a published clinical trial highlighted that at least a month of PS administration is required to see a significant decrement in cholesterol level [59]. In the 4^{th} or final week, on the other hand, liposomal PS (both in orange juice and water) showed significantly lower T-C compared to their respective control, orange juice, and water. Unlike liposomal PS, marketed PS orange juice did not impart any significant reduction in T-C compared to its control (orange juice). A higher concentration of VLDL-C in the marketed PS group could be a possible reason behind its higher T-C concentration. Regarding HDL-C in the final week, no significant differences were observed in the treatment

groups versus the control. This observation agrees with published works that highlighted that PS does not affect the HDL-C concentration [60, 61]. Finally, in the case of the primary endpoint, LDL-C, all three treatment groups showed significantly lower LDL-C compared to their controls. In addition, there was no significant difference between liposomal PS orange versus marketed PS containing orange juice in their ability in reducing LDL-C. This demonstrates that a unique PS combination of brassicasterol, campesterol, and β-sitosterol extracted from the CODD and formulated in a novel liposomal formulation with tocopherols significantly reduces LDL-C and is as efficient as marketed PS containing orange juice. Furthermore, reduction of LDL-C was similar among the groups, liposomal PS in orange juice versus liposomal PS in water. This finding indicates that the vehicles do not influence the efficacy of PS and is in agreement with previous report [62].

To sum, the developed liposomal formulation containing canola PS and antioxidants showed significant efficacy in reducing LDL-C. In addition, the superiority of developed liposomal formulation lies in its ability to prevent POPs ensuring the safety of the final product as well as its ability to entrap bioactives with high entrapment efficiency which is advantageous for commercial aspects.

6.2. Future directions

The long-term goal of the research is to utilize liposomal PS for therapeutic purposes. Therefore, it is crucial to assess its LDL-C lowering ability in humans by conducting clinical trials. The selection and design of formulation with FDA and Health Canada approved active components (phytosterols and tocopherols) and adjuvants (phosphatidylcholine) is conducive to direct use of the formulation in humans.

In addition to assessing the existing PS mixture composed of brassicasterol, campesterol, and β-sitosterol, it is also important to evaluate the efficacy of the individual PS. Such information is valuable in developing products with specific PS that are responsible for the therapeutic effect. We can isolate individual PS either from the CODD PS by using two approaches: solvent recrystallization method [345] and high-speed counter-current chromatography [346].

In my Ph.D. work, we developed LC-MS methods to quantify PS and POPs in liposomal formulation, which was applied to assess the role of liposomes as well as antioxidants in preventing autoxidation of PS. However, PS can also undergo enzymatic oxidation *in vivo* due to the activity of intestinal microflora [63]. Thus, it is valuable to assess if liposomes and tocopherols can prevent the endogenous generation of POPs. The experiment can be done using an animal model [64] or through *in vitro* experiments by incubating phytosterols with the gut microflora. The existing POPs quantification method can be modified to apply for such an assessment. For this, method has to be developed and validated in the biological matrices unlike our developed method where liposomal matrix was utilized. Also, the efficient strategy for extracting POPs from blood samples needs to be developed. The saponification followed by liquid-liquid extraction has been commonly used to isolate POPs from biological samples [65].

Similar to PS, other nutraceuticals, such as the red yeast rice (composed of monacolins) and berberine (isoquinoline alkaloid) are well known for their LDL-C lowering ability. development of liposomal formulations of these three nutraceuticals (PS, red yeast rice, and berberine) may impart synergistic effect in reducing LDL-C. This combined nutraceutical formulation could mitigate hypercholesterolemia by two different mechanisms: i) inhibiting the absorption of dietary cholesterol (by PS and berberine), and ii) inhibiting cholesterol synthesis (by red yeast rice). Therefore, a strong synergistic effect in LDL-C reduction is expected.

In addition to cholesterol-lowering ability, numerous epidemiological, preclinical and clinical studies have provided evidence of the anti-carcinogenic effects of PS, especially in prostate, esophageal, and breast cancer [66-68]. Even though several mechanisms, namely, the inhibition of carcinogen productions, modulation of sterol biosynthesis, altering testosterone mechanism (in case of prostate cancer), have been proposed, its exact mechanism for anti-carcinogenic effect is still unclear [67]. Thus, thorough pharmacodynamic studies as well as a structure-activity relationship assessment for the anti-cancer property of PS is definitely a gap that needs to be bridged.

6.3. Conclusions

In this work, a novel lipid-based formulation was developed and characterized to deliver canola PS and antioxidants. The formulation development was conducted in such a way that it is easy for the scale-up procedure. The entrapment of PS in liposomes and antioxidant mixtures was an excellent strategy in enhancing the oxidative stability of PS, thus preventing the generation of POPs. Therefore, this research provides preclinical evidence that liposomes accompanied with tocopherols are an excellent candidate to deliver oxidation-prone compounds. Furthermore, two different analytical platforms, which were developed for the determination of liposomal bioactives and the PS oxidation products, addresses the major challenges related to the analysis of these target analytes, such as i) longer run time, ii) complex gradient elution, and iii) poor baseline separation of analytes. The assessment of the developed formulation in hamsters showed a significantly higher LDL-C lowering ability relative to control. In addition, the PS mixture containing brassicasterol in liposomes is as efficient as marketed PS enriched orange juice. To sum, PS obtained from CODD can be an effective source for designing it as supplements or enriched food products.

6.4. References

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APPENDICES

Appendix I

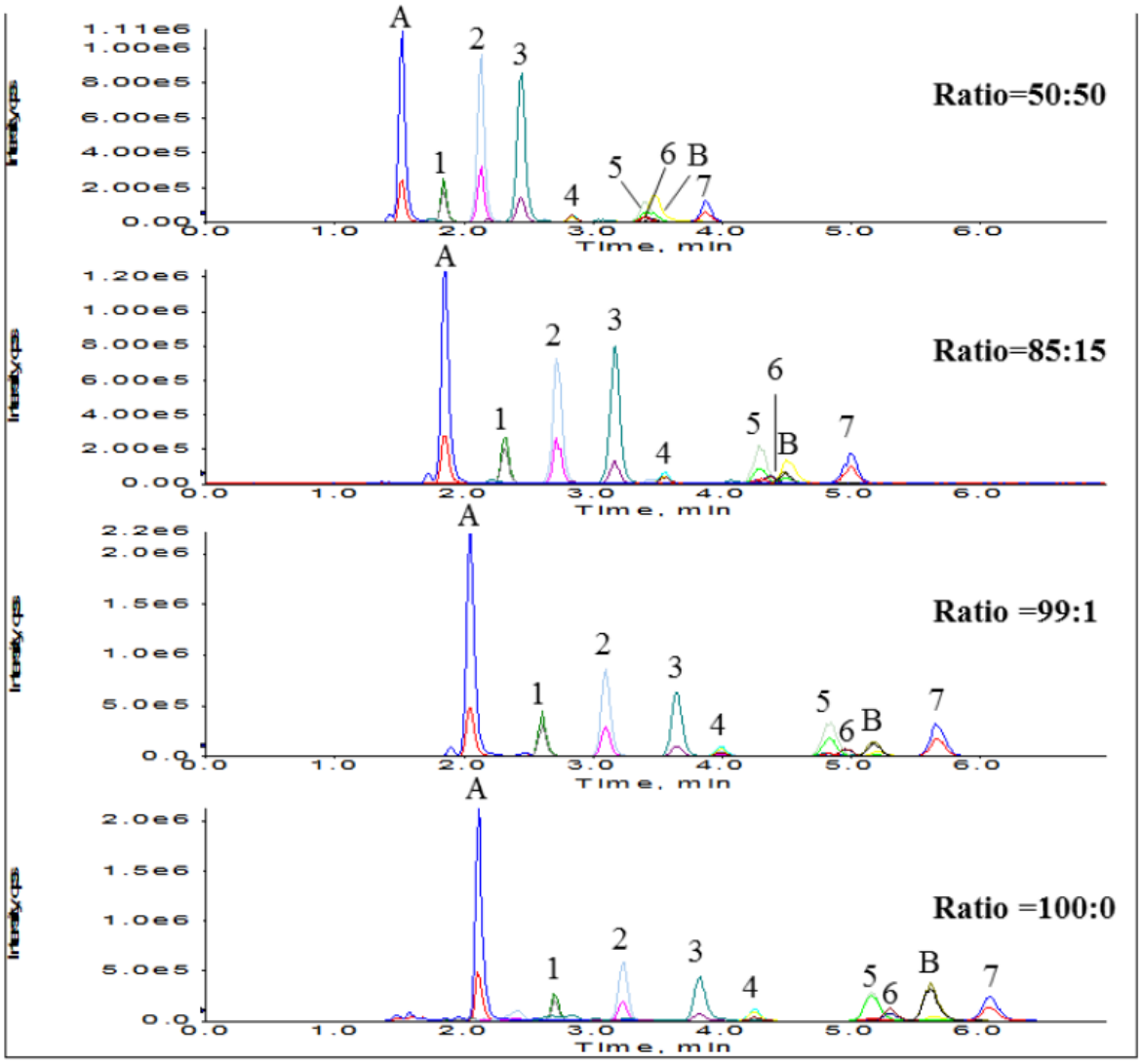
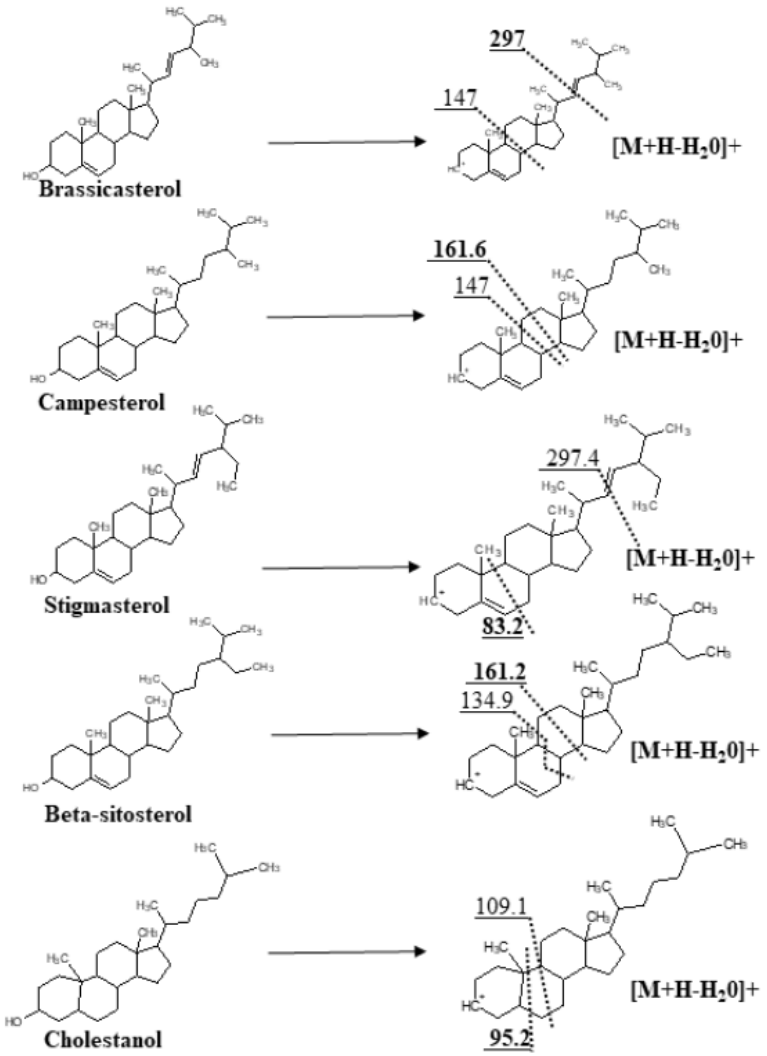
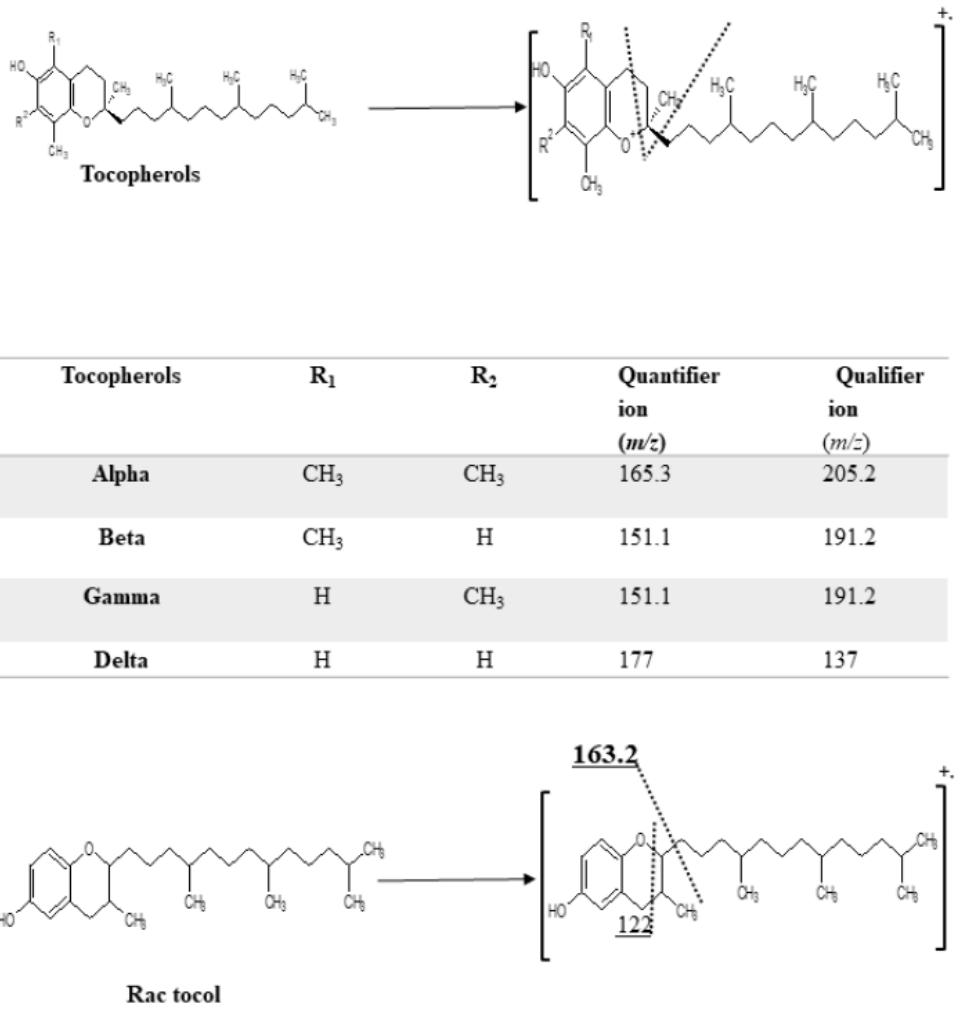


Figure S2.1. Chromatographic separation profile of tocopherols (1-δ tocopherol, 2-β+ γ tocopherol, 3-α tocopherol), PS (4-brassicasterol, 5-campesterol, 6-stigmasterol, and 7-β-sitosterol), rac tocol and cholestanol at varying ratios of acetonitrile: methanol.





Tocopherols	R_1	R_2	Quantifier ion (*m/z*)	Qualifier ion (*m/z*)
Alpha	CH_3	CH_3	165.3	205.2
Beta	CH_3	H	151.1	191.2
Gamma	H	CH_3	151.1	191.2
Delta	H	H	177	137

Figure S2.2. Structure of four PS and four tocopherols abundant in CODD along with internal standards cholestanol and Rac tocol showing quantifier (bolded *m/z*) and qualifier ions (non-bolded *m/z*) used during LC-MS/MS analysis.

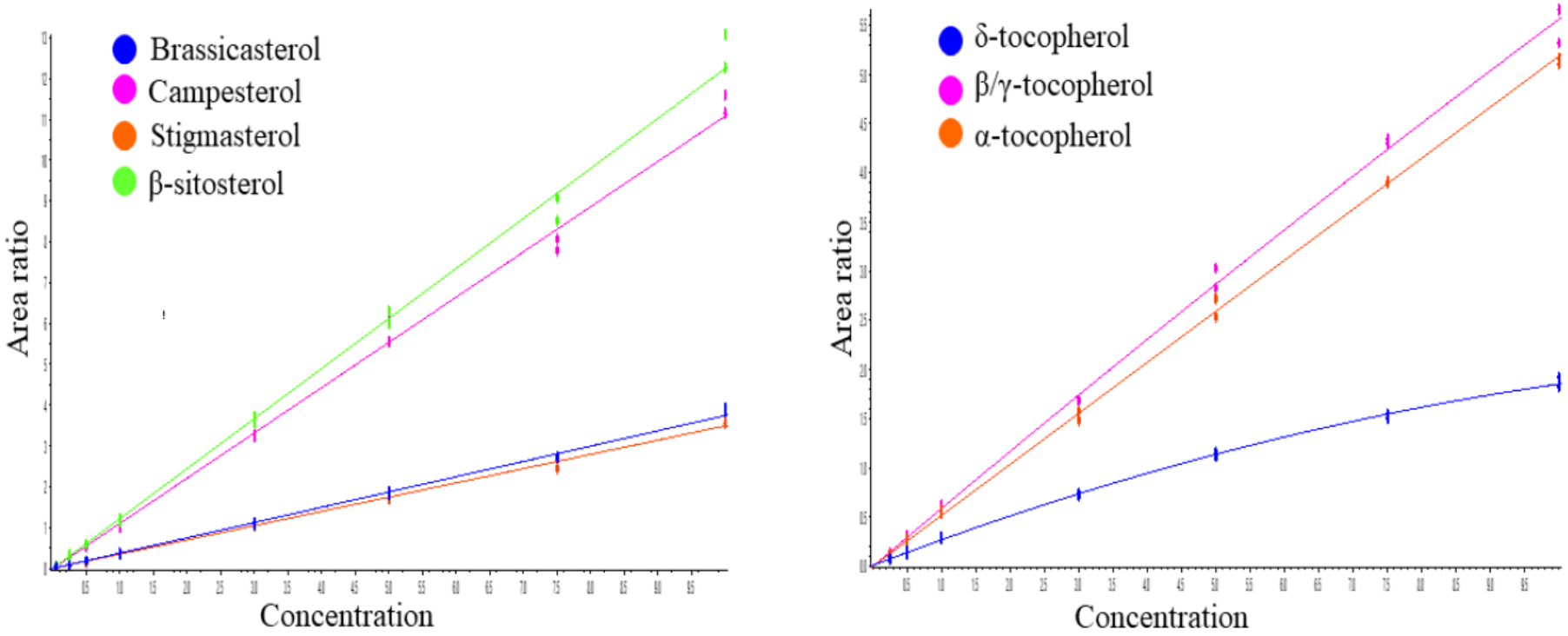


Figure S2.3. Calibration curves for PS (brassicasterol, campesterol, stigmasterol, β-sitosterol) and tocopherols (alpha tocopherol, beta/gamma tocopherol, delta tocopherol.

Table S2.1. Dilution integrity test for PS and tocopherols at 2.5 µg/mL (5 fold dilution) and 7.5 µg/mL (1.7 fold dilution).

Analyte	Dilution Integrity					
	Conc (µg/ml)	**Accuracy (%)**	**CV (%RSD)**	**Conc (µg/ml)**	**Accuracy %**	**CV (%RSD)**
δ-tocopherol	**2.5**	112.6	3.7	**7.5**	99	7.6
γ-tocopherol	**2.5**	104.1	3.5	**7.5**	93	0.9
α-tocopherol	**2.5**	92.8	1.1	**7.5**	85	2.3
Brassicasterol	**2.5**	99	4.9	**7.5**	96.4	4.2
Campesterol	**2.5**	100.4	2.7	**7.5**	98.5	3.5
Stigmasterol	**2.5**	104.7	2.9	**7.5**	102.1	3.5
β-sitosterol	**2.5**	107.3	3.7	**7.5**	104.7	3.3

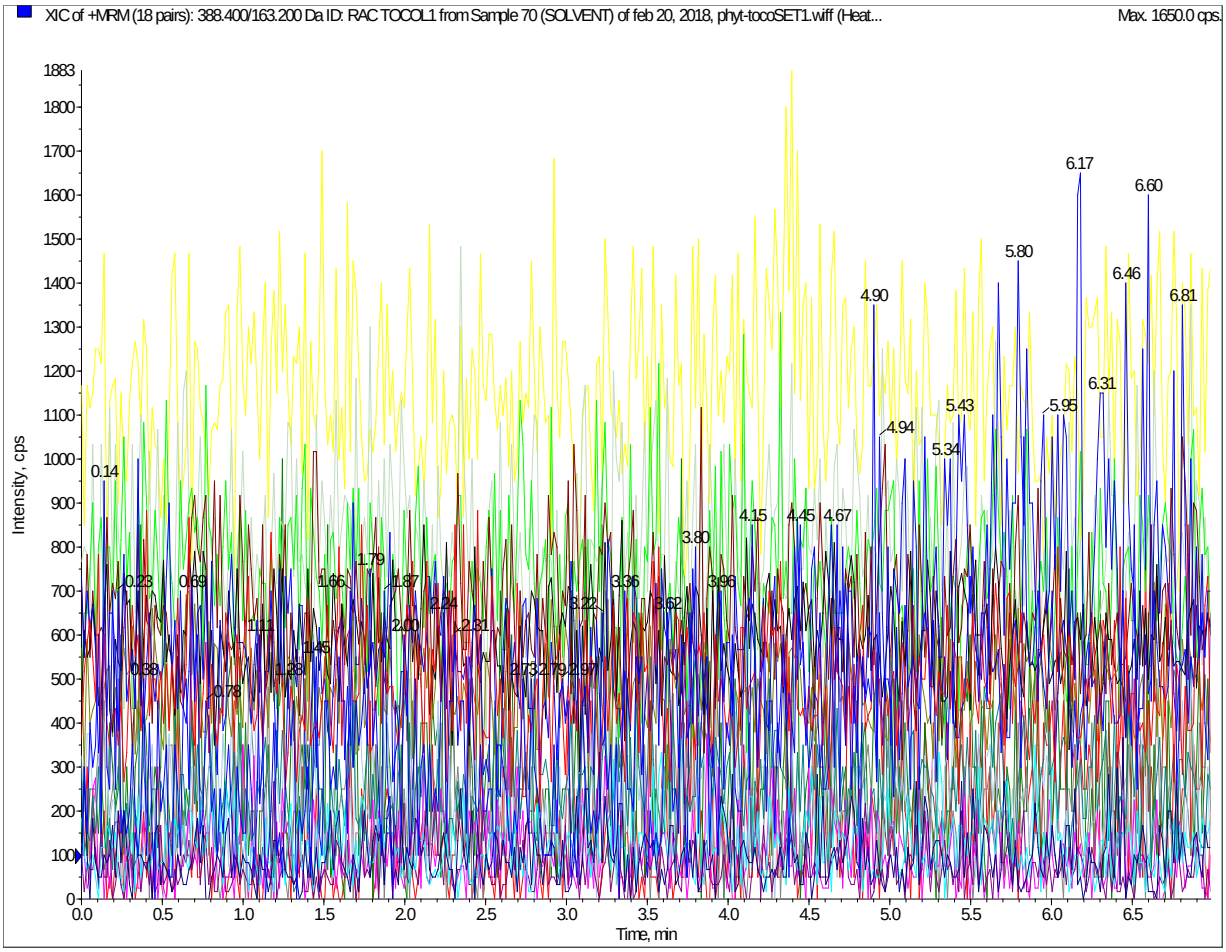


Figure S2.4. LC-MS/MS chromatogram of blank liposomal matrix for MRM transitions of PS and tocopherols

Table S2.2. One month stability (A), autosampler stability (B), benchtop stability (C) and freeze thaw stability (up to 3 cycle) of PS and tocopherols

A.Freeze thaw stability of phytosterols and tocopherols up to 3 cycles

Compound		**QCs**	**Concentration**	**Accuracy**	**Precision**
δ-Tocopherol	Cycle 1	LQC	0.75	112.96	1.34
		MQC	5.5	113.18	1.24
		HQC	9	114.34	2.76
	Cycle 2	LQC	0.75	107.38	5.36
		MQC	5.5	110.20	5.46
		HQC	9	106.58	4.29
	Cycle 3	LQC	0.75	114.28	0.79
		MQC	5.5	112.45	3.23
		HQC	9	114.79	0.89
β+γ- Tocopherol	Cycle 1	LQC	0.75	104.89	2.44
		MQC	5.5	94.00	3.75
		HQC	9	93.97	0.07
	Cycle 2	LQC	0.75	101.00	0.98
		MQC	5.5	105.23	2.98
		HQC	9	101.52	2.65
	Cycle 3	LQC	0.75	104.27	3.48
		MQC	5.5	101.02	0.04
		HQC	9	103.47	1.39
α- Tocopherol	Cycle 1	LQC	0.75	96.98	2.19
		MQC	5.5	92.34	10.06
		HQC	9	102.48	2.10
	Cycle 2	LQC	0.75	100.09	7.81
		MQC	5.5	103.15	9.65
		HQC	9	107.93	2.73
	Cycle 3	LQC	0.75	98.57	0.69
		MQC	5.5	100.15	11.55

		HQC	9	110.43	2.41
Brassicasterol	Cycle 1	LQC MQC HQC	0.15 5.5 9	86.51 89.25 102.19	3.47 2.76 4.03
	Cycle 2	LQC MQC HQC	0.15 5.5 9	93.52 86.03 97.57	0.45 0.77 2.33
	Cycle 3	LQC MQC HQC	0.15 5.5 9	86.94 96.42 87.50	1.62 7.63 4.60
Campesterol	Cycle 1	LQC MQC HQC	0.15 5.5 9	94.76 102.89 106.15	4.46 2.47 2.59
	Cycle 2	LQC MQC HQC	0.15 5.5 9	92.07 99.88 101.76	5.14 5.14 0.64
	Cycle 3	LQC MQC HQC	0.15 5.5 9	92.07 108.24 100.79	6.68 7.68 2.59
Stigmasterol	Cycle 1	LQC MQC HQC	0.15 5.5 9	102.22 104.52 113.45	1.48 7.47 3.69
	Cycle 2	LQC MQC HQC	0.15 5.5 9	94.2 101.34 109.53	3.81 7.68 3.07
	Cycle 3	LQC MQC HQC	0.15 5.5 9	94.90 103.81 109.42	0.32 12.45 2.10
β-Sitosterol	Cycle 1	LQC MQC HQC	0.15 5.5 9	95.65 94.15 98.35	4.31 5.91 0.71
	Cycle 2	LQC MQC HQC	0.15 5.5 9	94.95 95.51 97.37	2.35 0.12 5.91
	Cycle 3	LQC MQC	0.15 5.5	88.64 95.00	0.27 11.35

		HQC	9	93.88	2.60

B.One month stability

i. First week stability

Compound		Concentration (µg/mL)		
			Accuracy (%)	Precision (%RSD)
δ-Tocopherol				
	LQC	0.75	104.8	5.9
	MQC	5.5	97.4	6.2
	HQC	9	99.0	12.2
β+γ- Tocopherol				
	LQC	0.75	95.4	5.3
	MQC	5.5	98.3	3.6
	HQC	9	102.2	7.5
α- Tocopherol				
	LQC	0.75	91.2	2.8
	MQC	5.5	92.5	4.5
	HQC	9	92.8	5.3
Brassicasterol				
	LQC	0.15	110.6	1.8
	MQC	5.5	100.9	4.6
	HQC	9	99.4	5.0
Campesterol				
	LQC	0.15	104.4	1.3
	MQC	5.5	99.4	2.1
	HQC	9	97.3	4.0
Stigmasterol				
	LQC	0.15	105.4	1.9
	MQC	5.5	98.9	2.3
	HQC	9	100.2	4.6

β-Sitosterol				
	LQC	0.15	97.8	2.3
	MQC	5.5	94.3	5.1
	HQC	9	92.8	2.5

ii. <u>Second week stability</u>

Compound		**Concentration (µg/mL)**		
			Accuracy (%)	**Precision (%RSD)**
δ-Tocopherol				
	LQC	0.75	107.4	2.8
	MQC	5.5	104.8	7.9
	HQC	9	103.6	7.2
β+γ- Tocopherol				
	LQC	0.75	110.0	1.3
	MQC	5.5	104.8	3.9
	HQC	9	96.2	2.9
α- Tocopherol				
	LQC	0.75	103.3	1.7
	MQC	5.5	104.5	4.7
	HQC	9	104.2	3.3
Brassicasterol				
	LQC	0.15	108.3	9.5
	MQC	5.5	98.0	3.1
	HQC	9	97.8	6.5
Campesterol				
	LQC	0.15	97.2	8.8
	MQC	5.5	98.7	5.4
	HQC	9	97.4	6.7
Stigmasterol				
	LQC	0.15	104.7	10.3
	MQC	5.5	99.4	5.7
	HQC	9	97.3	6.6

β-Sitosterol				
	LQC	0.15	89.4	8.1
	MQC	5.5	91.9	4
	HQC	9	90.5	7.1

iii. Third week stability

Compound		**Concentration (µg/mL)**		
			Accuracy (%)	**Precision (%RSD)**
δ-Tocopherol				
	LQC	0.75	103.74	0.27
	MQC	5.5	101.89	9.86
	HQC	9	92.31	2.07
β+γ- Tocopherol				
	LQC	0.75	107.52	5.62
	MQC	5.5	101.35	3.33
	HQC	9	98.47	1.31
α- Tocopherol				
	LQC	0.75	113.93	0.07
	MQC	5.5	107.86	4.47
	HQC	9	101.18	1.24
Brassicasterol				
	LQC	0.15	99.80	5.01
	MQC	5.5	100.08	3.09
	HQC	9	95.17	4.59
Campesterol				
	LQC	0.15	105.83	6.60
	MQC	5.5	106.81	3.56
	HQC	9	100.87	3.66

Stigmasterol				
	LQC	0.15	103.14	12.51
	MQC	5.5	104.98	5.50
	HQC	9	99.02	7.14
β-Sitosterol				
	LQC	0.15	98.92	9.56
	MQC	5.5	98.92	5.00
	HQC	9	90.70	3.34

Iv Fourth week stability

Compound		**Concentration (µg/mL)**		
			Accuracy (%)	**Precision (%RSD)**
δ-Tocopherol				
	LQC	0.75	112.96	1.34
	MQC	5.5	113.18	1.24
	HQC	9	114.34	2.76
β+γ- Tocopherol				
	LQC	0.75	104.89	2.44
	MQC	5.5	94.00	3.75
	HQC	9	93.97	0.07
α- Tocopherol				
	LQC	0.75	96.98	2.19
	MQC	5.5	92.34	10.06
	HQC	9	102.48	2.10
Brassicasterol				
	LQC	0.15	86.51	3.47
	MQC	5.5	89.25	2.76
	HQC	9	102.19	4.03
Campesterol				
	LQC	0.15	94.76	4.46

	MQC	5.5	102.89	2.47
	HQC	9	106.15	2.59
Stigmasterol				
	LQC	0.15	102.22	1.48
	MQC	5.5	104.52	7.47
	HQC	9	113.45	3.69
β-Sitosterol				
	LQC	0.15	95.65	4.31
	MQC	5.5	94.15	5.91
	HQC	9	98.35	0.71

C. <u>Autosampler stability of analytes</u>

Auto sampler Compound		**Concentration (µg/mL)**		
			Accuracy (%)	**Precision (%RSD)**
δ-Tocopherol				
	LQC	0.75	113.47	0.92
	MQC	5.5	106.75	4.48
	HQC	9	95.53	2.98
β+γ- Tocopherol				
	LQC	0.75	109.48	1.07
	MQC	5.5	98.46	0.56
	HQC	9	92.13	2.56
α- Tocopherol				
	LQC	0.75	86.70	2.38
	MQC	5.5	88.22	2.11
	HQC	9	85.56	2.09
Brassicasterol				
	LQC	0.15	104.16	1.94
	MQC	5.5	102.36	1.72
	HQC	9	103.93	3.94

Campesterol				
	LQC	0.15	107.48	2.58
	MQC	5.5	99.48	4.47
	HQC	9	104.98	9.82
Stigmasterol				
	LQC	0.15	100.89	4.40
	MQC	5.5	99.68	2.21
	HQC	9	99.19	9.62
β-Sitosterol				
	LQC	0.15	106.05	1.36
	MQC	5.5	102.92	0.14
	HQC	9	100.58	9.34

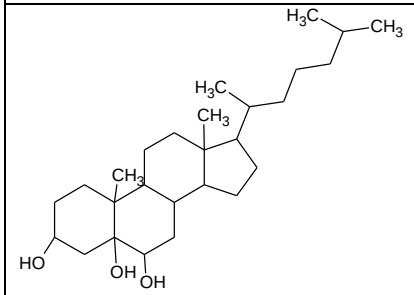
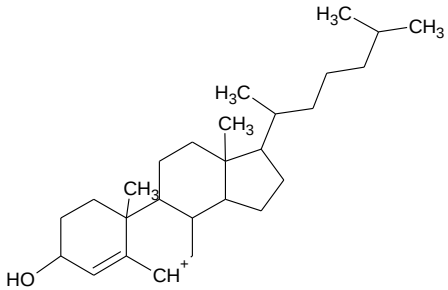
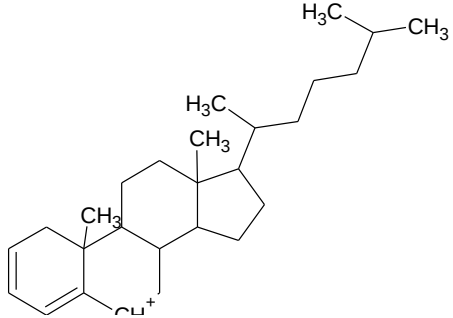
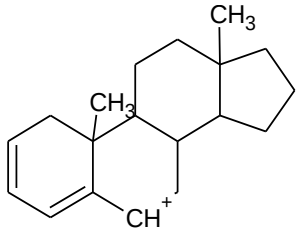
D. <u>Benchtop stability of analytes</u>

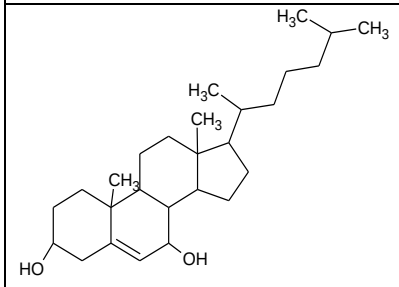
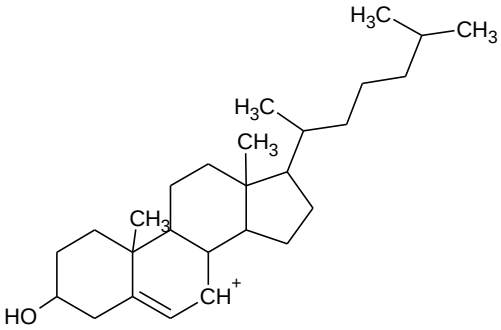
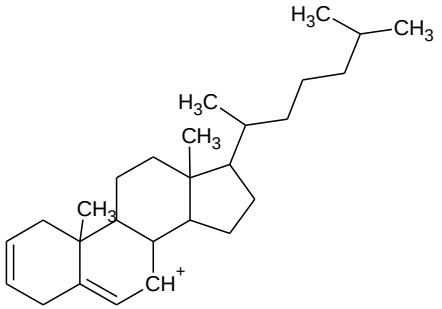
Compound		**Concentration (µg/mL)**		
			Accuracy (%)	**Precision (%RSD)**
δ-Tocopherol				
	LQC	0.75	109.95	2.35
	MQC	5.5	106.59	2.75
	HQC	9	98.64	12.44
β+γ- Tocopherol				
	LQC	0.75	113.23	2.33
	MQC	5.5	103.58	10.81
	HQC	9	98.31	9.92
α- Tocopherol				
	LQC	0.75	95.14	2.18
	MQC	5.5	88.83	6.55
	HQC	9	90.63	10.75
Brassicasterol				
	LQC	0.15	95.19	14.14

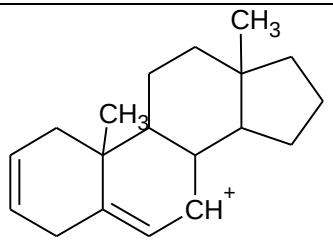
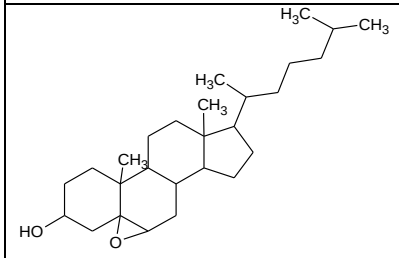
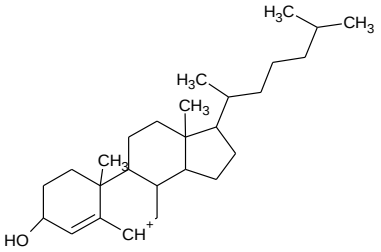
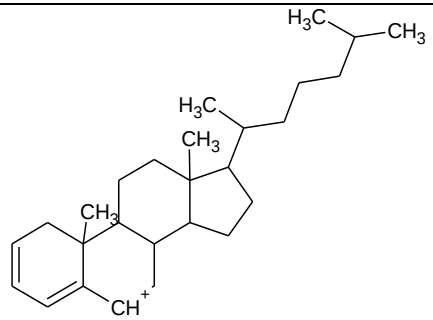
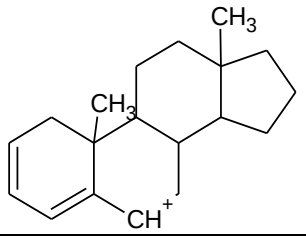
	MQC	5.5	99.89	0.71
	HQC	9	93.13	11.88
Campesterol				
	LQC	0.15	99.66	4.98
	MQC	5.5	99.26	2.64
	HQC	9	101.63	12.28
Stigmasterol				
	LQC	0.15	93.68	0.37
	MQC	5.5	91.71	0.75
	HQC	9	97.39	14.0
β-Sitosterol				
	LQC	0.15	90.38	2.27
	MQC	5.5	102.92	0.14
	HQC	9	93.93	13.33

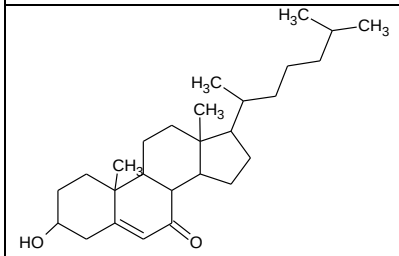
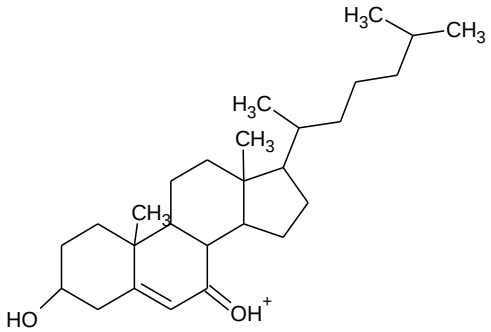
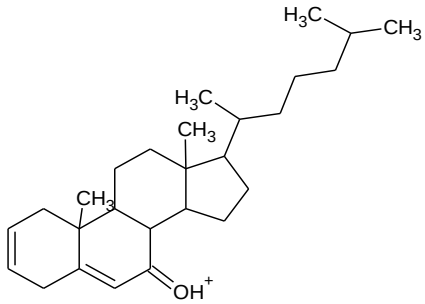
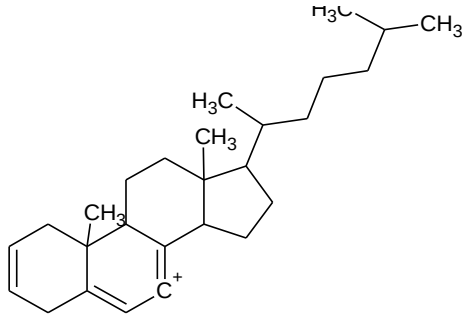
Appendix II

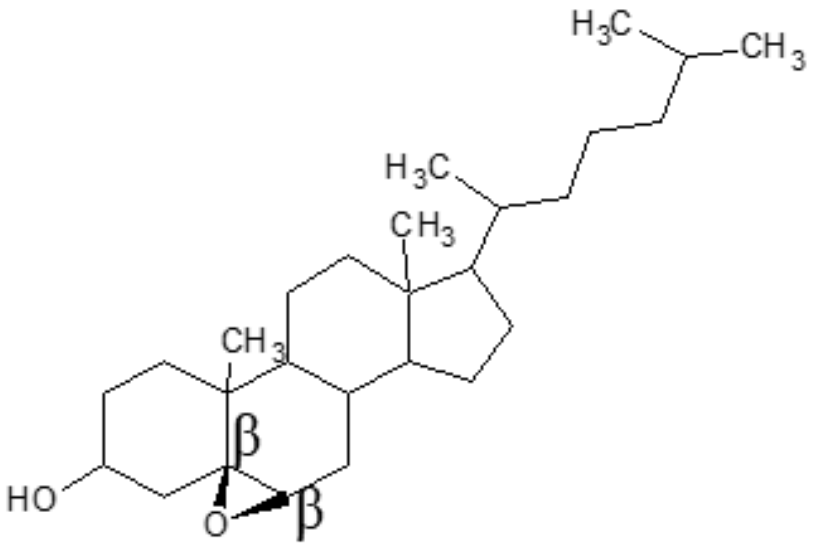
Table S4.1. Precursor and monitored product ions of cholesterol oxidation products

Cholesterol oxidation products	**Precursor ion**	**Product ion**
 **5,6-dihydroxycholesterol**	 *m/z* 385.3 **[M + H - 2H₂O]** $^{+}$	 *m/z* 367.3 **[M + H - 3H₂O]** $^{+}$  *m/z* 255.3 **[M + H - 3H₂O – side chain]** $^{+}$

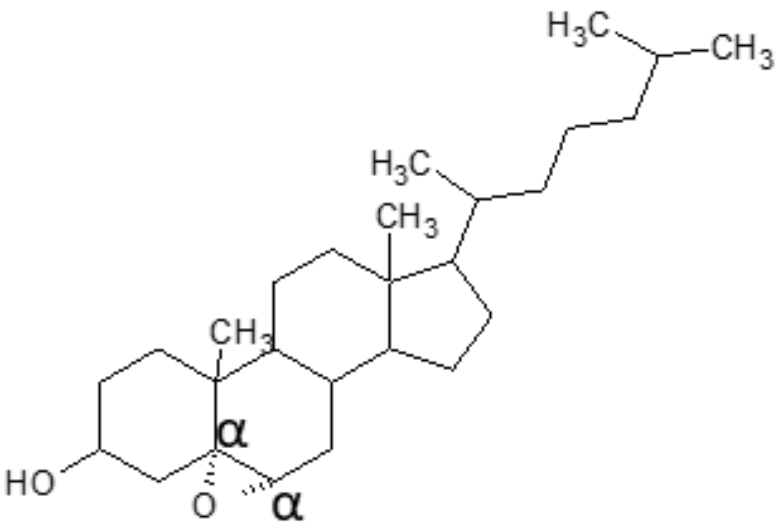
 **7-hydroxycholesterol**	 *m/z* 385.3 **[M + H - H_2O]** $^+$	 *m/z* 367.3 **[M + H - 2H_2O]** $^+$

		 *m/z* 255.3 **[M + H - 2H$_2$O – side chain]$^+$**
 **5,6-epoxycholesterol**	 *m/z* 385.3 **[M + H - H$_2$O]$^+$**	 *m/z* 367.3 **[M + H - 2H$_2$O]** 

		m/z 255.3 **[M + H - 2H$_2$O – side chain]$^+$**
 **7-ketocholesterol**	 *m/z* 401.3 **[M + H]$^+$**	 *m/z* 383.3 **[M + H - H$_2$O]$^+$**  *m/z* 365.3 **[M + H - 2H$_2$O]$^+$**



5β,6β epoxycholesterol



5α,6α epoxycholesterol

Figure S4.1. Structure of stereoisomers of 5,6 epoxycholesterol (5β, 6β epoxycholesterol and 5α, 6α epoxycholesterol.

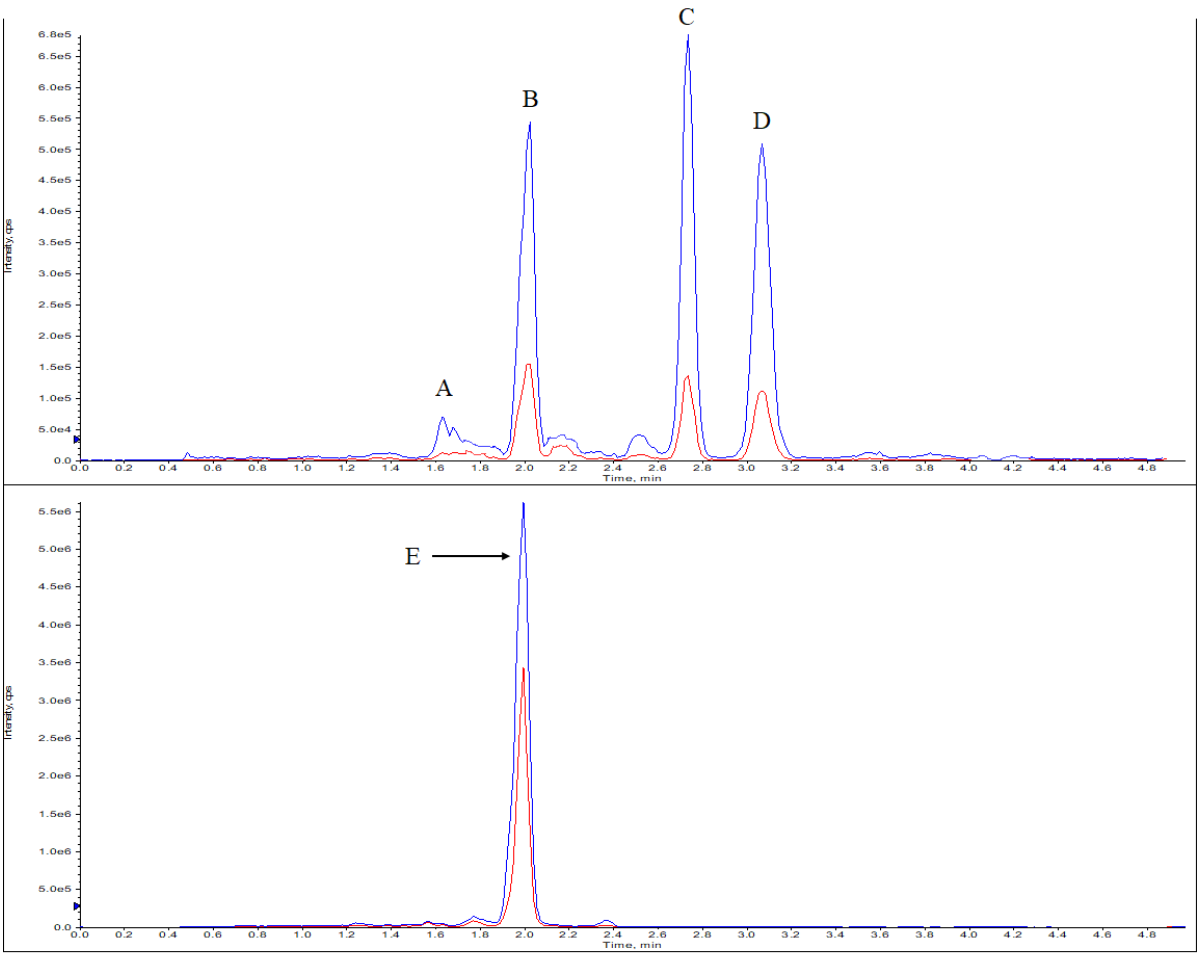


Figure S4.2. Extracted ion chromatogram of campesterol oxidation products; 5,6-dihydroxycampesterol (A), 7-hydroxycampesterol (B), 5,6-epoxycampesterol (C is 5β,6β-epoxy and D is 5α,6α-epoxy), and 7-ketocampesterol (E). A, B, C and D shares same MRM transition which makes them show up together in extracted chromatogram. It is only E with unique MRM transition.

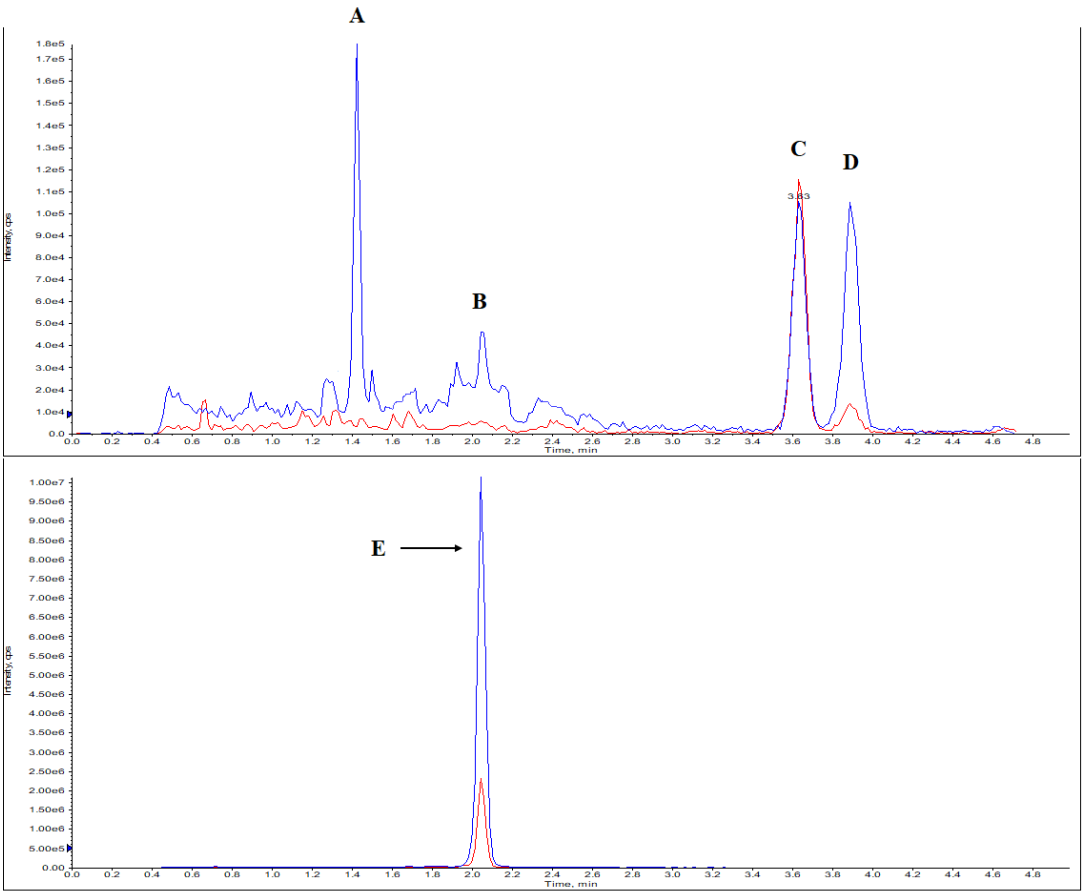


Figure S4.3. Extracted ion chromatogram of stigmasterol oxidation products; 5,6-dihydroxystigmasterol (A), 7-hydroxystigmasterol (B), 5,6-epoxystigmasterol (C is 5β,6β-epoxy and D is 5α,6α-epoxy), and 7-ketostigmasterol (E). A, B, C and D shares same MRM transition which makes them show up together in extracted chromatogram. It is only E with unique MRM transition.

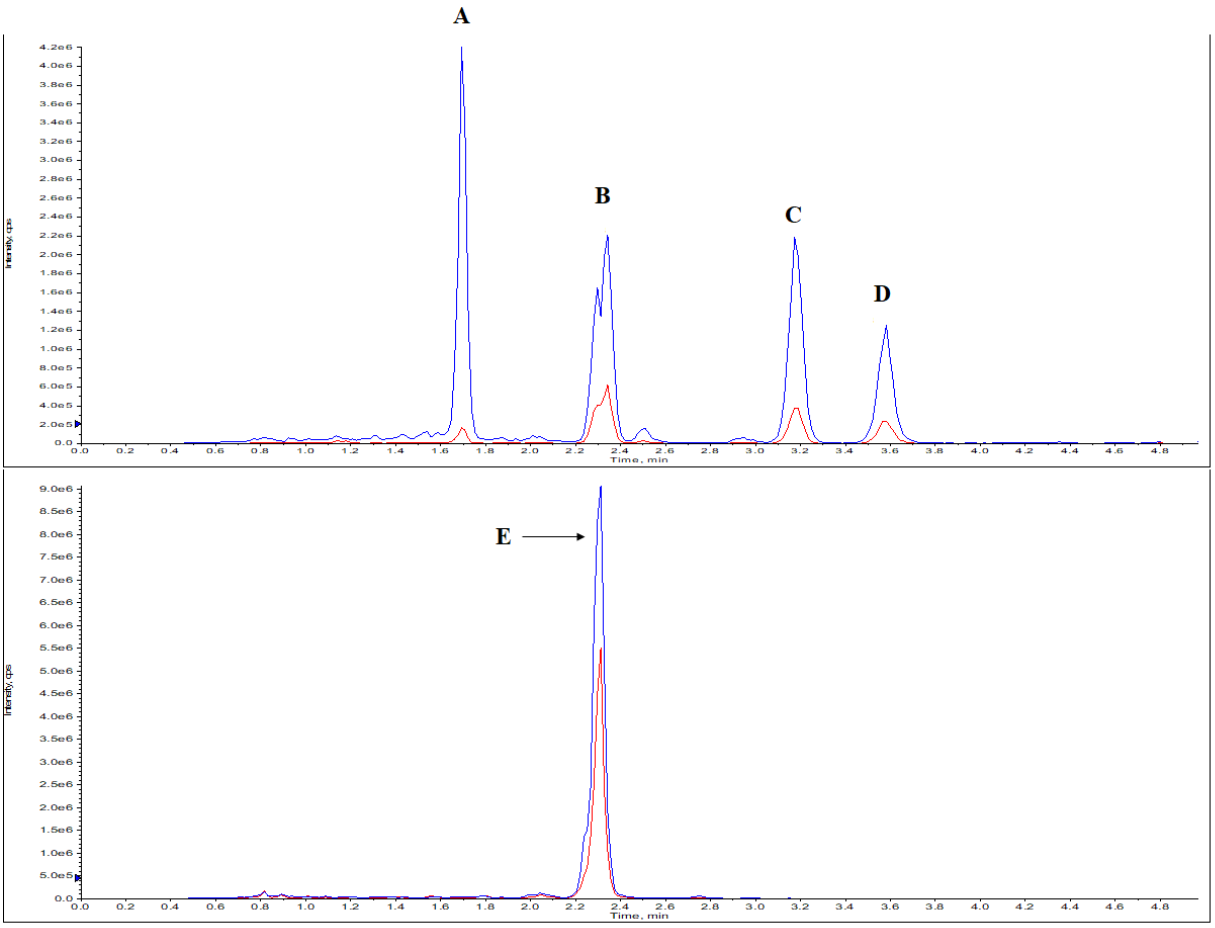


Figure S4.4. Extracted ion chromatogram of β-sitosterol oxidation products; 5,6-dihydroxysitosterol (A), 7-hydroxysitosterol (B), 5,6-epoxysitosterol (C is 5β,6β-epoxy and D is 5α,6α-epoxy), and 7-ketositosterol (E). A, B, C and D shares same MRM transition which makes them show up together in extracted chromatogram. It is only E with unique MRM transition.

Table S4.2. Inter-day and intra-day accuracy and precision of cholesterol oxidation products.

COPs		Concentration (μg mL^{-1})	Intra-day (n=6)		Inter-day 1 (n=6)		Inter-day 2 (n=6)		Inter-day 3 (n=6)	
			Accuracy (%)	Precision (%RSD)	Accuracy (%)	Precision (%RSD)	Accuracy (%)	Precision (%RSD)	Accuracy (%)	Precision (%RSD)
5,6-dihydroxy	LLOQC	0.05	94.62	2.88	85.16	5.15	103.86	6.21	96.19	4.03
	LQC	0.15	97.60	5.97	86.71	2.49	105.05	0.05	92.19	6.26
	MQC	2.5	101.81	5.04	96.59	2.24	106.12	3.82	93.75	2.86
	HQC	4	94.89	3.11	98.06	2.69	101.70	2.39	95.44	3.72
7-hydroxy	LLOQC	0.05	97.34	7.18	82.65	4.77	90.24	5.38	89.32	3.95
	LQC	0.15	108.9	6.09	90.19	1.52	103.90	4.17	97.22	3.91
	MQC	2.5	94.80	2.04	98.73	2.79	107.65	3.03	101.41	3.18
	HQC	4	97.04	1.89	97.05	2.37	104.28	2.05	100.51	2.74
7-keto	LLOQC	0.05	93.87	4.09	80.32	0.43	88.39	1.90	93.75	3.06
	LQC	0.15	106.26	2.96	85.37	2.59	104.89	0.57	101.39	3.45
	MQC	2.5	108.89	5.46	94.92	1.82	109.29	1.15	103.06	3.60
	HQC	4	95.20	2.00	91.20	0.74	107.98	2.75	102.10	4.02
5,6-epoxy	LLOQC	0.05	109.34	7.00	85.80	3.14	102.60	5.03	105.17	1.97
	LQC	0.15	107.26	4.51	87.41	2.11	103.43	7.02	100.15	3.49
	MQC	2.5	111.95	6.90	94.35	2.73	107.14	1.67	101.86	3.46
	HQC	4	100.68	2.63	93.18	2.31	106.37	3.39	106.42	3.24

Table S4.3. Dilution integrity assessment of cholesterol oxidation products at 2.5 µg/mL and 1.5 µg/mL (5-fold dilution).

Analyte	Dilution Integrity					
	Conc (µg mL^{-1})	**Accuracy (%)**	**CV (%RSD)**	**Conc (µg mL^{-1})**	**Accuracy %**	**CV (%RSD)**
5,6-dihydroxy	**1.5**	96.1	6.0	**2.5**	100.8	10.4
7-hydroxy	**1.5**	92.9	3.0	**2.5**	95.5	1.7
7-keto	**1.5**	103.7	4.6	**2.5**	94.3	3.2
5,6-epoxy	**1.5**	96.7	5.8	**2.5**	101.8	1.5

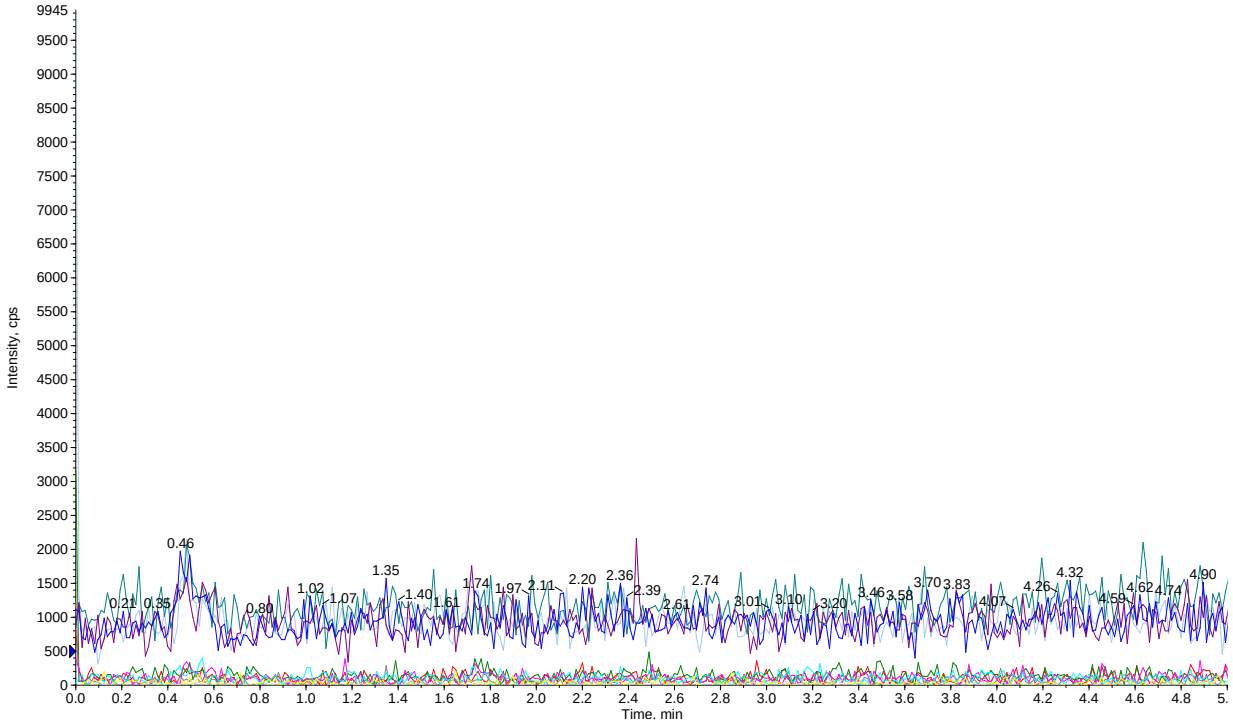


Figure S4.5. LC-MS/MS chromatogram of blank liposomal matrix for MRM transitions of cholesterol oxidation products and PS oxidation products.

Table S4.4. Matrix effect of cholesterol oxidation products at various QCs concentration.

Analytes	**QCs µg/ml**	**Matrix effect (%)**	**Mean Matrix effect (%)**
5,6-dihydroxy	0.15(LQC) 2.5(MQC) 4(HQC)	87.21 94.60 96.40	92.74 ± 4.87
7-hydroxy	0.15(LQC) 2.5(MQC) 4(HQC)	98.30 97.13 88.82	94.75 ± 5.17
7-keto	0.15(LQC) 2.5(MQC) 4(HQC)	98.63 88.18 96.30	94.37 ± 5.49
5,6-epoxy	0.15(LQC) 2.5(MQC) 4(HQC)	89.17 97.39 96.30	94.29 ± 4.46

Table S4.5. Stability evaluation of liposomal phytosterols formulations prepared with and without tocopherols stored at various temperatures.

PS	Phytosterols Oxidation Products (µg per 10mg of PS)	Initial POP content Day 0		Final POP content Day 30					
				-20 °C (Lyophilized)		4 °C (Lyophilized)		Bench top (Lyophilized)	
		A	B	A	B	A	B	A	B
Brassicasterol	5,6-dihydroxy	ND	ND	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
	7-hydroxy	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
	7-keto	11.60 ± 1.39	10.03 ± 0.84	12.50 ± 1.10	11.3 ± 1.43	12.03 ± 0.76	11.66 ± 0.93	11.78 ±1.25	11.16 ± 2.10
	5,6-epoxy	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
Campesterol	5,6dihydroxy	ND	ND	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
	7-hydroxy	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ

	7-keto	<LLOQ	<LLOQ	0.85 ± 2.30	0.90 ± 1.82	1.08 ± 1.61	1.05 ± 1.37	1.15 ± 1.81	1.05 ± 1.99
	5,6-epoxy	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
β-sitosterol	5,6-dihydroxy	ND	ND	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
	7-hydroxy	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ
	7-ketositosterol	<LLOQ	<LLOQ	1.26 ± 2.17	1.10 ± 0.91	1.00 ± 1.43	0.93 ± 1.33	1.08 ± 0.89	1.016 ± 1.72
	5,6-epoxy	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ	<LLOQ

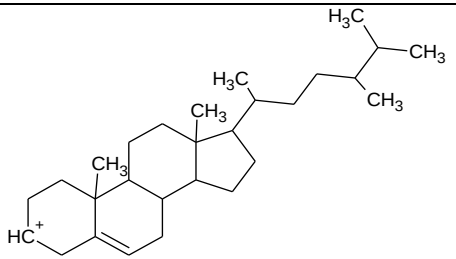
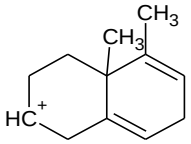
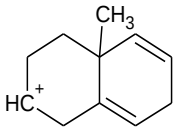
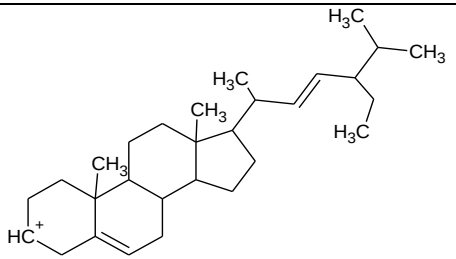
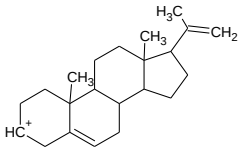
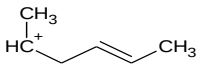
[A] Liposomes containing PS (µg per 10mg of PS)

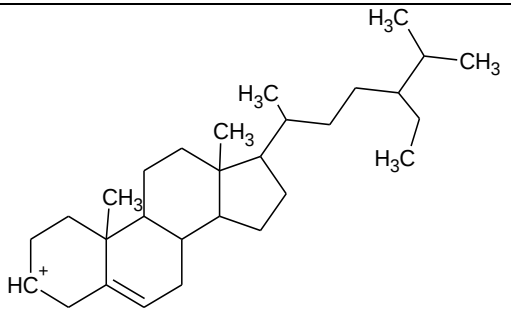
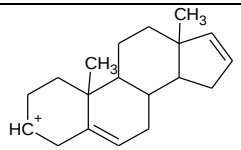
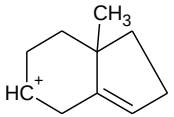
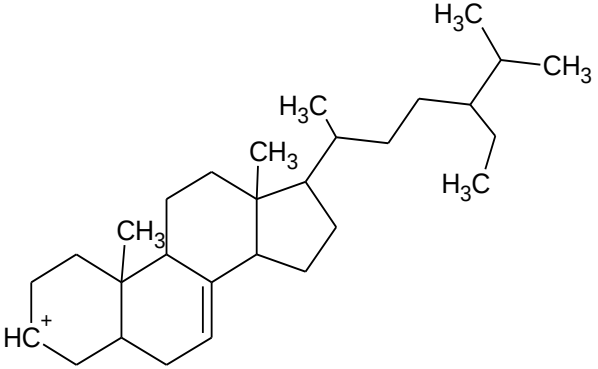
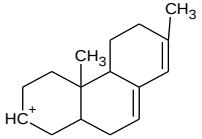
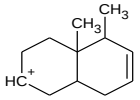
[B] Liposomes containing PS and tocopherols (µg per 10mg of PS)

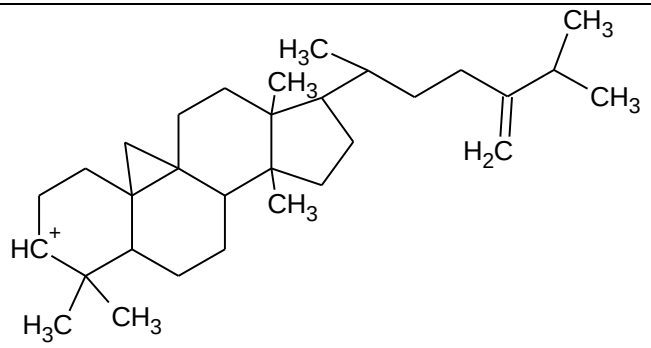
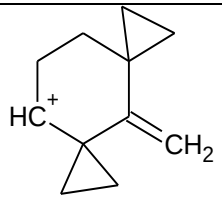
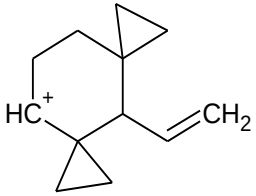
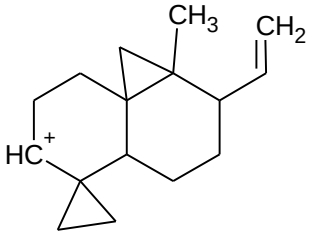
ND: No peak detected

LLOQ: Lower limit of quantification

Table S4.6. Theoretical diagnostic product ions of phytosterols that is expected to be present in extra virgin olive oil (EVOO).

Phytosterols	**Precursor ion**	**Diagnostic product ion**
Campesterol	 *m/z* 383.4	 *m/z* 161.1  *m/z* 147.1
Stigmasterol	 *m/z* 397.4	 *m/z* 297.4  *m/z* 83.2

β-sitosterol	 *m/z* 397.4	 *m/z* 255.2  *m/z* 135.1
Avenasterol	 *m/z* 397.4	 *m/z* 215  *m/z* 163

Methylenecycloartenol	 *m/z* 423.4	 *m/z* 147.1  *m/z* 161.13  *m/z* 215.2

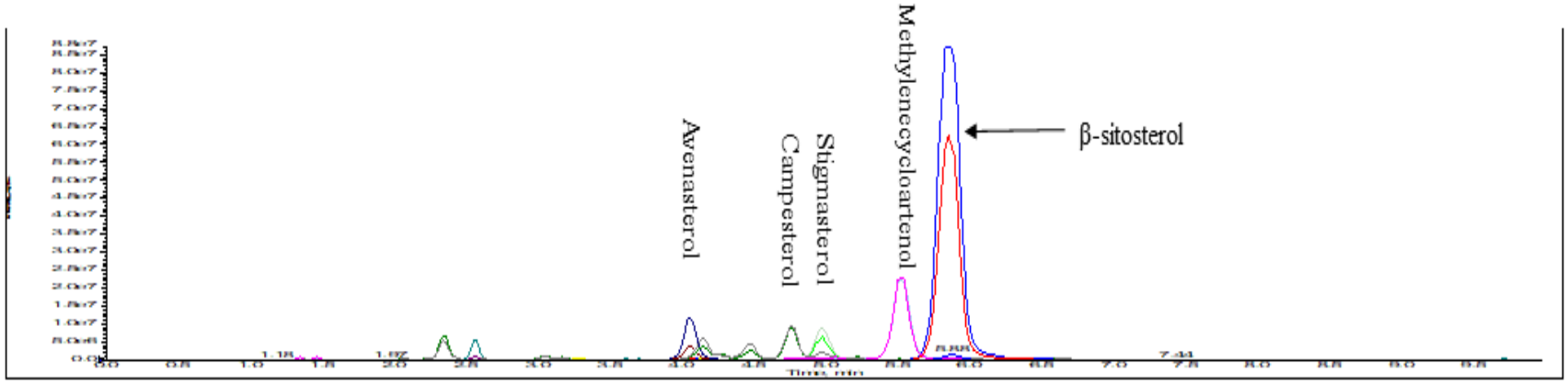


Figure S4.6. LC-MS/MS chromatogram of PS present in unsaponifiable fraction of extra virgin olive oil.

Appendix III

Table S5.1. Average entrapment efficiency of phytosterols in liposomes along with the average particle size and zeta potential of the developed liposomal formulations expressed as mean ± standard deviation.

PS entrapment efficiency (%)			**Size (nm)**	**Zeta potential (mV)**
Brassicasterol	Campesterol	β-sitosterol		
96.6 ± 2.3	95.6 ± 5.0	94.3 ± 4.8	186.3 ± 4.4	-13.0 ± 5.0

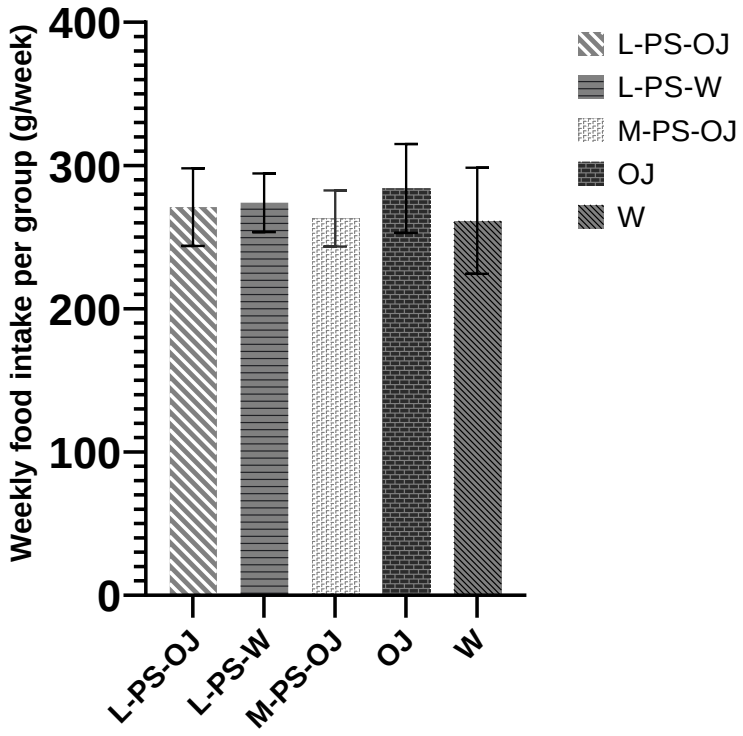


Figure S5.1. Weekly food intake (purified hamster diet containing high fat and 0.5% cholesterol) in the experimental groups during a month of experimental trial. Average of two cohorts were calculated to determine weekly food intake. No significant differences in the food intake among the groups were observed. Abbreviations: L= Liposomal, PS = Phytosterol, M = Marketed, OJ = Orange juice, and W = Water. N values: L-PS-OJ (N=2/3 animals per cage), L-PS-W (N=2/3 animals per cage), M-PS-OJ (N=2/3 animals per cage), OJ (N=2/3 animals per cage), and W (N=2/3 animals per cage).

Table S5.2. Absorbance of visible light of wavelength 530 nm by outliers and the normal samples in the hamster serum in week 4 of the experimental trial.

Absorbance of outliers [AU]	**Absorbance of random normal samples [AU]**
1.493 (Outlier 1)	0.402 (sample from group 2: liposomal phytosterols in water,1st cohort)
1.636 (Outlier2)	0.419 (sample from group 3: marketed phytosterols containing orange juice, 1st cohort)
1.64 (Outlier 3)	0.407 (sample from group 4: control water, 1st cohort)

Table S5.3. Calculated LDL-concentration in treatment and control groups containing outliers in week 4.

Liposomal phytosterols in water [mg/dL] (Cohort 1)	**Marketed phytosterols containing orange juice [mg/dL] (Cohort 1)**	**Control water [mg/dL] (Cohort 2)**
727.7	485.0	377.1
717.19	425.1	2888.6 (Outlier 3)
421.4	534.8	623.3
2541.1 (Outlier 1)	465.5	664.4
753.4	2917.7 (Outlier 2)	280.7
630.7	220.03	-