ENZYMATIC SYNTHESIS AND PROPERTIES OF SOME NOVEL PHYTOSTEROL DERIVATIVES

BY

© ZHULIANG TAN

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Memorial University of Newfoundland

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ABSTRACT

Phytosterols and their derivatives occur naturally in the cell membranes of plants and are present at low levels in grains, fruits, and vegetables as well as marine algae. In addition to their well-known effect in lowering low-density lipoprotein cholesterol, phytosterols possess other biological activities, such as antiinflammatory, antiatherogenicity, and anticarcinogenic potential. Thus, they have recently received much scientific and commercial attention for the production of nutraceuticals and ingredients for development of functional foods. While most of the research and commercial interest has been on phytosteryl esters with vegetable oil fatty acid mixtures of different chain length, the objectives of this work were to explore enzymatic preparation of phytosteryl esters with phenolic acids or specific fatty acids of interest, such as caprylic acid, oleic acid and docosahexaenoic acid. Phenolic acids are potent antioxidants that scavenge free radicals and other reactive oxygen species. Caprylic acid, oleic acid and docosahexaenoic acid are representatives of medium-chain, long-chain monounsaturated and long-chain polyunsaturated fatty acids, respectively. The health benefits of these fatty acids are well documented. Esterification of phytosterols with phenolic acids, caprylic acid, oleic acid or docosahexaenoic acid may render them better physiochemical properties such as lipid solubility, miscibility, oxidative stability and hence bioactivity and bioavailability. Thus, novel phytosteryl esters, prepared enzymatically, may offer the health benefits of phytosterols and those of phenolic acids, caprylic acid, oleic acid and docosahexaenoic acid, possibly in an additive or synergistic manner. This thesis describes methods for successful enzymatic or chemoenzymatic preparation of selected phytosteryl esters. The

identity of the phytosteryl esters so produced was confirmed by using different analytical methods. The enzymatic synthesis of phytosteryl caprylates was optimized using response surface methodology. The antioxidant activity of phytosteryl phenolates was determined using different *in vitro* assays in a variety of model systems. They exhibited antioxidant activity that was system-dependent and followed different antioxidant mechanisms. Phytosteryl phenolates, especially phytosteryl caffeates, provide an opportunity for future use as food antioxidants. The cholesterol-lowering effect of phytosteryl oleates and docosahexaenoates was evaluated using an apo-E deficient mouse model. Preliminary results indicated that they may be used as excellent ingredients in nutraceuticals and functional foods for cholesterol-lowering purposes. However, phytosteryl oleates and docosahexaenoates exhibited no triacylglycerol-lowering effect in this animal model; in fact they increased the total plasma triacylglycerols level, as expected for this model. Therefore, other animal models may be employed to determine their triacylglycerol-lowering effect. Further research on the evaluation of biological activities of phytosteryl esters so prepared is necessary in order to shed further light on the extent of the beneficial health effects of conjugated compounds reported in this thesis.

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List of Abbreviations

AA	- Arachidonic acid
AAPH	- 2,2'-Azobis(2-amidinopropane) dihydrochloride
ABCA1	- Adenosine triphosphate binding cassette A1
AH	- Antioxidants
AIBN	- α,α-Azobisisobutyronitrile
ALA	- α-Linolenic acid
ANOVA	- Analysis of variance
AOCS	- American Oil Chemists' Society
ATR	- Attenuated Total Reflectance
AUC	- Area under the curve
BDE	- Bond dissociation energy
B-B	- Box-Behnken
BHA	- Butylated hydroxyanisole
BHT	- Butylated hydroxytoluene
B-PE	- *β*-Phycoerythrin
CA	- Caffeic acid
CCD	- Central composite design
CCF	- Central composite face-centred
CD	- Conjugated dienes
CI-MS	- Chemical ionization-mass spectroscopy
COE	- Cholesterol esterase

COPS	- Cholesterol oxide products
CPA	- Caprylic acid
CVD	- Cardiovascular disease
DAD	- Diode array detector
DE	- Degree of esterification
DHASCO	- Docosahexaenoic acid single cell oil
DHA	- Docosahexaenoic acid
DMSO-d_6	- Dimethyl sulphoxide-d6
DPA	- Docosapentaenoic acid
DPPH	- 2,2-Diphenyl-1-picrylhydrazyl
EC_{50}	- Concentration to decrease concentration of test free radical by 50%
EFA	- Essential fatty acids
EI	- Electronic ionization
EPA	- Eicosapentaenoic acid
EPR	- Electron paramagnetic resonance
ESI-MS	- Electrospray ionization-mass spectroscopy
ET	- Electron transfer
FA	- Ferulic acid
FDA	- Food and Drug Administration
FFA	- Free fatty acids
FID	- Flame ionization detector
FP	- Free phytosterols

FTIR	- Fourier-transform infrared
GC	- Gas chromatography
GC-MS	- Gas chromatography-mass spectrometry
GRAS	- Generally recognized as safe
HAAs	- Heterocyclic aromatic amines
HAT	- Hydrogen atom transfer
HDL	- High density lipoprotein
H & E	- Hematoxylin and eosin
HLB	- Hydrophilic-lipophilic balance
HPLC	- High performance liquid chromatography
HPLC-MS	- High liquid chromatography-mass spectrometry
HPLC-MS/MS	- High performance liquid chromatography-mass spectrometry/mass spectrometry
IP	- Ionization potential
IT	- Induction time
IUB	- International Union of Biochemistry
IUPAC	- International Union of Pure and Applied Chemistry
LA	- Linoleic acid
LCFA	- Long-chain fatty acids
LDL	- Low-density lipoprotein
LDL-C	- Low-density lipoprotein cholesterol
LPL	- Lipoprotein lipase
MCFA	- Medium-chain fatty acids

Me	- Methyl group
MUFA	- Monounsaturated fatty acids
NI	- Negative ion
NMR	- Nuclear magnetic resonance
NP	- Normal phase
$ONOO^-$	- Peroxynitrite
ORAC	- Oxygen radical absorbance capacity
OVAT	- One-variable-at-a-time
PBS	- Phosphate buffered saline
PC	- Phytosteryl caffeates
PF	- Phytosteryl ferulates
PG	- Propyl gallate
PI	- Positive ion
PS	- Phytosteryl sinapates
PUFA	- Polyunsaturated fatty acids
PV	- Phytosteryl vanillates
RMCD	- Randomly methylated β-cyclodextrin
$R^{\cdot}$	- Lipid radical/alkyl radical
RH	- Unsaturated lipids
$RO^{\cdot}$	- Alkoxyl radical
$ROO^{\cdot}$	- Peroxyl radical
ROOH	- Hydroperoxides

ROS	- Reactive oxygen species
RP	- Reverse phase
RSM	- Response surface methodology
SA	- Sinapic acid
SCFA	- Short-chain fatty acids
SFA	- Saturated fatty acids
TAG	- Triacylglycerols
TBA	- Thiobarbituric acid
TBARS	- Thiobarbituric acid reactive substances
TBHQ	- Tert-butylhydroquinone
TC	- Total cholesterols
TCA	- Trichloroacetic acid
TCM	- Traditional Chinese Medicine
TLC	- Thin layer chromatography
TMP	- 1,1,3,3-Tetramethoxypropane
TMS	- Tetramethylsilane
USDA	- United States Department of Agriculture
VA	- Vanillic acid

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Chapter 1

General Introduction

1.1 Background

Cardiovascular disease (CVD) is a main cause of mortality and morbidity in the world and ranks first in this respect. In Canada, CVD is the leading cause of death accounting for at least 36% of all deaths (37% among women, 35% among men), or about 70,000 individuals each year (Statistics Canada, 2010). The cause of most CVD is atherosclerosis. Low-density lipoprotein cholesterol (LDL-C) is the major atherogenic component of plasma. CVD costs the Canadian economy more than $22.2 billion every year in physician services, hospital costs, lost wages and decreased productivity (Dai et al., 2009).

Research on the biological activities and health-promoting effects of phytosterols (or plant sterols) and their derivatives has yielded positive results, such as lowering LDL-C and anticancer activity. The cholesterol-lowering effect of phytosteryl esters enriched foods has been well documented. The substantial scientific evidence supporting the cholesterol-lowering properties of phytosteryl esters prompted the Uniterd States Food and Drug Administration (FDA) in 2003 to begin allowing specific heart-protective label claims for phytosteryl esters-containing food products. Health Canada in 2010 approved the health claim for phytosteryl esters-enriched food products for their beneficial cholesterol lowering effects. Thus synthesis of phytosteryl esters is of importance due to

their recent recognition and application in functional foods and nutraceutical industries as excellent cholesterol-lowering agents.

Although major commercial phytosteryl esters are those with fatty acids of different chain lengths up to 18 carbons from vegetable oils and most of the research reported in the literature has focused on free phytosterols and phytosteryl esters with saturated or long-chain monounsaturated fatty acids, less research has explored the synthesis of phytosteryl esters with phenolic acids (also called phytosteryl phenolates in this thesis) or long-chain omega-3 polyunsaturated fatty acids (PUFA).

Another topic that has received much attention in recent years is that of antioxidants. Antioxidants constitute one of the most important topics in food science and human nutrition due to the deleterious effects of lipid free radicals, both in food and in the human body. As for phytosteryl phenolates, they are particularly interesting due to their unique properties and natural occurrence in many food products. This group of compounds contains a phenolic acid moiety, which is always a powerful antioxidant, so it is proposed that in addition to reducing LDL-C levels, phytosteryl phenolates might also reduce the oxidation of LDL-C, hence further lowering the risk of CVD. Certain types of phytosteryl phenolates, namely phytosteryl ferulates, phytosteryl caffeates and phytosteryl coumarates, exist naturally in the outer layers of many cereals and are important phytosterol constitutes in bran fractions, especially in oils from rice bran, corn and wheat (Norton, 1994; 1995; Seitz, 1989; Hakala et al., 2002). A mixture of phytosteryl ferulates known as γ-oryzanol is commercially extracted from rice bran. Phytosteryl ferulates render several physiological effects and have been regarded as

cholesterol-lowering agents, antioxidants, and photoprotectors (Hakala et al., 2002; Wang et al., 2002). Such proposed physiological functions evoked our interest in the enzymatic synthesis of these compounds as well as other phytosteryl phenolates. Few attempts have been made to chemically synthesize phytosteryl ferulates (Kondo et al., 1988; 1991; Condo et al., 2001). However these processes often use harsh chemicals and require isolation of sensitive intermediates and protection/deprotection of the functional hydroxyl group in phenolic acids. The chemoenzymatic synthesis of this group of compounds may facilitate their application as functional food ingredients and as nutraceuticals.

Oleic acid is the main reason why eating a Mediterranean diet rich in fruits, vegetables and particularly olive oil is healthy. Evidence from epidemiological studies suggests that the Mediterranean diet with a high proportion of monounsaturated lipids may reduce the risk of coronary heart disease (Keys et al., 1986). Long-chain PUFA, especially omega-3 PUFA, have been demonstrated to possess cardioprotective and immune-enhancing effects (Shahidi and Finley, 2001; Shahidi and Wanasundara, 1998). Esterification of phytosterols with omega-3 PUFA or oleic acid may enhance the physiochemical properties such as solubility, miscibility, oxidative stability and hence bioactivity and bioavailability. Thus, phytosteryl esters with omega-3 PUFA may offer both the benefits of phytosterol and those of long-chain omega-3 PUFA, possibly in a synergistic manner. The enzymatic synthesis of phytosteryl esters with omega-3 PUFA may also render a novel group of compounds as functional food ingredients and nutraceuticals with enhanced health promoting effects.

1.2 Objectives

The objectives of this study are described below.

A) To optimize the reaction conditions (reaction time, substrate mole ratio, amount of enzyme) using response surface methodology (RSM) for production of phytosteryl caprylates using the lipase-catalyzed esterification reaction between phytosterols and caprylic acid.

B) To chemoenzymatically synthesize phytosteryl phenolates using a two-step approach. In the first step phenolic acids were chemically modified to produce vinyl phenolates. In the second step, the vinyl phenolates so produced were esterified with phytosterols via lipase assisted alcoholysis in a binary organic solvent mixture of hexane and 2-butanone.

C) To evaluate the antioxidant activity of phytosteryl phenolates using multiple assays.

D) To enzymatically synthesize phytosteryl oleates and docosahexaenoates.

E) To study the cholesterol-lowering effect of phytosteryl oleates and docosahexaenoates in a mouse model.

This thesis consists of eight chapters.

The first two chapters describe the relevant background and objectives as well as the related literature review.

Chapter 3 presents materials and methods, while results and discussions are presented in Chapters 4-7.

Chapter 4 reports optimization of enzymatic synthesis of phytosteryl caprylates using RSM.

Chapters 5 and 6 present the result of chemoenzymatic synthesis of phytosteryl phenolates and evaluation of their antioxidant activity in different model systems

Chapter 7 describes the enzymatic synthesis of phytosteryl oleates and docosahexaenoates and evaluation of their cholesterol-lowering effect in apo-E deficient mice model.

Finally, conclusions and perspectives for future studies are given in Chapter 8.

Chapter 2

Literature Review

2.1 Phytosterols

Phytosterols (also known as plant sterols) are a group of steroid alcohols naturally occurring in plants and are part of the broad group of isoprenoids. They are important structural components of plant cell membranes, a role which in mammalian cells is played by cholesterol. The main functions of phytosterols are to stabilize phospholipid bilayers in cell membranes and regulate membrane fluidity in plant cells (Moreau et al., 2002). In addition, they are substrates for the synthesis of numerous secondary plant metabolites such as glycoalkaloids and saponins and act as biogenetic precursors of compounds involved in plant growth. They may also play other physiologic functions associated with plant biology (Hartmann, 1998). Although structurally similar to cholesterol, phytosterols are differentiated by their side chain configuration at the C_{24} position and by their degree of saturation. Phytostanols are saturated phytosterols. They are not abundant in nature and only occur in high levels in tissues of a few cereal species and some trees (Piironen et al., 2002) and can be produced by chemical hydrogenation of their unsaturated phytosterol counterparts. Phytosterols are relevant in pharmaceuticals, nutrition, and cosmetics and are well known for their lowering of total blood cholesterol and low-density lipoprotein cholesterol (LDL-C). In the past few years, consumers in the United States, Australia, and many European countries have introduced phytosterol-enriched food products in their diet

as novel means to reduce their serum LDL-C concentrations. Health Canada in 2010 approved the health claim for phytosterols containing foods, which offers Canadians a new way to lower their total cholesterol. In addition other biological activities such as antiinflammatory, antiantherogenicity, anticarcinogenicity and antioxidative activities have also been reported for phytosterols and their derivatives. Thus, they have recently received much scientific and commercial interest in the production of nutraceuticals and ingredients for development of functional foods due to their health benefits.

2.1.1 Structures and derivatives

The steroid skeleton for nomenclature of phytosterols following the International Union of Pure and Applied Chemistry-International Union of Biochemistry (IUPAC-IUB) recommendation of 1989 is shown in Figure 2-1. Phytosterols possess a hydroxyl group at C_3 and a branched aliphatic chain of 8-10 carbon atoms at C_{17} of the steroidal skeleton. Over 250 different types of phytosterols and related derivatives have been identified in various plants and marine materials, with the most common ones being sitosterol, stigmasterol, campesterol and sitostanol. Minor ones are avenesterols, and fucosterols, among others. Brassicasterol is a characteristic phytosterol only found in cruciferous plants of the *brasscaseae* family, such as canola. Ergosterol occurs only in yeasts and many other fungi.

Phytosterols can be divided into three groups based on the number of methyl groups on C_4, namely two (4-dimethyl), one (4-monomethyl) and none (4-desmethyl) phytosterol. 4-Dimethyl phytosterols and 4-monomethyl phytosterols are metabolic

intermediates in the biosynthesis of 4-desmethyl phytosterols. Cycloartanol and gramisterol are example of 4-dimethyl phytosterols and 4-monomethyl phytosterols, respectively, as shown in Figure 2-2. Both of these phytosterols are present at low levels in most plant tissues. Cycloartanol and its esters are abundant in rice bran oil. They are one of the main ingredients in γ-oryzanol, a commercialized natural health product derived from rice bran oil. The most frequently encountered phytosterols belong to the group of 4-desmethyl phytosterols. They include 27, 28 and 29-carbon 4-desmethyl phytosterols. Representative phytosterols are shown in Figures 2-3, 2-4 and 2-5. Among them, 28 and 29-carbon 4-desmethyl phytosterols are the major membrane structural components in plant cells. It is reported that over 100 of the 250 indentified phytosterols and their derivatives are 4-desmethyl phytosterols (Akihisa et al., 1991). The 27-carbon 4-desmethyl phytosterols are ubiquitous and predominant in animals, but also generally present in plants at low levels. The general public and even chemists have had a misconception that plant tissues are devoid of cholesterol (Moreau et al., 2002). This is due to the fact that cholesterol often contributes only 1-2% to the total content of plant sterols, but may constitute 5% or more to the total phytosterols in selected plant families, species, organs, or tissues. Thus, cholesterol is also classified into the subgroup of phytosterols, which is the 27-carbon 4-desmethyl phytosterols in some literature (Behrman and Gopalan, 2005). Most 4-desmethyl phytosterols have a double bond between C_5 and C_6 of ring B in the sterol nucleus and thus are known as Δ^5 phytosterols. Another group of common 4-desmethyl phytosterols are Δ^7 phytosterols which have a double bond between C_7 and C_8 instead of C_5 and C_6. They are also abundant in certain

plants. Both Δ^5 and Δ^7 4-desmethyl phytosterols may include a second double bond in the alkyl side chain, most often between C_{22} and C_{23} or C_{24} and C_{24}. Examples of Δ^5 and Δ^7 4-desmethylsterols are presented in Figures 2-4 and 2-5, respectively.

In addition to the free form, phytosterols may occur as four types of ''conjugates'', in which the C_3-OH group is esterified to a fatty acid (phytosteryl fatty acid esters) or a phenolic acid (phytosteryl phenolates), or glycosylated with a hexose, usually glucose (phytosteryl glycosides), or a 6-fatty acyl hexose (acylated phytosteryl glycosides). Representative structures of each group of conjugates are given in Figure 2-6. The occurrence of these conjugates varies among food products and may also be distributed in different proportions in different parts of food products. Norton (1995) and Hakala et al. (2002) have indicated that phytostanols predominate in corn ferulates. Seitz (1989; 1990) showed that phytosteryl ferulates of corn grains were highly localized within the inner pericarp layer. Therefore, phytosteryl phenolates were regarded as candidates for kernel pathogen resistance for both fungi and insects (Norton, 1994).

Phytostanols are naturally-occurring, especially in tall oil phytosterols, where the level of phytostanols can be as high as 15% of the total phytosterols (Cantrill and Kawamura, 2008). They can also be prepared by hydrogenating phytosterols. Phytosterols are high melting powders and their esters are chemically stable materials, having comparable chemical and physical properties to edible fats and oils. They are insoluble in water, but soluble in solvents such as hexane and *iso*-octane. The esters are also soluble in vegetable fats and oils.

2.1.2 Health effects and health claims

The cholesterol-lowering properties of phytosterols were first demonstrated 50 years ago by Peterson (1951). They were used as pharmaceutical agents for hypercholesterolaemia in the late 1950s. However, due to their both low water and lipid solubility and the resulting low bioavailability, a daily intake of 18 g of sitosterol was needed to achieve a reduction of 15% in serum cholesterol levels. The product was unmarketable as a pharmaceutical agent and production was stopped (Thompson and Grundy, 2005). A resurgence of interest in cholesterol-lowering effects of phytosterol happened after researchers esterified phytosterols with fatty acids in the 1990's. As discussed by Wester (1999), esterification of phytostanols with fatty acids derived from vegetable oils converted them from a crystalline powder with low lipid solubility into a fatty substance that could easily be incorporated into a variety of foods. This process was patented by a Finnish company in 1989 and resulted 6 years later in their first successfully marketed Benecol margarine in Finland (Moreau, 2004). The phytosteryl esters made it possible to greatly expand the market for phytosterols as dietary supplements, leading to a rapidly growing world-wide market for functional foods containing phytosteryl esters (Salo and Wester, 2005). Except for spreads, there are numerous other foods approved in which phytosteryl esters have been added, for example, salad dressing, milk, soy, yoghurt, cheese-based products, soy and fruit drinks, and even sausages and breads. The main physiological response to ingesting of phytosterols is suppressed cholesterol absorption. Different mechanisms have been proposed for the cholesterol-lowering effects of phytosterols; these include competition

with cholesterol for solubilization in the micelles, co-crystallization with cholesterol to form insoluble crystals (Ikeda et al., 1988; 1989), and competitive reactions during hydrolysis with cholesterol esterases (Trautwein et al., 2003). At the epithelial cell level, phytosterols can further diminish cholesterol absorption due to an upregulation of the adenosine triphosphate binding cassette A1 (ABCA1) protein and subsequent increased cholesterol efflux (Plat and Mensink, 2002). Although equally effective in lowering plasma cholesterol, phytosteryl esters may have higher absorption compared with phytostanyl esters. The resulting increased serum phytosterols levels may occasionally approach values seen in phytosterolemia, a strongly atherogenic hereditary metabolic abnormality. Thus, phytostanyl esters may be preferred for incorporation into foods (Miettinen, 2001; Jones et al., 1999). However, more research is needed to confirm this controversial report.

Compared with the commercialized phytosteryl esters with fatty acids, another group of phytosteryl esters namely phytosteryl phenolates have attracted much less attention. These compounds exist naturally in the outer layers of many cereals and are important phytosterol constitutes in the bran fractions of cereals and especially, in the oils from bran of rice, corn and wheat (Hakala et al., 2002; Norton, 1994; 1995; Seitz, 1989). Several physiological roles for phytosteryl ferulates have been proposed. They have been regarded as cholesterol-lowering agents, antioxidants, and photoprotectors (Hakala et al., 2002; Wang et al., 2002). A mixture, mainly consisting of phytosteryl ferulates, known as γ-oryzanol, is commercially extracted from rice bran and has been the subject of research and patents for a wide range of applications and activities for nearly 30 years (Cicero and

Gaddi, 2001). The beneficial effects of γ-oryzanol on hyperlipidemia in a variety of subjects, including humans have been reported (Kahlon et al., 1991; Yoshino et al., 1989). Sitostanyl ferulates from corn fibre oil were also very effective in lowering cholesterol in an animal model in a preliminary study (Nicolosi et al., 2001).

In addition to their cholesterol-lowering properties, phytosterols and their derivatives possess other biological activities, namely antiinflammatory, anticarcinogenic and antioxidative activities (Jones and AbuMweis, 2009). Several mechanisms have been proposed for anticarcinogenicity of phytosterols and their derivatives; these include their effect on membrane structure and function of tumour and host tissue, signal transduction pathways that regulate tumour growth and apoptosis, immune function of the host and cholesterol metabolism by host (Awad and Fink, 2000).

Antioxidant activity of free phytosterols has been reported in a few publications. Avenasterol and fucosterol are effective as antioxidants, while other phytosterols, including cholesterol and stigmasterol, are ineffective against the oxidation of a triacylglycerols mixture at 180 °C (Gordon and Magos, 1983). Its antioxidant activity has been attributed to the formation of an allylic free radical and its isomerization to other relatively stable free radicals. Yoshida and Niki (2003) reported that phytosterols chemically act as antioxidant, a mild radical scavenger, and physically as a stabilizer in the membranes.

The cholesterol-lowering effect of phytosteryl esters-enriched foods has been well documented. Phytosteryl esters are clinically proven to lower cholesterol up to 15 percent with a health claim approved by the U.S. Food and Drug Administration (FDA, 2000).

This was the twelfth health claim approved by FDA at that time. Some fifty percent of the general Canadian population is considered to be moderately to highly hypercholesterolemic, which is believed to be a major risk factor for coronary heart disease (Health Canada, 2010). On May 21, 2010, Health Canada also released health claim guideline to food manufacturers who wished to make such claims for foods containing added plant sterols. These health claims of food labelling about the beneficial effects of phytosterols on LDL-C and thus on cardiovascular health are based on a large number of clinical and experimental studies.

2.1.3 Sources

Phytosterols occur widely, at varying concentrations, in the fat soluble fractions of seeds, roots, stems, branches, leaves and blossoms. Dietary sources of phytosterols are vegetable oils, cereals, nuts, berries and other fruits. Among these, vegetable oils are the major sources of daily intake of phytosterols as they may contain 0.1-1% of phytosterols and their derivatives (Verleyen et al., 2002). Some of the specialized oils, such as those extracted from corn fibre, may contain phytosterols up to 10 g/ 1000 g of oil (Moreau, 2005). Cereals are generally regarded as good sources of phytosterols. Some differences may exist in the phytosterol compositions and their total amount in different cereals. Although the total content of phytosterols in fresh vegetables is low, their significance as dietary phytosterol sources is considerable (Pillow et al., 1999). Table 2-1 shows the phytosterol contents in selected common food products. Many other fruits also contain relatively the same amount of phytosterols compared to vegetables except for avocado,

which contains significantly more phytosterols (752 mg/kg) (Piironen et al., 2003). As indicated in the Table 2-1, sitosterol is the most important 4-desmethyl phytosterol and dominates the phytosterols in all selected food products. It accounts for 40-90% of the total phytosterols content. Other 4-desmethyl phytosterols occurring in a significant level include campesterol and stigmasterol. Δ^5Avenasterol and phytostanols are also reported to be present in most of these food products. The estimated average daily phytosterol consumption varies from 140 to 400 mg per day in various populations depending on the dietary pattern and on the way in which the food products are consumed (Ostlund, 2002; Pirronen and Lampi, 2004).

Tall oils and vegetable oils are the two major sources for large scale isolation for commercial exploitation of phytosterols (Fernandes and Cabral, 2007). The common vegetable oils for commercial isolation of phytosterols include soybean oil, rapeseed (canola) oil, sunflower oil and corn oil. Tall oil is a by-product of the manufacture of wood pulp. Total phytosterols, mostly in esterified form, may contain 3–7% by weight in the crude tall oil (Huibers et al., 2000; Wong et al., 1999). A relatively higher level of phytostanols in tall oil than in vegetable oils has been reported (Cantrill and Kawamura, 2008). Phytosterols isolated from both sources have been used in health, pharmaceutical and food applications (Kritchevesky and Chen, 2005). Wastes produced during fermentation and processing of sugarcane juice in rum factories have also been used to produce phytosterol-rich oil (Nuissier et al., 2002). Cocoa hulls, a waste by-product of the roasting of cocoa beans with little value in chocolate manufacturing, have been identified as another, although minor, source of phytosterols (Romanczyk and McClelland, 2001).

2.2 Lipid oxidation

The importance of lipid oxidation in biological systems and foodstuff has been widely recognized. Oxidative processes involving free radicals in membrane lipids are thought to have destructive cellular effects in *vivo*; whereas oxidative changes in foods during processing, distribution, storage, and final preparation may result in loss of sensory desirability, nutritional value, safety, and aesthetic appeal (Shahidi, 1994).

2.2.1 Mechanisms of lipid oxidation

In the presence of catalytic systems such as light, heat, enzymes, metals, and metalloproteins, lipids are susceptible to oxidation. Four different pathways of lipid oxidation are reported, namely autoxidation, photo-oxidation, thermal oxidation and enzymatic oxidation. Among them autoxidation is the most common process occurring spontaneously when lipids are exposed to atmospheric oxygen and is through a chain reaction of free radicals. The mechanism of autoxidation of unsaturated lipids can be described in terms of 1) initiation or the formation of free radicals; 2) propagation or the free-radical chain reactions; and 3) termination or the formation of non-radical products.

Initiation:

$$RH \xrightarrow{\text{Initiator}} R^{\bullet} + H^{\bullet} \quad (1)$$

Propagation:

$$R^{\bullet} + O_2 \longrightarrow ROO^{\bullet} \quad (2)$$

$$ROO^{\bullet} + RH \longrightarrow R^{\bullet} + ROOH \quad (3)$$

Termination:

$$R^{\bullet} + R^{\bullet} \longrightarrow RR \text{ (non-radical product)} \quad (4)$$

$$R^{\bullet} + ROO^{\bullet} \longrightarrow ROOR \text{ (non-radical product)} \quad (5)$$

$$ROO^{\bullet} + ROO^{\bullet} \longrightarrow ROOR + O_2 \text{ (non-radical product)} \quad (6)$$

In the initiation stage, unsaturated lipids (RH) lose a hydrogen atom to form a lipid radical ($R^{\bullet}$). Reaction (1) is thermodynamically unfavourable since the activation for this reaction is about 35 kcal, or 146 kJ/mol (Naway, 1996).

During the propagation step, the highly reactive alkyl radical of unsaturated lipids ($R^{\bullet}$) can readily undergo propagation reaction (2) by reacting with molecular oxygen to form peroxyl radical ($ROO^{\bullet}$) or reaction (3) to abstract a hydrogen atom from the α-position adjacent to a double bond to form hydroperoxides (ROOH). The rate-constant (k_1) of reaction (2) and rate constant (k_2) of reaction (3) are 3×10^8 M^{-1} s^{-1} and 10 M^{-1} s^{-1}, respectively (Buettner, 1993). Hydroperoxides are one of the major initial oxidation

products known as primary oxidation products. Four different hydroperoxides are produced during the autoxidation of oleic acid. The mechanism of oleic acid autoxidation is shown in Figure 2-7.

At the last stage of autoxidation, the accumulated peroxyl radicals (ROO˙) and alkyl radicals of unsaturated lipids (R˙), which are at relatively high concentration, could interact with each other to form non-radical products through reactions (4)-(6). The accumulated hydroperoxides can then decompose to secondary oxidation products, such as aldehydes, ketones, alcohols, hydrocarbons, furans and acids, among others.

2.2.2 Influences of lipid oxidation

Lipid oxidation compromises both the nutritional value and the sensory quality of foods. The loss of nutrients is mainly due to the oxidized PUFA. The lipid hydroperoxides formed during autoxidation could decompose liposoluble vitamins, such as vitamin E and its pro-vitamin carotenes (Addis, 1986). Lipid hydroperoxides could also decompose to form compounds responsible for off-flavour and off-odour, as mentioned earlier. The free radicals so produced during lipid oxidation may participate in the development of atherosclerosis (Kubow, 1990 and 1993). Hence food products containing oxidized fat are considered to be a risk factor for human health. In particular, a few cholesterol oxide products (COPS) are recognized as atherogenic agents and appear to have mutagenic, carcinogenic and cytotoxic properties (Guardiola et al., 1996). Lipid peroxides and oxidized cholesterol may be involved in tumour promotion and in atherosclerosis, whereas malonaldehyde, a secondary product of lipid oxidation, has been

implicated as a catalyst in the formation of *N*-nitrosamines and also as a cause for mutagenesis (Sanders, 1987). Lipids and their oxidation products may also interact with the products from Maillard reaction in a number of ways and influence the formation of several carcinogenic/mutagenic heterocyclic aromatic amines (HAAs) during cooking of muscle foods products, particularly at high temperatures (Skog, 1993).

2.3 Antioxidants

Lipid oxidation is a major concern of the food industry because oxidation products influence the flavour and nutritional value of food products and some may render damage to human health. Antioxidants have thus attracted the interest of food scientists, medical and nutritional experts as well as the general public due to their health benefits.

In 1921, Henry A. Mattill (George, 2005) first discovered the antioxidant function of vitamin E through his investigation of milk as the "perfect food". Then it revolutionized the field and led to the realization of the importance of antioxidants in biology. The possible mechanisms of action of antioxidants were first explored thoroughly by Moreau and Dufraisse (Denny, 1927), who recognized that a substance with antioxidative activity is likely to be one that is itself a target for oxidation. Halliwell (1990) gave a broader definition of antioxidant, which states "any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delays or prevents the oxidation of' that substrate. The term "oxidizable substrate" includes every type of molecule found *in vivo*". However, in the food industry it is implicitly restricted to chain-breaking inhibitors of lipid peroxidation, such as α-

tocopherol (Halliwell, 1995). According to the United States Department of Agriculture (USDA) Code of Federal Regulations (21, CFR 170.3) (USDA, 2006), the following definition was given: "antioxidants are substances used to preserve food by retarding deterioration, rancidity or discolouration due to oxidation." Antioxidants were first used before World War II for food preservation. These early antioxidants were natural products, but were soon replaced by synthetic substances, which were more cost effective (Pokorny, 1999). The most commonly used synthetic antioxidants are BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), PG (propyl gallate) and TBHQ (tert-butylhydroquinone). However, some common synthetic antioxidants have also become controversial due to their potential adverse effects on health (Barlow, 1990; Shahidi and Zhong, 2010). Therefore, replacing synthetic antioxidants with natural alternatives, or simply replacing the synthetic food additives with natural choices, has attracted great interest over the past two decades.

2.3.1 Types of antioxidants

Antioxidants could simply be classified into two groups, namely synthetic and natural (López-Vélez, 2003), or they could be classified according to their diverse mechanisms of action as summarized in Table 2-2. Antioxidants that break the chain reaction of autoxidation by hydrogen donation and generation of more stable radicals are called primary antioxidants. Other antioxidants, which slow the oxidation rate by several mechanisms, including chelation of metal ions, regeneration of primary antioxidants,

decomposition/stabilization of hydroperoxides and scavenging of oxygen, among others, can then be classified as secondary antioxidants.

2.3.2 Mechanisms of action of antioxidants

As can be seen from Table 2-2, there are several types of antioxidants that may protect against autoxidation of lipids, but only the compounds known as primary antioxidants (AH), inhibit oxidation by inactivating free radicals, thus break the chain reaction in autoxidation . The first detailed kinetic study of the mechanism of this type of antioxidants was conducted by Bolland (Fennema, 1996) using a model system of autoxidizing ethyl linoleate containing hydroquinone as an inhibitor. The primary antioxidants (AH) interfere with either chain propagation or initiation by electron transfer and by readily donating hydrogen atoms to lipid alkyl, alkoxyl and peroxyl radicals, to inhibit or retard lipid oxidation. The main reactions are shown below:

$$ROO^{\cdot} + AH \longrightarrow ROOH + A^{\cdot} \quad (7)$$

$$RO^{\cdot} + AH \longrightarrow ROH + A^{\cdot} \quad (8)$$

$$ROO^{\cdot} + A^{\cdot} \longrightarrow ROOA \quad (9)$$

$$RO^{\cdot} + A^{\cdot} \longrightarrow ROA \quad (10)$$

$$A^{\cdot} + A^{\cdot} \longrightarrow A\text{-}A \quad (11)$$

To be effective primary antioxidants, the resulting phenoxyl radical produced by reactions (7) and (8) should be relatively stable. At the same time, reaction (7) should be

faster than reaction (3), and so inhibit the chain reaction during the propagation phase of lipid autoxidation. Reaction (8) becomes important only at low oxygen pressure and elevated temperatures because at the atmospheric pressure the alkoxyl radical (RO˙) could quickly react with oxygen to produce peroxyl radical (ROO˙) by reaction (2). The produced antioxidant radicals A˙ could either react with RO˙ to form a non-radical product (ROA) or react with peroxyl radical (ROO˙) to form stable peroxides (ROOA) by reactions (9) and (10) or dimerize with another antioxidant radical (A˙) to produce another non-radical product (A-A). The above reactions are exothermic in nature, so they are thermodynamically favourable. The efficiency of primary antioxidants (AH) increases with decreasing A-H bond strength. The activation energy increases with increasing A-H and R-H bond dissociation energy (Shahidi and Naczk, 2004).

There are also other kinds of antioxidants with different mechanisms. They are briefly introduced in Table 2-2.

2.3.3 Methods for the assessment of antioxidant activity

Oxidative processes involving free radicals in membrane lipids are thought to have destructive cellular effects *in vivo*; whereas oxidative changes in foods result in flavour and nutritional quality deterioration that may also affect their safety. Antioxidants protect cells and foods against oxidative stress. The effectiveness of antioxidants or antioxidant activity depends on many factors, such as structure, composition, antioxidant concentration, temperature, oxygen pressure, and the presence of other antioxidants and many common food components, e.g. proteins and carbohydrates. Many methods have

been developed for evaluating the activity of antioxidants. These may be classified into two major categories: measuring the ability of antioxidants in inhibiting oxidation reactions in a model system and radical scavenging assays. A brief introduction of relevant methods used in this work is provided below.

In the first category, the ability of antioxidants in inhibiting oxidation reactions in a model system is evaluated by monitoring the associated changes using sensory, physical, chemical or instrumental means. A large number of tests for determining antioxidant activity has been proposed and used by measuring inhibition of oxidation of a suitable substrate under prescribed conditions of a model system in the presence or absence of an antioxidant (Van den Berg et al., 1999). The process of lipid oxidation can be monitored by evaluating changes in the substrates, oxidant/initiator, intermediates or final products. Methods used to determine the extent of lipid oxidation include sensory evaluation, peroxide value, conjugated dienes (CD), thiobarbituric acid reactive substances (TBARS) value, volatile acids by Rancimat method, and volatiles by gas chromatography, among others. As the effectiveness of antioxidants in protecting foods against oxidative deterioration is a complex phenomenon, many factors had to be considered when selecting a model system for measuring antioxidant activity (Frankel, 2005). These include firstly the substrates; the use of different lipid substrates has a significant impact on the activity of various antioxidants according to their hydrophilic and hydrophobic nature. Normally, most assessments of antioxidant activity are performed using triacylglycerols (oils) or phospholipids, in bulk oil, emulsion, or liposome system because they are most important in foods and biological systems. If bulk lipid/oil model system is

used, the stripping of the oil may be necessary as the endogenous antioxidants present may influence the interpretation of the final results with respect to the efficiency of antioxidants. The starting oils should also be devoid of high levels of oxidation products as some oxidation products such as hexanal and 2,4-decadienal have been reported to exhibit pro-oxidative effect (El-Magoli et al., 1979). Secondly, conditions such as temperature, oxygen supply, and presence of metal catalysts. Different results may be obtained at different temperatures because the mechanisms of oxidation and hydroperoxides decomposition change with temperature. Thirdly, the concentration is an important factor and selection of the concentration of antioxidants for test is necessary as certain compounds may play a dual role of being antioxidant or pro-oxidant depending on their concentration. Fourthly, the analytical strategy is important when analyzing at different time points of the development of primary and secondary oxidation products as the effects of different antioxidants vary in their kinetic of oxidation. It is also very important to assess antioxidant activity at an appropriate end point. Finally, the calculation and results expression, the basis of induction period, percentage inhibition, rate of oxidation production formation/decomposition and EC_{50} (concentration to decrease concentration of test free radical by 50%), among others, are normally used in quantification analysis. A wide range of parameters were used to express the resulting antioxidant activity, which is shown in Table 2-3.

Radical scavenging assays include methods based on hydrogen atom transfer (HAT) or electron transfer (ET) mechanisms. Oxygen radical absorbance capacity (ORAC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assays are discussed as examples for

HAT and ET mechanisms, respectively, in the subsequent section.

The ORAC assay is based on the measurement of the radical chain breaking ability of antioxidants by monitoring the inhibition of peroxyl radical induced oxidation by the tested antioxidant compounds. In this method, peroxyl radical reacts with a fluorescent probe resulting in the loss of fluorescence, which is detected with a fluorometer. *β*-Phycoerythrin (B-PE), an oxidizable protein isolated from *Porphyridium cruentum*, was initially employed as the fluorescent probe in early ORAC studies. However, it was found that using fluorescent probe B-PE suffers from several disadvantages such as its large lot-to-lot variability in reactivity with peroxyl radicals as it is derived from red algae of various species, its interaction with polyphenols due to nonspecific protein binding, and its being photobleached under excitation light (Ou et al., 2001). Therefore, a more stable and less reactive nonprotein probe fluorescein (FL: 3'6'-dihydroxyspiro[isobenzofuran-1[3H], 9'[9H]-xanthen]-3-one) was used as a replacement of B-PE (Ou et al., 2001). The oxidation products of fluorescein with peroxyl radicals have been characterized, and their reaction mechanism verified as being a classic HAT one. Azo compounds including the lipophilic AIBN (α,α-azobisisobutyronitrile) and AMVN (2,2'-azobis(2,4-dimethylnaleronitrile) and the hydrophilic AAPH (2,2'-azobis(2-amidinopropane) dihydrochloride) have been used as peroxyl radical generators. The added antioxidants can rapidly react with peroxyl radicals and thus inhibit the loss of fluorescence intensity, and this inhibition is proportional to the antioxidant activity. A fluorescence decay curve (fluorescence intensity versus time) can be constructed (Figure 2-8). It is the only method that takes the free radical action to completion and uses the

area under the curve (AUC = AUC_{sample}-AUC_{blank}) for quantification; it thus combines both the percentage of inhibition and the length of inhibition of free radical formation by antioxidants into a single quantity instead of following the extension of the lag phase only. A series of trolox solutions with varying concentrations is used as antioxidant standard to obtain a standard curve (trolox concentration versus AUC), and ORAC values are normally reported as trolox equivalents (Huang et al., 2005).

The ORAC method was first reported by Glazer (1990) and modified by Cao and Prior (1998). The original assay and its modified versions were mainly developed for hydrophilic test environment. Huang et al. (2002) made a further modification to develop a lipophilic ORAC measurement method that employed randomly methylated β-cyclodextrin (RMCD) as a solubility enhancer. The newly modified method allowed one to measure the antioxidant capacity of lipophilic and hydrophilic components separately for a given sample, but based on the same peroxyl radical (Wu et al., 2004). The ORAC assay is temperature sensitive (Huang et al., 2002), as well as pH sensitive due to the fact that the intensity of fluorescence decreases sharply in situations when the pH is below 7.0 (Ou et al., 2001). Incubation of the reaction buffer at 37°C prior to the dissolving AAPH is recommended in order to reduce the intra-assay variability. The ORAC assay is currently considered to be a more reliable assay because it takes into account both the percentage and the duration of inhibition of free radicals by antioxidants on the target indicator molecule. In general ORAC is a very effective method to assess the antioxidant potential of various biological samples. These vary from being pure compounds such as flavonoids and melatonin (Aejmelaeus et al., 1997; Cao et al., 1997; Sharma et al., 1995;

Wang et al., 1997), to complex matrices such as teas, fruits, vegetables and animal tissues (Cao et al., 1996; Wang et al., 1996; Yu et al., 1995).

DPPH radical scavenging assay is another commonly used method for evaluating the total antioxidant activity of foods, especially foods containing phenolic compounds. The DPPH method is believed to be mainly based on ET reaction, and HAT mechanism is a marginal reaction pathway in this assay. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) (Figure 2-9) is a synthetic stable chromogen radical which has a deep purple colour. It is commercially available and does not need to be generated prior to the assay. In the DPPH test, the scavenging of DPPH radicals is followed by monitoring the decrease in the absorbance at 515 to 517 nm, which occurs due to the reduction by antioxidants or reaction with radical species (Brand-Williams, 1995). Most papers in which the DPPH method has been reported results as EC_{50}, which is the concentration of antioxidant required for 50% scavenging of DPPH radicals in a specified time period, or percentage of inhibition of free radicals. However, the absorbance of DPPH at 517 nm is influenced by several other factors, such as light, oxygen, pH, and type of solvents, in addition to the antioxidant. Therefore, these factors should be considered when performing this assay or interpreting the corresponding data (Ozcelik et al., 2003). Alternatively, electron paramagnetic resonance (EPR) can be used to quantitatively monitor the remaining DPPH radicals in the sample as it is a stable and well-characterized solid radical source. Because radials have unpaired electrons, they display different paramagnetic properties or energy levels (measured as magnetic moment) under a varying magnetic field. DPPH is the traditional and perhaps the most popular standard of the position (*g*-marker) and intensity

of EPR signals in the laboratory research. This method has been successfully employed in our laboratory for testing the antioxidant activity of different compounds or plant extracts (Alvarez-Parrilla et al., 2011; Madhujuth and Shahidi, 2008; Wettasinghe and Shahidi, 2000).

The above described methods are useful for assessing the total antioxidant activity. However, each antioxidant evaluation should be carried out under various conditions of oxidation. Several methods should also be used to measure different products of oxidation related to real food quality. There is no single method that can serve as a universal standard for quantitative analysis of antioxidant activity. Therefore, the total antioxidant as a nutritional index of food label is not available.

2.4 Phenolic acids

Phenolic acids are a subclass of a larger category of plant metabolites commonly referred to as "phenolics". The name "phenolic acids", in general, describes phenols that possess one carboxylic acid group. Among naturally occurring phenolic compounds, phenolic acids are of particular interest because of the wide spectrum of their potential biological properties, such as antiinflammatory, antiallergic, antimicrobial, anticarcinogenic, antiviral and antioxidative activities (Castelluccio et al., 1996; Rice-Evans et al., 1996).

Phenolic acids and their derivatives are widely distributed in plants, many being essential secondary metabolites. Hydroxybenzoic acids and hydroxycinnamic acids are two subgroups of phenolic acids. Hydroxybenzoic acids (Table 2-4) are aromatic

compounds with basic skeleton C6–C1, with gallic, *p*-hydroxybenzoic, protocatechuic, vanillic and syringic acids being the most common members. Hydroxycinnamic acids are the most widely distributed group of phenolic acids, also known as phenylpropanoids. These compounds include caffeic, ferulic, *p*-coumaric and sinapic acids, which have a C6–C3 structure (Table 2-4).

Phenolic acids are potent antioxidants against free radicals and other reactive oxygen species (ROS), which are the major cause of many chronic human diseases such as cancer and cardiovascular diseases. They can act as free radical scavengers, reducing agents and metal chelators (Shahidi and Naczk, 2004). Molecular structure of phenolic acids, especially the availability of phenolic hydrogens and the possibility for stabilization of the resulting phenoxyl radicals, plays a key role on their antioxidant activity. The role of the ethylene moiety of hydroxycinnamic acids in their radical scavenging properties remains controversial. Some studies suggest that this structural feature is important for the activity because it could participate in the stabilization by resonance of the phenoxyl radical formed in the process, whereas others claim that the conjugated olefinic double bound is not a requirement for their efficacy (Chen and Ho, 1997; Cuvelier et al., 1992; Moon and Terao, 1998; von Gadow et al., 1997). The presence of the -CH=CH-COOH group in the hydroxycinnamic acids is considered to be key for the significantly higher antioxidative efficiency than the -COOH in the hydroxybenzoic acids (Rice-Evans et al., 1996; White and Xing, 1997). Shimoji et al. (2002) reported that "side-chain-saturated" dihydroferulic acid and dihydrosinapic acid had more potent radical scavenging activity than ferulic acid and sinapic acid, respectively.

Four hydroxycinnamic acids, namely caffeic, ferulic, *p*-coumaric acid, and sinapic acids and four hydroxybenzoic acid, namely gallic, gentisic, syringic and vanillic acid, were selected for esterification with phytosterols in this work. However, only phytosteryl caffeates, ferulates, sinapates and vanillates were successfully synthesized. Thus a brief introduction about these four parent phenolic acids is given as follow:

2.4.1 Caffeic acid

Caffeic acid and some of its derivatives are widely distributed in the plant kingdom. A broad spectrum of biological activities including antibacterial (Kujumgiev et al., 1993), anticarcinogenic (Tanaka et al., 1993), antioxidant (Kikujak et al., 2002), antiviral (Fesen et al., 1993), antiinflammatory (Orban et al., 2000), antiHIV (Burke et al., 1995), anticarcinogenic (Kuenzig et al., 1984) and antitumour (Nagaoka et al., 2002) activities, have been attributed to caffeic acid and its derivatives. Vinyl caffeate has been identified as a natural compound in the leaves of *Perilla frutescens Britton*, which are used as a folk medicine and a detoxicant, antitussive, antibiotic, and antipyretic agent and for treating intestinal disorders and allergies in Japan and China (Tada et al., 1996). Other caffeic acid derivative reported in the literature include caffeoyl quinate (also known as chlorogenic acid) (Michael, 2000), rosmarinic acid (Schuler, 1990) and benzyl caffeate (also known as caffeic acid phenethyl ester) in bee propolis (Yamauchi et al.,1992), methyl caffeate in the leaves of *Melissa officinalis L.* (lemon balm) (Tagashira and Ohtake, 1998) and *Lonicera japonica* (Chang and Hsu, 1992), and ethyl caffeate in the seeds of *Ipomoea muricata* (Ysrael and Nonato, 1999). Campesteryl *trans*-caffeate

has been identified in rice bran oil (Fang et al., 2003). A study by Takagi and Lida (1980) showed that an ether extract of canary seed was a potent antioxidant in lard and sardine oil; the major antioxidants found in this extract were esters of caffeic acid with phytosterols, such as sitosterol, gramisterol, campesterol and cycloartenol.

2.4.2 Ferulic acid

Ferulic acid is a ubiquitous plant constituent found naturally in plant cell walls, leaves and seeds. Oats, brown rice, whole wheat, peanuts, apples, pineapples and some other fruits and herbs are good sources of ferulic acid and its derivatives. It is the most abundant hydroxycinnamic acid in the plant kingdom and occurs both in the free and conjugated forms. Much of the ferulic acid occurs as esters in many plants. It is covalently conjugated with monosaccharides and disaccharides, plant cell wall polysaccharides, glycoproteins, lignin, betacyanins, and other insoluble cell wall carbohydrate biopolymers.

Ferulic acid exhibits a wide range of therapeutic effects against various diseases such as cancer, diabetes, cardiovascular and neurodegenerative diseases (Zhao and Moghadasian, 2008). A wide spectrum of beneficial activities for human health has been advocated for this phenolic compound, at least in part, because of its strong antioxidant activity. Due to its phenolic nucleus and an extended side chain conjugation, it readily forms a resonance stabilized phenoxyl radical which accounts for its potent antioxidant potential. Anticarcinogenic activity of ferulic acid has also been reported (Kuenzig et al., 1984).

2.4.3 Sinapic and vanillic acids

Sinapic acid is a cinnamic acid derivative, which possesses 3,5-dimethoxyl and 4-hydroxyl substitutions in the phenyl group of cinnamic acid. Sinapic acid is a widely prevalent substance in the plant kingdom and is obtained from various sources such as rye, fruits, and vegetables (Andreasen et al., 2001; Lu et al., 2001). Sinapic acid, the main phenolic compound of rapeseed, constitutes over 73% of its free phenolic acids. Various biological activities of sinapic acid have been reported, for example, antioxidative (Wanasundara et al., 1996; Zou et al., 2002), anxiolytic-like effects (Yoon et al., 2007), antiinflammatory (Yun et al., 2008) and antibacterial activity (Tesaki et al., 1998). Sinapic acid is also known to have peroxynitrite ($ONOO^-$)-scavenging activity (Niwa et al., 1999) and can be utilized for protection of the cellular defence activity against diseases involving $ONOO^-$ (Zou et al., 2002).

Vanillic acid is a hydroxybenzoic acid, which is generally used as a flavouring agent. It is an intermediate in the production of vanillin from bioconversion of ferulic acid (Lesage-Meessen et al., 1996). The root of *Angelica sinensis*, commonly used as Traditional Chinese Medicine (TCM), has been reported to contain the highest amount of vanillic acid (Duke, 1992). Various studies have provided evidence for the effectiveness of vanillic acid in the management of immune and inflammatory responses (Chiang et al., 2003; Itoh et al., 2009; Kim et al., 2010).

2.5 Fatty acids

Fatty acids can be saturated or unsaturated (monounsaturated or polyunsaturated) with different chain length. They can be bound or attached to other molecules, such as in triacylglycerols or phospholipids. Three types of fatty acids were chosen as acyl donors for the synthesis of phytosteryl esters in this work. These included a medium-chain fatty acid, caprylic acid; a monounsaturated fatty acid, oleic acid; and a polyunsaturated fatty acid (PUFA), docosahexaenoic acid (DHA).

2.5.1 Medium-chain fatty acids (caprylic acid)

Saturated fatty acids (SFA) contain only single carbon-carbon bonds in the aliphatic chain and hydrogen atoms occupy all other available sites. SFA fall into three classes, based on the number of carbon atoms in the aliphatic chain, short-chain fatty acids (SCFA; C2 to C4), medium-chain fatty acids (MCFA; C6-C12) and long-chain fatty acids (LCFA; > 12 carbon atoms). MCFA and long-chain fatty acids (LCFA) have different absorption rates because MCFA are quickly oxidized for energy and LCFA are oxidized very slowly. Thus, MCFA exhibit unique structural and physiological features and can be rapidly cleared from the blood. They are easily absorbed and metabolized, similar to glucose (Babayan, 1987).

Caprylic acid is a MCFA with eight-carbon atoms and is also known by its systematic IUPAC name as octanoic acid. It is found naturally in the milk of various mammals as well as coconut oil and palm kernel oils. It is a food-grade compound generally recognized as safe (GRAS) by the US Food and Drug Administration (FDA). It

can easily penetrate fatty cell wall membranes due to its relatively short chain length, hence its effectiveness in combating certain lipid-coated bacteria, such as *Salmonella enteritidis* (Vasudevan et al., 2005), *Escherichia coli*, *Staphylococcus aureus* and various species of S*treptococcus* (Nair et al., 2005). Therefore, it is used in the treatment of some bacterial infections. Caprylic acid is also used as an antimicrobial pesticide, algaecide, bactericide, and fungicide. Caprylic acid was used as starting material for enzymatic esterification of phytosteryl caprylates in this research work. The process was optimized using response surface methodology (RSM) in this study.

2.5.2 Monounsaturated fatty acids (oleic acid)

Monounsaturated fatty acids (MUFA) are fatty acids that have a single double bond in their hydrocarbon chain and all of the remaining carbon atoms in the chain are single-bonded. By contrast, polyunsaturated fatty acids (PUFA) have more than one double bond.

Oleic acid (C18:1) is a monounsaturated omega-9 fatty acid found in various animal and vegetable sources, such as olive oil, olives, avocados, almonds, peanuts, sesame oil, pecans, pistachios, cashews, hazelnuts, and macadamia nuts, among others. It makes up 60-80% of olive oil, safflower oil as well as many nut oils. Oleic acid is the main reason why eating a Mediterranean diet rich in fruits, vegetables and particularly olive oil is healthy. Evidence from epidemiological studies suggests that the Mediterranean diet with a high proportion of monounsaturated lipids may reduce the risk of coronary heart disease (Keys et al., 1986). Reports have also shown that the

Mediterranean diet which contains a high content of oleic acid may protect against breast cancer (Martin-Moreno et al., 1994; Trichopoulou et al., 1995). This may be one of the reasons why oleic acid is of interest to many epidemiologists.

2.5.3 Polyunsaturated fatty acids (PUFA)

PUFA with two or more double bonds in their backbone structures cannot be made in the body and hence are considered essential fatty acids (EFA). They are known to provide unique health benefits related to cardiovascular disease (Conquer and Holub, 1997), inflammation (Mori and Beilin, 2004), cancer (Cave, 1991), immune response (Puertollo et al., 2004), diabetes (Stene and Joner, 2003), hypertension (Aguilera et al., 2004) and mental disorders (Plotnick, 1996), among others. There are two groups of EFA, the n-3 and the n-6 fatty acids; the n-3 PUFAs are of particular interest. These include α-linolenic acid (ALA), stearidonic acid, eicosapentaenoic acid (EPA; 20:5n-3), docosapentaenoic acid (DPA; 22:5n-3), and docosahexaenoic acid (DHA; 22:6n-3). Epidemiological studies have linked the low incidence of coronary heart disease in Greenland Eskimos with their high dietary intake of n-3 PUFA (Bang and Dyerberg, 1972 and 1986). Studies have shown that DHA is essential for the development of the grey matter of the brain as well as the retina of the eye and the components of the heart of the fetus (Kyle, 2001; Shahidi and Finley, 2001). The n-3 PUFA are essential for normal growth and development throughout the life cycle of humans and therefore should be included in the diet. Neurological disorders associated with decreased levels of DHA have been reported in patients with schizophrenia, Alzheimer's disease, and depression, among others (Conquer and Holub, 1997). The n-3 PUFA have

been extensively studied for their influence on cardiovascular disease (CVD). While the exact mechanism by which these effects are rendered remains unknown, research results have shown that these FA in marine oils may prevent CVD by decreasing serum triacylglycerols and acting as antiatherogenetic and antithrombotic agents (Newton, 2001). ALA (18:3 n-3) is a main constituent of flaxseed oil (50-60%). Marine oils are rich sources of n-3 PUFA, especially EPA and DHA. Cod liver, menhaden, and sardine oils contain approximately 30% EPA and DHA (Kyle, 2001). ALA, the parent of the n-3 FA family, can be metabolically converted to DHA via a series of desaturation and elongation reactions.

In short, PUFA exhibit multifunctional roles in health promotion and disease risk reduction, however, they are highly susceptible to oxidation during processing and storage, therefore it is very important to stabilize PUFA by employing different means and approaches.

2.6 Synthesis of phytosteryl esters

Free phytosterols are poorly soluble in most foods, and this has led to problems with formulations and for consistent efficacy (Condo et al., 2001). To solve this problem, phytosterols are usually esterified with fatty acids to make them soluble in fatty food products. Compared with the limited solubility of the free phytosterols, phytosteryl esters provide a means for administering effective amounts of these hypocholesterolemic agents to food products due to their improved solubility.

2.6.1 Phytosteryl esters with fatty acids

Phytosterols were first esterified with fatty acyl chlorides and the synthesized products were subsequently used to study their cholesterol absorption lowering effects (Mattson et al., 1977; 1982). The results indicated that both free phytosterols and their esters significantly decreased cholesterol absorption, with a consequent decrease in plasma cholesterol. The method of Mahadevan and Lundberg (1962) for preparing cholesteryl esters was adopted by Hsieh et al. (1980) for preparing campesteryl and sitosteryl palmitates. In this method, phytosteryl acetate and methyl palmitate were transesterified in the presence of sodium ethoxide under reduced atmospheric pressure. Methyl acetate generated during the reaction was trapped in a cold propanol bath. Pouilloux et al. (2003) reported a method for the synthesis of phytosteryl esters from natural phytosterols and fatty acids methyl esters in the presence of alkali oxide catalysts. They concluded that alkali oxides were less corrosive, more selective and effective than homogeneous catalysts such as alkali hydroxides and carbonates.

Although phytosteryl esters with FA can be synthesized by chemical means, the chemical methods suffer from problems such as the formation of side products (3,5-diene derivative or oxyphytosterol) (Negishi et al., 2003). To avoid such problems, several enzymatic procedures, using lipases as catalysts, for the preparation of phytosteryl esters of FAs have been developed in recent years. Osanai (1986) reported the synthesis of cholesteryl oleate using lipase and solvents such as cyclohexane, benzene, or toluene. Weber et al. (2001a; 2001b; 2002) synthesized phytosteryl fatty acid esters by carrying out the enzymatic reaction in solvent-free oil under reduced pressure to remove the water

and the alcohol from the reaction system. The lipase-catalyzed esterification and transesterification reaction were also carried out successfully in organic solvent systems (Villeneuve et al., 2005; Vu et al., 2004). Lipase derived from *Burkholderia cepacia*, Chirazyme L-1, was used to catalyze the reaction between sitostanol and different fatty acids with chain length C8-C18 in supercritical carbon dioxide as a reaction medium (King et al., 2001). Yields of 92% for sitostanyl caprylate and 99% for sitostanyl palmitate were achieved.

The synthesis of phytosteryl esters with n-3 PUFA in both chemical and enzymatic methods has been reported in the literature. Shimada et al. (1999) detailed the synthesis of a DHA ester of cholesterol in a biphasic medium containing 30% water. Edwart et al. (2002) reported the chemical synthesis of phytosteryl esters with n-3 PUFA by using sodium methoxide as a catalyst. The synthesized products were then used in Guinea pig model studies. The results indicated that the TAG-lowering and eicosanoid-modifying properties of n-3 PUFA in these esters were retained. A similar procedure was employed to synthesize phytosteryl esters with n-3 PUFA (Russell et al., 2002). The authors found that phytosteryl esters with fish oil at a high dose (2.6 g/kg) had a significant hypolipidemic effect and, at a very low dose (86 mg/kg), had a beneficial effect on endothelial and vascular smooth muscle cell function.

Gako-Golan et al. (2003) in their patent disclosed a process related to products of alcoholysis, esterfication and/or interesterification of phytosterol(s) and/or phytostanol(s) with different oils using lipase. Although fish oil was included in their list of oils used and phytosterol and phytostanyl esters with C14-C22 acids were claimed, they stated that

preferred phytosteryl esters were those with C16-C18 saturated or unsaturated fatty acids, particularly oleic, linoleic, linolenic, palmitic and stearic acids. The gas chromatograms attached in their patent showed no proof of the presence of phytosteryl and phytostanyl esters with DHA, EPA and DPA. As a matter of fact we found that gas-chromatography (GC) is not an ideal structure analysis tool for phytosteryl esters with DHA, EPA and DPA; this is because such esters are highly hydrophobic and difficult to volatilize. They also have low oxidative stability, especially at high temperature. Thus, HPLC is the best of choice for structure elucidation of phytosteryl esters with PUFA. Therefore, phytosteryl esters with omega-3 PUFA (EPA, DPA and DHA) were not properly evidenced in the patent disclosed by Gako-Golan et al. (2003).

2.6.2 Phytosteryl esters with phenolic acids

Phytosteryl esters with phenolic acids (named as phytosteryl phenolates in this thesis) exist naturally in the plants; one of the most reported series is phytosteryl ferulates. As one of the mainly functional ingredients in γ-oryzanol, phytosteryl ferulates have been widely studied for their antioxidant activites as well as other pharmacological properties. Gamma-oryzanol components including cycloartenyl ferulate, 24-methylenecycloartanyl ferulate, and campesteryl ferulate purified from rice bran oil showed higher inhibition of cholesterol oxidation than that of α-tocopherol, α-tocotrienol, γ-tocopherol, and γ–tocotrienol found in rice bran oil. Thus, they may contribute to the potential hypocholesterolemic properties of rice bran oil (Xu et al., 2001).

There are very limited literature reports on the synthesis of phytosteryl phenolates. Kondo et al. (1988) found that *trans*- and *cis*- feruloyl phytosterols were the major ovulatory active compounds in crops of Job's tears; they successfully isolated *trans*-feruloyl stigmastanol and *trans*-feruloyl campestanol. In the same study, they also reported a method to synthesize *trans*-feruloyl stigmastanol using 4-*O*-acetylferuloyl chloride as the acylating agent. The method has also been described in the US patent disclosed by Kondo et al. (1991). The limitations of this method are: (1) the required preparation of *trans*-4-*O*-acetylferuloyl chloride, which is difficult to purify and handle due to its high reactivity, and (2) the deprotection step that uses sodium borohydride to remove the acetyl protective group on the *trans*-feruloylated product. An improved method reported by Condo et al. (2001), instead of employing highly reactive *trans*-4-*O*-acetylferuloyl chloride, used *trans*-4-*O*-acetylferulic acid by acetylation of ferulic acid with acetic anhydride/pyridine, followed by condensation of *trans*-4-*O*-acetylferulic acid with phytostanol in the presence of *N,N*-dicyclohexylcarbodiimide and 4-(dimethylamino)-pyridine. Finally, selective deacetylation of the feruloyl acetate was achieved. Both methods used harsh chemicals, and required isolation of highly reactive intermediates, protection and deprotection of the hydroxyl groups in the phenolic acid. To avoid these problems, enzymatic methods that use lipase may be developed to make the synthesis of this group of compounds more environmentally friendly, economical and workable, especially for future industrial production.

Guyot and coworkers (1997) reported, for the first time, the enzymatic esterification of phenolic acids and alcohols with lipases from *Candida antarctica* lipase

B. Meanwhile, Buisman et al. (1998) studied the esterification of cinnamic acid and some benzoic acid derivatives with fatty alcohols with varying chain lengths of 4 to 12 carbon atoms (C4-C12).

Vinyl esters were chosen as acyl donors in this study because they have previously been shown to be useful for lipase-catalyzed preparation of enantiomerically pure compounds (Wang et al., 1989). The vinyl alcohol formed during the transesterification reaction tautomerizes to acetaldehyde, thus making the process irreversible (Santaniello et al., 1993). Vinyl esters have been reported as acylating agents for enzymatic synthesis of menthyl acetate and primary terpenyl esters (Akoh et al., 1998; Wu et al., 1996).

2.7 Response surface methodology (RSM) for optimization

In order to optimize the process conditions for production of phytosteryl caprylates, RSM was employed. RSM is an optimization procedure that determines optimal process conditions by combining particular experimental designs with modelling using first or second order polynomial equations and was first introduced by Box and Wilson (1951). RSM consists of a group of mathematical and statistical procedures that can be used to investigate correlations between one or more responses and a number of independent variables. RSM identifies the effect of the independent variables, alone or in combination, on the process. In addition to analyzing the effects of independent variables, this experimental methodology produces a mathematical model that accurately describes the overall method (Box and Wilson, 1991). It has been a popular and efficient statistical

procedure for studying complex processes. In product development and optimisation, RSM can be used to model and optimise any response affected by levels of one or more quantitative factors, such as ingredients or process variables (Dean and Voss, 1999).

Traditionally, a one-variable-at-a-time (OVAT) method has been used for optimization of chemical processes. In this approach, a limited number of OVAT experiments are carried out in which the levels of one variable are changed at a time while the others are kept constant (Bezerra et al., 2008). A major disadvantage of the OVAT approach is that the results do not indicate real changes in the environment as they ignore interactions among variables. As a consequence, it may not lead to the true optimum and may even lead to different end results depending on the starting point (Tye, 2004). More experiments need to be performed in order to reach the true optimum; therefore, it will be more costly in terms of analysis time and consumption of chemicals (Bezerra et al., 2008).

In contrast, RSM allows for a more efficient and more accurate determination of the optimum conditions because of several advantages over the OVAT method. First it changes combinations of variables simultaneously which does allow for incorporation of the interaction effects (Gooding, 2004). Second it is the concurrent optimization of multiple responses in order to find the optimal compromise between them. Additionally, RSM requires only a small subset of experiments from all possible variable combinations to cover the design space, which significantly reduces the number of necessary experiments (Mason et al., 1989).

Hill and Hunter (1966) first reported the origin of RSM and its application in food research. RSM has successfully been employed in determination of optimal conditions for enzymatically synthesis of acetylated tyrosol (Aissa et al., 2007) and phytosteryl esters of oleic acid (Kim and Akoh, 2007).

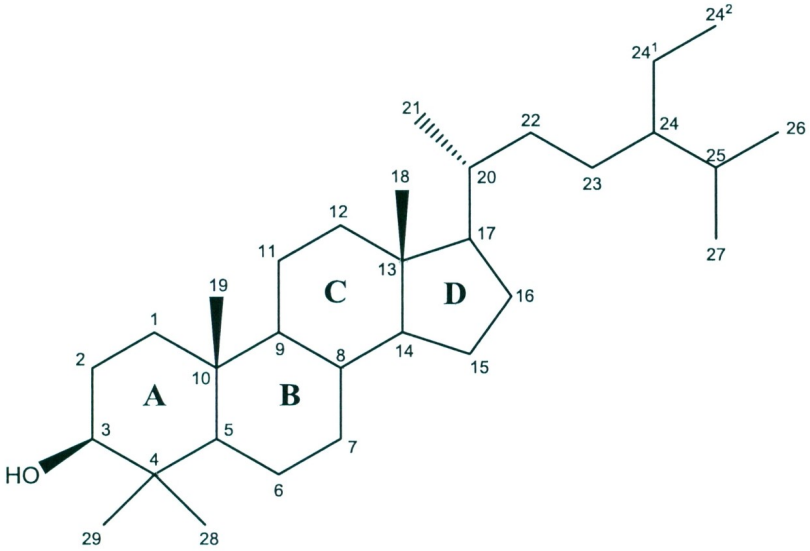
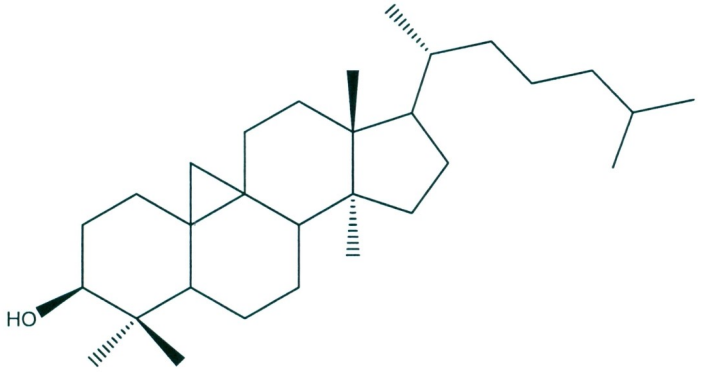
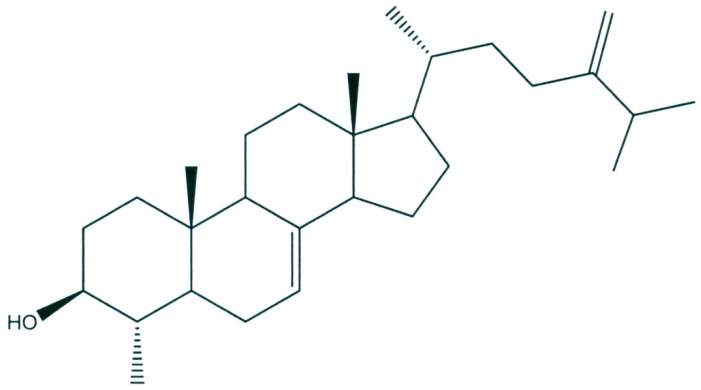


Figure 2-1. Skeletal structure of phytosterols (IUPAC-IUB recommendation of 1989).

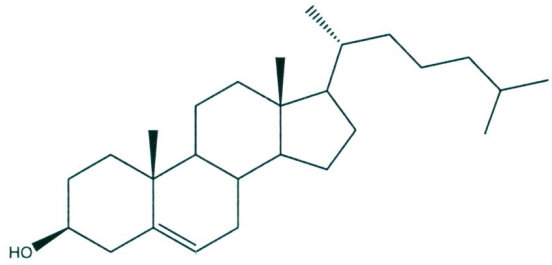


Cycloartanol

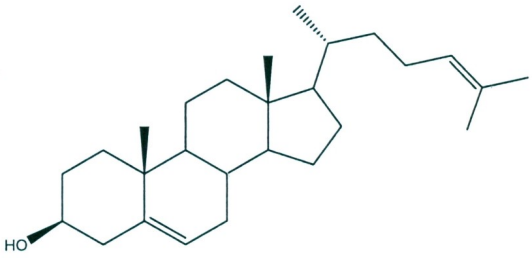


Gramisterol

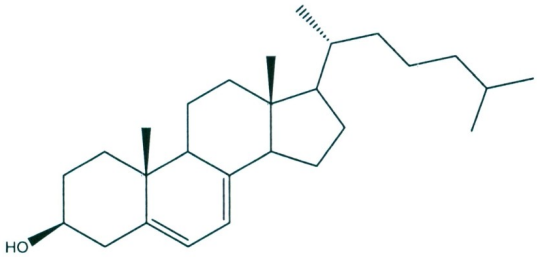
Figure 2-2. Examples for 4,4-dimethyl phytosterols (cycloartanol) and 4-monomethyl phytosterols (gramisterol).



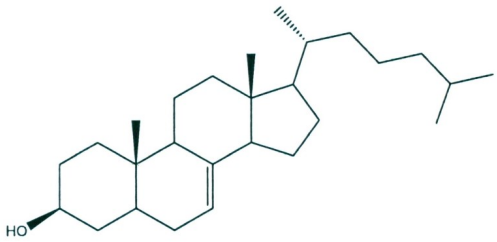
Cholesterol



Desmosterol



Cholesta-5,7-dien-3β-ol



Lathosterol

Figure 2-3. 27-carbon 4-desmethyl phytosterols.

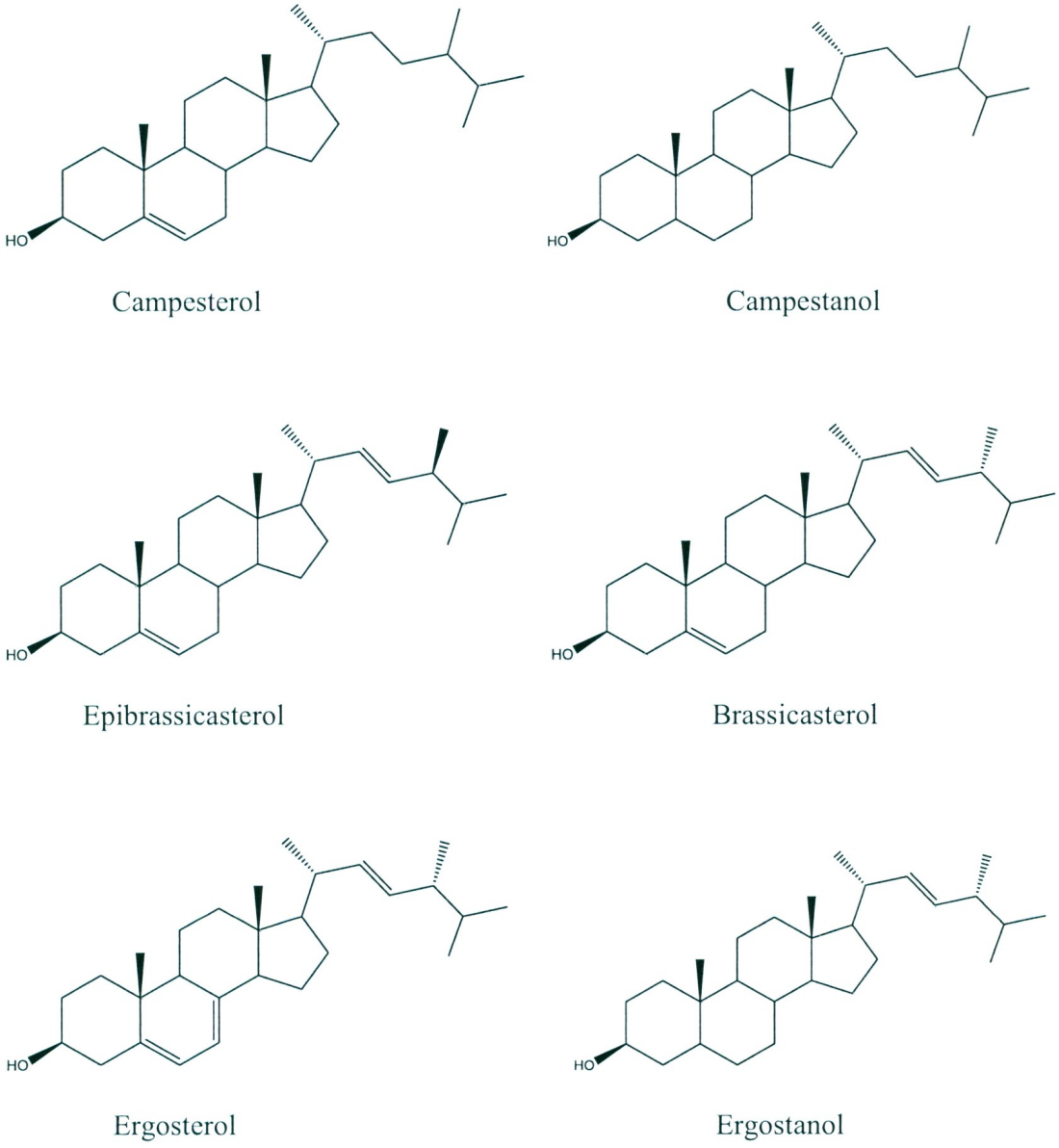
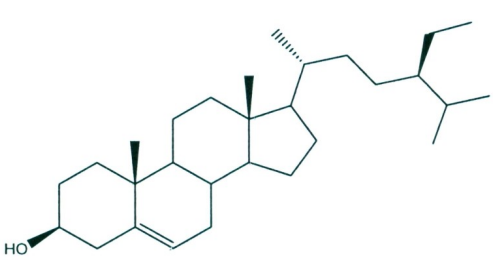
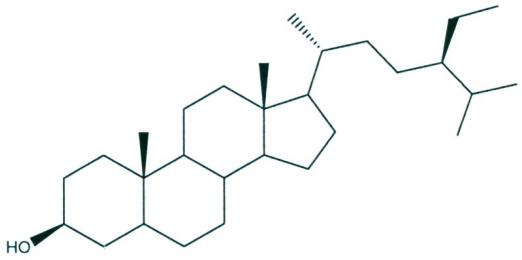


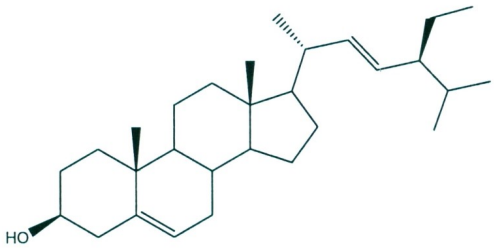
Figure 2-4. 28-carbon 4-desmethyl phytosterols.



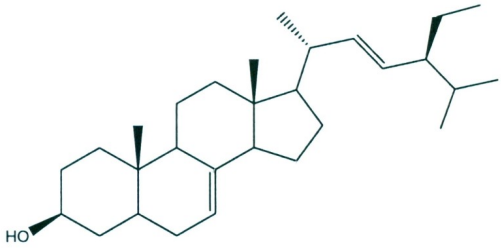
Sitosterol



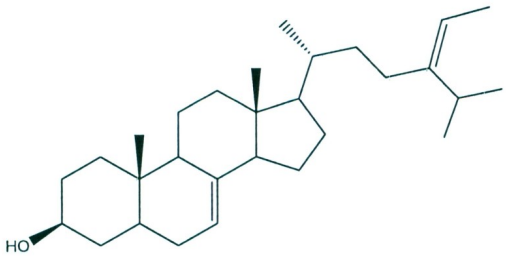
Sitostanol/Stigmastanol



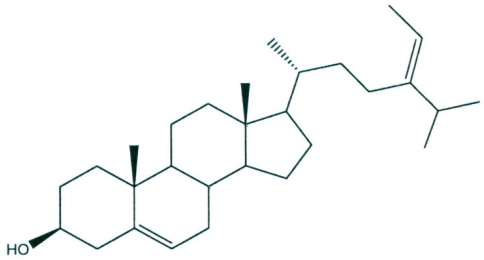
Stigmasterol



Δ^7 Stigmasterol/Spinasterol

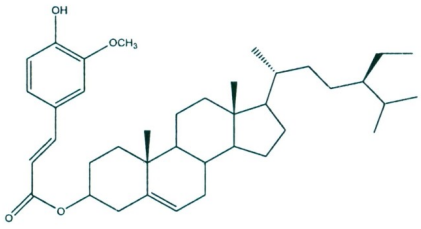


Δ^7-Avenasterol

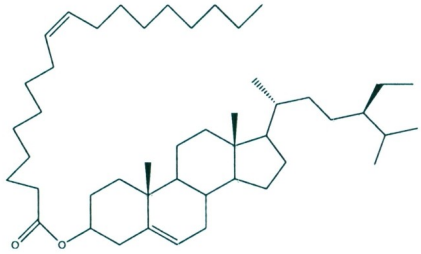


Fucosterol

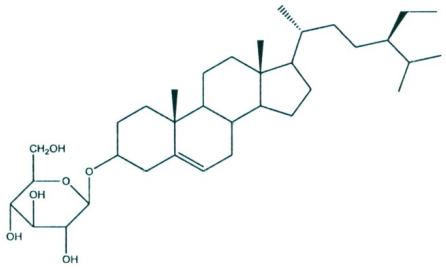
Figure 2-5. 29-carbon 4-desmethyl phytosterols.



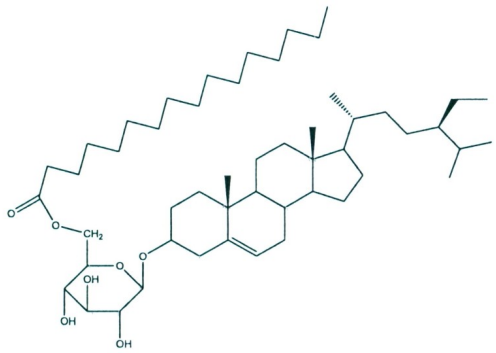
Phytosteryl phenolic acid ester (sitosteryl ferulate)



Phytosteryl fatty acids ester (sitosteryl oleate)



Phytosteryl glycoside (sitosteryl *β*-D-glucoside)



Acylated phytosteryl glycoside (sitosteryl (6'-*O*-stearoyl) *β*-D-glucoside)

Figure 2-6. Representative structures of phytosteryl conjugates.

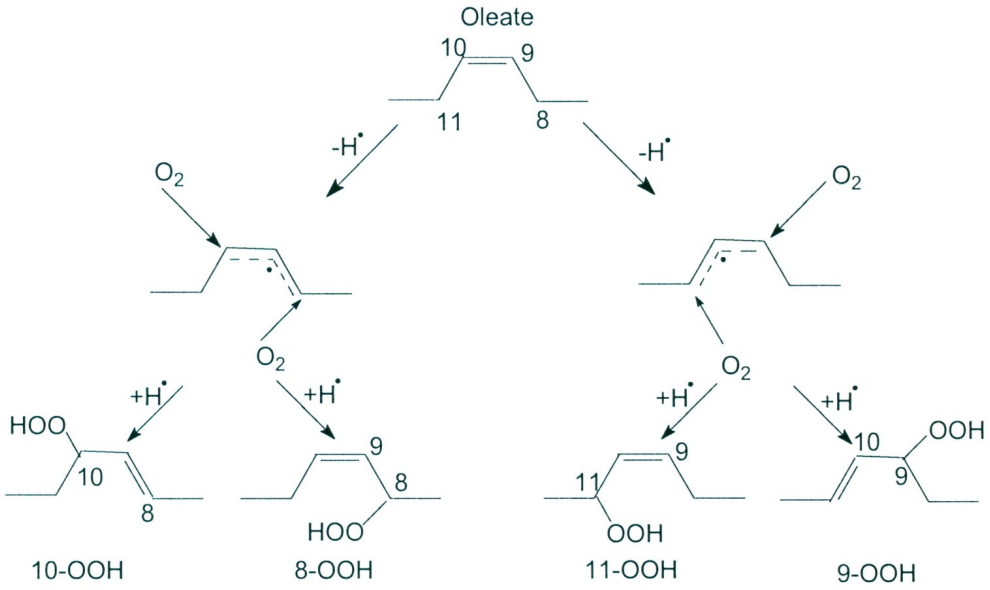


Figure 2-7. Formation of hydroperoxides from autoxidation of oleic acid.

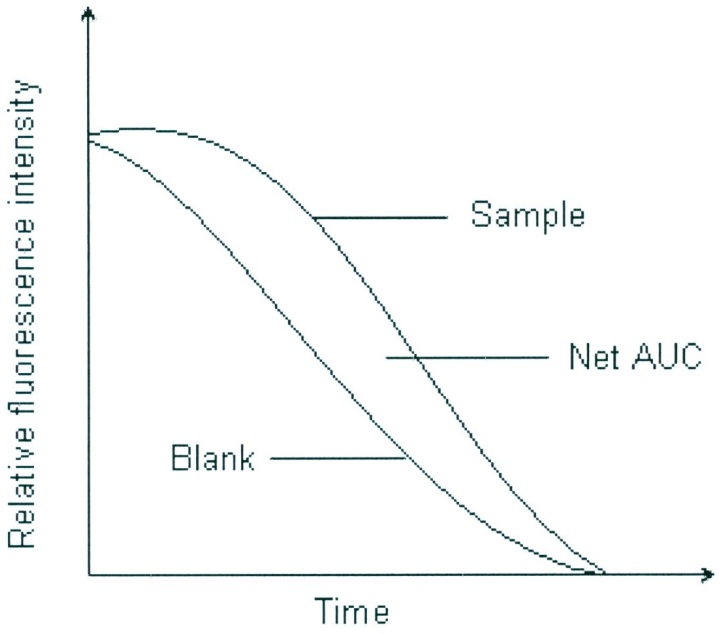
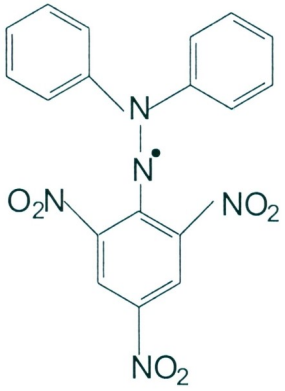


Figure 2-8. Kinetic curves in oxygen radical absorbance capacity (ORAC) assay. AUC: area under the curve.



$$DPPH^{\cdot} + AH \longrightarrow DPPH\text{–}H + A^{\cdot}$$

$$DPPH^{\cdot} + A^{\cdot} \longrightarrow DPPH\text{–}A$$

Figure 2-9. Stable 2,2-diphenyl-1-picryhydrazyl radical ($DPPH^{\cdot}$) and its reaction with antioxidants (AH).

Table 2-1. Phytosterols content in selected foods (mg/kg).

Sample	Major phytosterols			Total phytosterols
	Campesterol	Sitosterol	Stigmasterol	
Refined vegetables oils				
Corn oil	1230-1640	4540-6900	460-760	6860-9520
Rapeseed oil	760-3000	1380-3950	nd-16	2500-7670
Soybean oil	340-820	1230-1730	370-640	2030-3280
Olive (extra virgin)	40-50	1180-1330	9-13	1440-1620
Palm oil	140-180	350-410	70-100	600-680
Cereals				
Corn	320	1200	210	1780
Millet	112	371	18	770
Rice	146	375	104	723
Oats	32-51	237-323	23-38	480-611
Wheat	108-270	288-486	15-24	447-830
Vegetables				
Broccoli	67-69	285-310	40855	367-390
Brussels sprout	61-80	170-340	nd-3.8	240-430
Cauliflower	30-95	120-260	16-37	180-400
Carrot	10-22	70-110	27-30	120-160
Lettuce	6-11	37-106	24-45	85-174

nd- not detected

Sources: Weihrauch and Gardner, 1978; Verleyen et al., 2002; Reina et al., 1997 and 1999; Philips et al., 2002; Dutta and Aappelqvist, 1996; Toivo et al., 1998; Scherz et al., 2000; USDA, 2000; Piironen et al., 2002 and 2003; Maatta et al., 1999; Zambiazi et al., 1998; Hakala et al., 2002; Jonker et al., 1985; Normen et al., 1999.

Table 2-2. Types of antioxidants and their mechanisms of action.

Antioxidant class	Mechanism of antioxidant activity	Examples of antioxidants
Primary antioxidants	Inactivating lipid free radicals	Phenolic compounds
Hydroperoxide stabilizers	Preventing decomposition of hydroperoxides into free radicals	Phenolic compounds
Synergists	Promoting activity of primary antioxidants	Citric acid, ascorbic acid
Metal chelators	Binding heavy metals and pro-oxidant metals into inactive compounds	Phosphoric acid, citric acid Phenolic compounds, Maillard browning compounds
Singlet oxygen quenchers	Transforming singlet oxygen into triplet oxygen	Carotenes, phenolic compounds
Reducing substances hydroperoxides	Reducing hydroperoxides in a non-radical way	Proteins, amino acids

Sources: Pokorny et al., 1999; Cheeseman and Slater, 1993

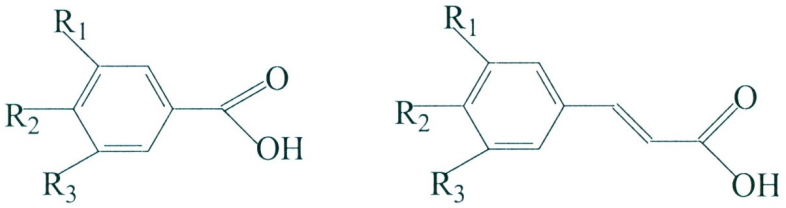
Table 2-3. Major methods used for reporting results of antioxidant activity tests.

Methods	Dimensions
Induction period	h (hour), d (day)
Time to reach a set level of oxidation (pre-induction period)	h, d
Rate of oxidation (pre-induction period)	mol kg^{-1} h^{-1}, gL^{-1} d^{-1}
Concentration of oxidation product after a set time period	mg kg^{-1} (ppm, w/w)
Scale reading after a set time period	Absorbance, conductivity, etc.
Concentration to produce equivalent effect to reference antioxidant (pre-induction period)	mol kg^{-1}, gL^{-1}
ORAC (oxygen radical absorbance capacity)	μmol of Trolox equivalents
Free stable radical quenching	Percentage inhibition; EC_{50}, concentration to decrease concentration of test free radical by 50%; T_{EC50}, time to decrease concentration of test free radical by 50%;

Sources: Huang et al., 2005; Shahidi and Zhong, 2007.

Table 2-4. Structures of phenolic acids.

R_1	R_2	R_3	Hydroxybenzoic acid	Hydroxycinnamic acids
H	OH	H	*p*-Hydroxybenzoic	*p*-Coumaric
OH	OH	H	Protocatechuic	Caffeic
OH	OH	OH	Gallic	
OCH_3	OH	H	Vanillic	Ferulic
OCH_3	OH	OCH_3	Syringic	Sinapic



Chapter 3

Materials and Methods

3.1 Materials

Phytosterols used in this study were kindly provided by Forbes Medi-Tech Inc. (Vancouver, BC). The composition of phytosterol mixtures was analyzed by both GC-MS and HPLC-MS. Results showed that it contained 75.6% sitosterol, 12.2% sitostanol, 8.1% campesterol, and 4.1% of other minor phytosterols. The relevant GC-MS and HPLC-MS data of starting free phytosterols are presented in the results and discussions part (Section 4.4 and 5.4). Docosahexaenoic acid single cell oil (DHASCO) containing approximately 40% Docosahexaenoic acid (DHA), was obtained from Martek Bioscience Corporation (Columbia, MD, USA). DHA was prepared via urea complexion as reported by Wanasundara and Shahidi (1999); the purity of the final DHA products was >97%. Caprylic acid, linoleic acid, and oleic acid were obtained from Nu-Chek (Elysian, MN, USA). Eicosapentaenoic acid (EPA) methyl ester (>99% pure) was from Fuso Pharmaceutical Industries LTD (Osaka, Japan) and kindly provided by Dr. Kazuo Miyashita of Hokkaido University, Japan.

Thirteen lipases from different commercial sources were used in this study. Novozyme 435 (lipase acrylic resin from *Candida antarctica*) and *Mucor miehei* (Lipozyme-1M) were purchased from Novo Nordisk (Franklinton, NC, USA). Three lipases were purchased from Sigma-Aldrich Canada (Oakville, ON) which included Amano lipase from *Pseudomanos fluorescens*; Amano lipase PS from *Burkholderia cepacia*

(Pseudomanos cepacia) and lipase from *Candida rugosa* type VII. Five lipases were purchased from Toyobo CO., LTD (Osaka, Japan), including lipoprotein lipase (LPL) 311, LPL 314, cholesterol esterase (COE) 301, COE 311 and COE 313. Other lipases, namely *Pseudomonas spp* (PS-30), *Aspergillus niger* (AP-12), and *Candida rugosa* (AY-30) were obtained from Amano Enzyme (Troy, VA, USA).

Ground pork and baking powder were purchased from local supermarket. Helium, hydrogen, nitrogen and compressed air were purchased from Canadian Liquid Air Ltd. (St. John's, NL). Stripped corn oil was purchased from Acros Organics (Rutherford, NJ, USA). C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). PicoLab mouse diet 20 was purchased from Ren's Feed and Supply Ltd (Aberfoyle, ON). *Baker-flex*® silica gel IB-F (2.5 X 7.5 cm) pre-coated flexible thin layer chromatography (TLC) sheets were purchased from J.T. Baker (J.T. Baker, Phillipsburg, NJ, USA). Silica gel (particle size 100-200) was purchased from Selecto Scientific (Suwannee, GA, USA).

The following chemicals were purchased from Sigma-Aldrich Canada (Oakville, ON): vinyl acetate, mercury acetate, sulphuric acid, formic acid, sodium acetate, dimethyl sulphoxide-d_6 (DMSO-d_6), tetramethylsilane (TMS), formalin, fluorescein, molecular sieves (0.3 nm), β-carotene, low-density lipoprotein (LDL) from human plasma, Tween 40, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane (TMP), Whatman No 1 filter paper, Fisherbrand filter paper P5, cupric sulphate, hydrogen peroxide, ferric sulphate, potassium hydroxide, hydrochloric acid, cholesterol, hematoxylin and eosin (H & E), anhydrous sodium sulphate, phenolic acids (caffeic acid, ferulic acid, *p*-coumaric acid, sinapic acid, gallic acid, gentisic

acid, syringic acid and vanillic acid), sodium hydroxide, anhydrous monosodium phosphate, anhydrous disodium phosphate, randomly methylated β-cyclodextrin (RMCD), trolox, fluorescein sodium salt, 2,2'-azobis(2-aminopropane) dihydrochloride (AAPH), 2,2-diphenyl-1-picrylhydrazyl (DPPH), β-carotene, tris-acetic acid, ethylenediaminetetraacetic acid (EDTA), agarose, SYBR safe gel stain, supercoiled plasmid DNA (pBR322 RRI, 43kbp), bromophenol blue, xylene cyanol, glycerol, potassium ferricyanide, trichloroacetic acid (TCA), ferric chloride and ascorbic acid. All other chemicals and solvents, if their suppliers are not specified, were purchased from Sigma-Aldrich Canada (Oakville, ON) or Fisher Scientific (Nepean, ON).

3.2 Methods

3.2.1 Optimization of enzymatic synthesis of phytosteryl caprylates

3.2.1.1 Experimental design for RSM

Response surface methodology (RSM) is a collection of techniques that is developed as a means to finding optimum setting of input factors that maximize, minimize or target measured response or outcome variables. Central composite design (CCD) and Box-Behnken (B-B) designs are two of the most common RSM models. CCD was adopted in this optimization process. It is a 2^k factorial design with star points and centre points.

To establish the model coefficient, a three-factor and three-level CCD with 17 individual design points was selected in this study. Since the rotatable and orthogonal CCD cannot be practically achieved, on face (axial value $\alpha = \pm 1$) CCD was chosen in this study. Central composite face-centred (CCF) design matrix with the star points being at the centre

of each face of factorial space was used, so $\alpha = \pm 1$. This variety requires three levels of each factor.

In the present study, the experiment points are located in the middle of a cube ridge (14 experiments) and at the centre of the cube (3 experiments). To avoid bias, 17 runs were performed in a totally random order. A Central composite face centred (CCF) design for three variables X_1, X_2, and X_3 with three levels are presented in Table 3-1. Triplicate experiments were conducted at all design points.

The second-order polynomial regression model was assumed for predicting Y variable (DE = degree of esterification of phytosteryl caprylates). The model proposed for the response of Y fitted the equation as follows:

$$Y = \beta_o + \sum_{i=1}^{3} \beta_i X_i + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum\sum_{i<j=1}^{3} \beta_{ij} X_i X_j$$

Where, Y is response variable (degree of esterification), β_o, β_i, β_{ii} and β_{ij} are the regression coefficients for intercept, linear, quadratic, and interaction terms, respectively, and X_i and X_j are the independent variables (reaction time, enzyme load and substrate mole ratio).

The fitted model was used to study the effect of three chosen variables to the response and to look for the optimum experimental conditions which gave the highest esterification degree. Response surfaces and contour plots were attained using the fitted model by keeping the least effective independent variable at a constant value while changing the other two variables. To validate the estimated model, 3 confirmation experiments were performed using the optimum reaction conditions.

In this study, the design of the experiment and the data treatment of the CCD design were performed using statistical software *JMP* version 6.0.0. (SAS Institute Inc. Cary, NC, USA)

3.2.1.2 Lipase-catalyzed esterification of phytosteryl caprylates

The esterification reactions were carried out in 20 ml screw-capped test tubes. Phytosterols (60 mg) with different quantities of caprylic acid, corresponding to the different substrate mole ratios, were weighted into the test tubes, followed by the addition of different amounts of powdered *Candida rugosa* lipase as indicated in Table 3-3, and 2 ml of n-hexane were subsequently added. The test tubes were flushed with nitrogen to remove the oxygen inside the tube before they were sealed with screw-caps. The reactions were performed in an orbital shaking water bath at 45 °C at 200 rpm for different time periods as indicated in Table 3-3. The reaction was stopped by cooling under running tap water. Then 5 ml of n-hexane were added to the test tubes. The mixtures were vortexed for 20 seconds, and then the solvents containing reactants were passed through a filter paper (Fisherbrand filter paper P5), on which a layer of sodium sulphate was placed in order to remove the lipase and water generated during the esterification reaction. Two more 5 ml of n-hexane portions were added to recover all the reaction mixtures. The solvents were collected into a 50 ml round bottom flask and completely removed with rotary evaporator (BÜCHI Labortechnik, Flawil, Switzerland) at 45 °C. The sample was redissolved in 10 ml chloroform. From the above solution, a final 1 mg/ml solution was made for gas-chromatographic-mass-spectroscopic (GC-MS) analysis.

3.2.1.3 Gas-chromatographic-mass spectrometric (GC-MS) analysis

The composition of the reaction mixtures was analyzed by GC-MS. An Agilent Technologies 6890N series gas chromatography (Agilent Technologies, Inc., Santa Clara, CA, USA) equipped with an Agilent Technologies 5973 *inert* mass selective detector and a fused silica capillary column (J122-5032 DB-5, 30 m × 0.25 mm I.D., film thickness 0.25 μm. J&W Scientific., Folsom, CA, USA) was used. The carrier gas was helium and the total gas flow rate was 54 ml/min. The injector and detector temperatures were maintained at 280 °C. The column was heated initially at 180 °C and programmed to increase to 280 °C at the rate of 8 °C /min. The column temperature was then programmed to increase to 325 °C at rate of 10 °C /min and held at 325 °C for 10 min (Kinter et al., 1988). The detector temperature was set at 280 °C and the data were obtained in the scan mode by electron impact ionization under positive mode.

3.2.1.4 Calculation of degree of esterification

The following equation was employed to calculate the degree of esterification from the GC profile:

$$\text{Degree of Esterification (\%)} = \frac{A_{tpe}}{1.305 * A_{tp} \quad A_{tpe}}$$

Where A_{tp} is the total peak area of phytosterols, A_{tpe} is the total peak area of phytosteryl caprylates, and 1.305 is the ratio of the average molecular weight of phytosteryl caprylates to average molecular weight of phytosterols.

3.2.1.5 Fourier-transform infrared (FTIR) spectroscopic analysis

The synthesized products were separated by column chromatography and the purified phytosteryl caprylates were characterized using a Fourier-transform infrared spectrometer. The IR spectra were recorded using FTIR Bruker Tensor 27 spectrometer (Bruker Optik GmbH, Ettlingen, Germany). This spectrometer has a spectral range of 7,500 to 370 cm^{-1} and is equipped with a MIRacle Attenuated Total Reflectance (ATR) accessory allowing rapid and easy analysis of both liquid and solid samples.

3.2.2 Chemoenzymatic synthesis of phytosteryl phenolates

Eight types of phenolic acids were selected as acyl donors for the synthesis of phytosteryl phenolates. Four of them were hydroxycinnamic acids, namely caffeic, ferulic, *p*-coumaric and sinapic acids. The other four hydroxybenzoic acids were gallic, gentisic, syringic and vanillic acids. These eight phenolic compounds are referred to phenolic acids in subsequent chapters of this thesis.

3.2.2.1 Chemical synthesis of vinyl phenolates

Vinyl phenolates were chemically synthesized via the vinyl interchange reaction of vinyl acetate and phenolic acids according to the method described by Swern and Jordan (1963) and Gao et al. (2001). Phenolic acid (0.01 mol), 15 ml vinyl acetate (0.16 mol), mercury acetate (4%, w/w) and 10 ml tetrahydrofuran (THF) were added into a 125 ml Erlenmeyer flask and capped. The reaction mixture was stirred with a magnetic stirring bar under a nitrogen blanket for 30 min, and then 2 µl sulphuric acid (0.04 mmol) were added to

start the reaction. The reaction mixture was flushed again with nitrogen and then put into a 40 °C shaking water bath (200 rpm). After 12 hours reaction, an excess of sodium acetate (20 mg) was added to neutralize the sulphuric acid and to stop the reaction. The solvent and the excess of vinyl acetate were removed, using a rotary evaporator (BÜCHI Labortechnik, Flawil, Switzerland) at 40 °C. The residue obtained was subjected to column chromatography on silica gel using an isocratic elution with hexane/ethyl acetate (4:1, v/v). Thin layer chromatography (TLC) was carried on *Baker-flex*® silica gel IB-F (2.5 X 7.5 cm) pre-coated flexible TLC sheets (J.T. Baker, Phillipsburg, NJ, USA). Products were visualized under UV light at 254 nm using Spectroline® (Spectronics Corporation, New York, NY, USA).

3.2.2.2 NMR analysis of phenolic acids and vinyl phenolates

^{1}H-NMR analysis of purified vinyl phenolates were carried out in order to confirm the formation of vinyl esters. NMR spectra of pure phenolic acids and isolated vinyl phenolates were recorded on a Bruker AVANCE 500 spectrometer (Bruker Biospin Co., Billerica, MA, USA). Proton spectra were recorded at 500 MHz using a solvent field lock. The samples were dissolved in DMSO-d6 containing tetramethylsilane (TMS) as internal standard. Signal processing and interpretation were performed with the software Topspin 1.3 (Bruker Biospin Co., Billerica, MA, USA) and *MestRe Nova* (Mestrelab Research SL, Santiago De Compostela, Spain). Chemical shifts were expressed in δ (parts per million) values relative to TMS as internal reference. Structure elucidation was accomplished by comparing the chemical shifts of vinyl phenolates with that of the parent

phenolic acid molecules. *Chem & Bio Draw* 11.0 (CambridgeSoft Corporation, Cambridge, MA, USA) was used to predict the chemical shift values of protons in both phenolic acids and vinyl phenolates. The predicted values were also compared with the experimental values.

3.2.2.3 Screening enzyme for synthesis of phytosteryl phenolates

Vinyl phenolate (0.015 g) was placed into a test tube together with 0.06 g phytosterol mixtures (mole ratio of vinyl phenolate to phytosterol mixture, 1:2) and different lipases (8% of the total weight of both substrates). Three ml of solvent (hexane/2-butanone, 9:1, v/v) were added to the test tube which was then flushed with nitrogen before being sealed with screw cap. The reaction mixture was then shaken in a gyratory water bath shaker at 200 rpm and 45 °C (New Brunswick Scientific Co., Inc, New Brunswick, NJ, USA). The following 9 enzymes were used for screening the best enzyme for the synthesis of phytosteryl phenolates, namely Amano lipase from *Pseudomonas fluorescens*; Amano lipase PS from *Burkholderia cepacia (Pseudomonas cepacia)*; lipase from *Candida rugosa*; Novozyme 435 (lipase acrylic resin from *Candida antarctica*); lipoprotein lipase (LPL) 311; LPL 314; cholesterol esterase (COE) 301; COE 311; and COE 313. The reaction was monitored by using *Baker-flex*® silica gel IB-F (2.5 X 7.5 cm) pre-coated flexible TLC sheets (J.T. Baker, Phillipsburg, NJ, USA) with eluents of hexane/ethyl acetate/formic acid (80:20:1, v/v/v). After 10 days the reaction was stopped by placing them under the running tap water. The enzyme was filtered through a filter paper. Solvent was evaporated with rotary evaporator (BÜCHI Labortechnik, Flawil,

Switzerland) at 40 °C and the solid residue was then subject to column chromatography. The structure of the phytosteryl phenolates were then elucidated by FTIR and HPLC-MS/MS analysis.

3.2.2.4 Enzymatic synthesis of phytosteryl phenolates

In a small, gram-scale, experiment, substrates (0.5 g vinyl phenolate and 2.08 g phytosterol mixtures in mole ratio of 1:2) were weighted into a screw-capped 250 ml conical flask together with the enzyme (10% of total weight of both substrates), followed by the addition of 100 ml of solvent (hexane/2-butanone, 9:1, v/v) into the flask. The flask, flushed with a stream of nitrogen before sealing with screw cap, was then put into a gyratory water bath shaker at 200 rpm and 45 °C (New Brunswick Scientific Co., Inc, New Brunswick, NJ, USA). The reactions were carried out for 10 days. The reaction was monitored by TLC on *Baker-flex*® silica gel IB-F (2.5 X 7.5 cm) pre-coated flexible TLC sheets (J.T. Baker, Phillipsburg, NJ, USA) with eluents of hexane/ethyl acetate/formic acid (80:20:1, v/v/v) and the reaction was then stopped by cooling under running tap water until the band for the starting material-vinyl phenolate disappeared on the TLC plate. The enzyme was filtered through a filter paper (Fisherbrand filter paper P5). Solvent was evaporated using a rotary evaporator (BÜCHI Labortechnik, Flawil, Switzerland) at 40 °C. The solid residue was then subjected to column chromatography as explained below.

3.2.2.5 Colum chromatographic separation of phytosteryl phenolates

In a typical example, five grams of reaction mixtures were dissolved in a minimum amount of n-hexane/ethyl acetate (4:1, v/v) and applied to a column (40 cm × 5 cm I.D.) packed with silica gel (Selecto Scientific, Suwannee, GA, USA) as a slurry in n-hexane, subsequently eluted with n-hexane/ethyl acetate (4:1, v/v). The fractions were collected in test tubes. TLC on *Baker-flex*® silica gel IB-F (2.5 X 7.5 cm) pre-coated flexible TLC sheets (J.T. Baker, Phillipsburg, NJ, USA) was employed to monitor the different fractions.

3.2.2.6 FTIR analysis of phytosteryl phenolates

The above purified phytosteryl phenolates were characterized using a Fourier-transform infrared spectrometer. The IR spectra were recorded using FTIR Bruker Tensor 27 spectrometer (Bruker Optik GmbH, Ettlingen, Germany). This spectrometer has a spectral range of 7,500 to 370 cm^{-1} and is equipped with a MIRacle ATR accessory allowing rapid and easy analysis of liquid and solid samples.

3.2.2.7 HPLC-MS/MS analysis of phytosteryl phenolates

The identities of the phytosteryl phenolates were confirmed by high performance liquid chromatography-mass spectrometry/mass spectrometry (HPLC-MS/MS) with atmospheric pressure chemical ionization (APCI) using both negative ion (NI) and positive ion (PI) modes. The analysis was performed using an Agilent 1100 HPLC-MSD system (Agilent, Palo Alto, CA, USA) with an on-line solvent degasser, binary solvent

delivery system, autosampler and UV-Vis diode array detector (DAD). The MS detector has electrospray or atmospheric pressure chemical ionization capability with a mass range of *m/z* 50-3000.

Separation was achieved on a C18 column (250 mm length, 4.6 mm i.d., 5 μm particle size, Sigma-Aldrich Canada Ltd., Oakville, ON) coupled with a guard column. Phytosteryl phenolates were eluted using an isocratic solvent system containing methanol/water (95:5, v/v) at a flow rate of 1.0 ml/min. Fifty microlitres of each sample were injected to the system. For better separation and ionization of phytosteryl caffeates, formic acid was added to the above mentioned mobile phase at 1% level. All phytosteryl phenolates were detected at 325 nm by UV detection except phytosteryl vanillates which were detected at 280 nm. LC flow was analyzed on-line by a mass selective detector system (LC-MSD-Trap-SL, Agilent, Palo Alto, CA, USA) with both positive and negative ion APCI. The operating conditions used were 121v for the fragmentor voltage, 350°C for drying temperature, 400 °C for APCI temperature, 60 psi for the nebulizer pressure, and 7 l/min for the drying gas flow.

3.2.3 Evaluation of the antioxidant activity of phytosteryl phenolates

The antioxidant activity of phytosteryl phenolates, namely phytosteryl caffeates, phytosteryl ferulates, phytosteryl sinapates and phytosteryl vanillates, were evaluated using DPPH radical scavenging capacity test, oxygen radical absorbance capacity (ORACFL) assay, Rancimat test, β-carotene-linoleate model system, cooked pork model system, reducing power test, LDL cholesterol oxidation assay, and DNA scission test,

among others, together with their starting phenolic acids and intermediate vinyl phenolates, as describe below.

3.2.3.1 DPPH radical scavenging capacity test using electron paramagnetic resonance (EPR) spectrometry

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay was performed according to the method described by Shahidi et al. (2007). Two ml of a 0.18 mM solution of DPPH in ethanol were added to test tubes with 0.4 ml of various concentrations of samples/standard (trolox) dissolved in ethanol. Ethanol without sample was used as a blank. Mixtures were then vortexed and transferred to a syringe and then passed through the capillary tubing which guides the sample through the sample cavity of a Bruker e-scan EPR spectrometer (Bruker E-scan, Bruker Biospin Co. Billercia, MA,USA). Measurements started exactly after 1 min. The spectrum was recorded using the software *E-Scan* analyzer (Bruker Biospin Co. Billercia, MA, USA). The parameters for the test were set as follows; 5.02 x 10^2 receiver gain, 1.86 G modulation amplitude, 2.62 s sweep time, 8 scans, 100.0 G sweep width, 3495.26 G centre field, 5.12 ms time constant, 9.79 GHz microwave frequency, 86.0 kHz modulation frequency, and 1.86 G modulation amplitude. DPPH radical scavenging capacities of the samples (%) were calculated using the following equation.

$$\text{Inhibition \%} = \frac{\text{EPR signal intensity for blank - EPR signal intensity for sample}}{\text{EPR signal intensity for blank}} \times 100$$

The final results were expressed as trolox equivalents per millimoles of sample using appropriate standard curves.

3.2.3.2 Oxygen radical absorbance capacity (ORAC) assay

The $ORAC_{FL}$ assay for lipophilic antioxidant described by Wu et al. (2004) was adapted with modifications. The determination of $ORAC_{FL}$ was carried out using a Fluostar Optima plate reader (BMG LABTECH, Durham, NC, USA) equipped with an incubator and injector pump. Fluorecsein was used as the probe and AAPH was employed as the radical generator. The reaction was carried out in a 96-well Costar black plate (Corning Incorporated, Corning, NY, USA) with a final reaction mixture of 275 μl. Twenty microlitres sample were dissolved in 7% (w/v) randomly methylated *β*-cyclodextrin (RMCD) in acetone/water (1:1, v/v) and 200 μl fluorescein (0.11 mM in distilled water) were injected manually into each wells. The mixture was incubated for 20 min at 37°C in the built-in incubator and subsequently 75 μl 2,2'-azobis(2-aminopropane) dihydrochloride (AAPH) solution (63.4 mM in PBS), equilibrated at 37°C, were rapidly injected into the wells using the injector pump. The plate was shaken for 4 s after each addition at 4 mm shaking width. Other conditions for Fluostar Optima plate reader were set as follow: cycle No, 25; cycle time, 210 s; position delay, 0.3 s; shaking, 8 s; orbital, 4 mm width; shaking before each cycle; injection, pump 1; and speed, 420 μl/s. To optimize signal amplification in order to obtain maximum sensitivity, gain adjustment was performed at the beginning of each measurement. No more than 36 wells of the 96-well plate were used due to increased cycle time. Fluorescence was determined and

recorded and the antioxidant activity of the extracts was calculated as trolox equivalents using a standard curve prepared with concentrations in the range of 6.25-50 μM trolox in 7% (w/v) RMCD in acetone/water (1:1, v/v), blank 7% (w/v) RMCD in acetone/water (1:1, v/v), fluorescein and AAPH. Filters with an excitation wavelength of 485 nm and an emission wavelength of 520 nm were used.

3.2.3.3 Antioxidant activity in bulk oil model system

Measurement of the antioxidant potency of the synthesized phytosteryl phenolates in bulk oil model system, under accelerated oxidative conditions was performed, using the well-established Rancimat method. Rancimat® apparatus (743 Rancimat®, Metrohm Ion Analysis Ltd., CH-9101, Herisau, Switzerland) was operated at 120 ºC. One millilitre of sample (1.5mM) in ethanol was added into each reaction vessels of the Rancimat® apparatus. The solvent was evaporated under a stream of nitrogen and then 3 g of stripped corn oil were weighted into the reaction vessels. A constant stream of dry air (20 l/h), obtained by passing laboratory air through molecular sieves (0.3 nm), was blown through the oil mixtures containing sample antioxidants throughout the experiment. The volatile oxidation products, arising from the oxidation of the stripped corn oil, were collected in the measuring vessels containing 60 ml of deionized water. The conductivity of the aqueous solution was monitored continuously and recorded. The inflection point was calculated by the software 743 Rancimat® PC software version 1.0, 2000 (Metrohm Ion Analysis Ltd., CH-9101, Herisau, Switzerland) and recorded. The time (in hours) taken to reach a specific conductivity value, corresponding to the inflection point of the

peroxidation curve, was considered as the induction time (IT). The longer the induction time, the greater was the antioxidant potency of the compound. All tests were performed in triplicates. A blank containing pure stripped corn oil devoid of any sample was used. Results were reported as protection factor.

$$\text{Protection factor} = \frac{IT_{sample}}{IT_{blank}}$$

Where, IT_{sample} is induction time of oil mixtures containing the sample; and IT_{blank} is that of corn oil alone as a blank.

3.2.3.4 Antioxidant activity in β-carotene-linoleate model system

A β-Carotene-linoleate model system was used to evaluate the antioxidant activity of synthesized phytosteryl phenolates as described by Amarowicz and Shahidi (1995) with minor modifications. A Fluostar Optima (BMG LABTECH Inc., Durham, NC, USA) plate reader in absorbance mode was used for this purpose. The assay was based on the ability of the samples to decrease the oxidative bleaching of β-carotene in a β-carotene/linoleic acid emulsion system. The emulsion was prepared as follow: a 20 mg sample of crystalline β-carotene was dissolved in 10 ml of chloroform; a 1 ml portion of this solution was transferred into a 100 ml round-bottom flask containing 20 mg linoleic acid and 200 mg of Tween 40. After removal of chloroform under a nitrogen stream, 50 ml aerated distilled water were added to the flask and the mixture was stirred vigorously using a magnetic stirring bar. A blank emulsion devoid of β-carotene was also prepared

(20 mg of linoleic acid + 200 mg Tween 40 + 50 ml oxygenated distilled water) for background subtraction.

Test compounds (30 µl, 0.75 mM in ethanol) or ethanol as control and 270 µl blank emulsion without *β*-carotene were manually injected into the Costar 96-well white plates (Corning Incorporated, Corning, NY, USA). The *β*–carotene/linoleic acid emulsion was injected by the built-in injector pump. The absorbance was measured immediately after the addition of emulsion to sample. The temperature of the built-in incubator was set at 45 °C. A filter with excitation wavelength of 450 nm was used. Other parameters of Fluostar Optima were set as follow: cycle No, 12; cycle time, 60 s; position delay, 0.3 s; shaking, 8 s; orbital, 4 mm width; shaking before each cycle; injection, pump 1; and speed 420 µl/s.

Antioxidant activity of test compounds in protecting *β*-carotene/linoleic acid oxidation was calculated using the following equation.

$$AA\% = [1-(A_{s0}-A_{st})/(A_{c0}-A_{ct})]*100$$

Where A_{s0} and A_{st} are corrected absorbance values for test samples measured at zero time and after incubation, respectively; while A_{c0} and A_{ct} are corrected absorbance values for control at zero time and after incubation, respectively.

3.2.3.5 Antioxidant activity in cooked pork model system

A cooked pork model system was employed for assessing the antioxidant activity of phytosteryl phenolates. The meat model system described by Shahidi and Alexander (1998) was adopted. Forty g of fresh ground pork and 10 g deionized water were mixed in a 200 ml Mason jar. Samples and the reference antioxidant compound (BHA), dissolved in ethanol, were added to meat at a level of 100 μmol/kg. A control without any antioxidant was also prepared. The mixture was thoroughly homogenized with a glass rod and cooked at 80 ºC in a thermostated water bath for 40 min with intermittent stirring. The cooked meat was cooled to room temperature and homogenized with glass rod again. The homogenate was then transferred into plastic bags and stored at 4 ºC for 14 days. The meat samples were taken on day 0, 3, 5, 7, and 14 for measurement of TBARS (thiobarbituric acid reactive substances) values as indicator of the formation of secondary oxidation products.

TBARS values were determined as described by Shahidi and Pegg (1994). A series of 1,1,3,3-tetramethoxypropane (TMP) standard solutions at different concentrations was mixed with thiobarbituric acid (TBA) in screw-capped tubes and heated in a boiling water bath for 45 min. After cooling down to room temperature, the absorbance at 532 nm was recorded and a standard curve was constructed (absorbance versus concentration). For determination of TABRS in the cooked meat model system, 2 g of meat were mixed with 5 ml of trichloroacetic acid (TCA, 10% in deionized water) in a centrifuge tube, followed by addition of 5 ml of the TBA reagent (0.02 M in deionized water). The mixture was centrifuged at 3000 x g for 10 min and the supernatant was

filtered using a Fisherbrand P5 filter paper. The filtrate was heated in a boiling water bath for 45 min and the absorbance was read at 532 nm using a Hewlett-Packard 8452A diode array spectrophotometer (Hewlett-Packard Company, Palo Alto, CA, USA) after cooling to room temperature. TBARS values in meat samples were obtained using the standard curve as μmols malonaldehyde equivalents/kg of pork. All experiments were carried out in triplicates.

3.2.3.6 Reducing power test

The reducing power of samples was determined according to method explained by Shahidi and Naczk (1995) with slight modifications. The assay medium contained 1 ml of sample solution (0.375-1.5 mM in 95% ethanol), 2.5 ml phosphate buffer solution (PBS, 0.2M, pH 6.6) and 2.5 ml of 1% potassium ferricyanide. After incubating the above mixture at 50°C in a water bath with shaking at 200 rpm for 20 min, 2.5 ml of 10% trichloroacetic acid (TCA) were added followed by centrifugation at 3000 x g for 10 min. A portion of the supernatant (2.5 ml) was transferred into a 10 ml test tube containing 2.5 ml of deionized water and 0.5 ml of 0.1% ferric chloride in water. The absorbance was read at 700 nm using a Hewlett-Packard 8452 A diode array spectrophotometer (Hewlett-Packard Company, Palo Alto, CA, USA) and the results were expressed as millimoles of ascorbic acids equivalents/millimole of sample using an appropriate standard curve.

3.2.3.7 LDL cholesterol oxidation test

The method for LDL cholesterol oxidation test described by Liyana-Pathirana et al. (2006) was employed in this study. LDL cholesterol was dialysed in 10 mM PBS (phosphate buffered saline) (pH 7.4) at 4 °C under a nitrogen blanket in the dark for 24 h. LDL cholesterol (0.08 mg LDL/ml) was mixed with samples (0.2 mg/ml). Ferulic acid was used as the reference antioxidant compound. The reaction was initiated by adding a solution of cupric sulphate (50 μM) and then, samples were incubated at 37 °C for 22 h. The formation of conjugated dienes (CD) was recorded at 234 nm using a Hewlett-Packard 8452 A diode array spectrophotometer (Hewlett-Packard Company, Palo Alto, CA, USA). The inhibitory effect of tested samples on the formation of conjugated dienes (% inhibition CD) was then calculated using the equation given below; a separate blank that contained all of the reagents except LDL was used for each sample:

$$\% \text{ Inhibition}_{CD} = [(\text{Abs}_{control} - \text{Abs}_{sample})] / [(\text{Abs}_{control} - \text{Abs}_{native})] \times 100$$

Where $\text{Abs}_{control}$ is the absorbance of LDL +$CuSO_4$ + PBS; Abs_{sample} is the absorbance of LDL + $CuSO_4$ + sample/standard; Abs_{native} is the absorbance of LDL + PBS.

3.2.3.8 DNA scission assay

Two types of radicals were employed in this test; hydroxyl radical generated through Fenton reaction and peroxyl radical generated by AAPH. In a 0.5 ml Eppendorf

tube, 2 µl sample in ethanol were added, and the solvent was evaporated under a stream of nitrogen followed by the addition of 2 µl distilled water, 2 µl PBS, 2 µl of supercoiled pBR322 DNA (50 µg/ml in PBS), 2 µl hydrogen peroxide (0.5 mM) and 2µl ferric sulphate (0.5 mM) in the order given. As for the AAPH generated peroxyl radical, 4 µl of AAPH solution were used. The reactants were mixed well and incubated at 37 ºC for 1 h in the dark (Hu et al., 2000). Upon completion of incubation, 1 µl loading dye, consisting of 0.25% bromophenol blue, 0.25% xylene cyanol and 50% glycerol, was added to the sample. The mixture was then loaded to a 0.7% (w/v) agarose gel prepared in tris-acetic acid- ethylenediaminetetraacetic acid (EDTA) buffer (40 mM tris acetate, 1 mM EDTA, pH 8.5). SYBR safe gel stain was then added (1:10000) before solidification of the gel (when the gel was cooled to 60 ºC). Horizontal gel electrophoresis was performed at 80 V for 1.5 h. DNA strands were visualized under ultraviolet light. Images were analyzed using Gel Doc or AlphaEase TM stand-alone software (Alpha Innotech Co., San Leandro, CA, USA). Antioxidant activity was calculated as % retention of DNA based on the following equation.

$$\text{DNA retention (\%)} = \left\{ \frac{\text{Intensity of supercoiled DNA in sample}}{\text{Intensity of supercoiled DNA in control}} \right\} \text{X } 100$$

3.2.4 Synthesis of phytosteryl oleates and docosahexaenoates

3.2.4.1 Enzymatic synthesis of phytosteryl oleates

The method described by Kim and Akoh (2007) was adapted, with modifications, for gram-scale preparation of phytosteryl oleates. Phytosterols (6.4 g) with oleic acid (3.5 g, at a mole ratio phytosterols to oleic acid of 1.2) were weighed into a 250 ml conical flask. Lipase from *Candida rugosa* (9% of the total substrate weight) was added together with 100 ml n-hexanes. The flask was flushed with nitrogen to remove any oxygen before being sealed with a screw cap and then placed into an orbital shaking water bath at 45 °C at 200 rpm for 24 h. The reaction was stopped by cooling the flask under running tap water. Then 50 ml of n-hexane were added to the test tube, and then the solvents containing reactants were passed through a filter paper (Fisherbrand filter paper P5) with a layer of sodium sulphate to remove the lipase and water generated during the esterification reaction. Two more 50 ml portion of n-hexane was added to recover all the reaction mixtures. The solvents containing the sample were collected into a 500 ml round bottom flask and all solvents were then completely removed using rotary evaporator (BÜCHI Labortechnik, Flawil, Switzerland) at 45 °C. The sample was then subjected to flash column chromatography for separation.

3.2.4.2 Column chromatographic separation of phytosteryl oleates

In a typical example, Five g of reaction mixtures were dissolved in a minimum amount of n-hexane and applied to a column (40 cm × 5 cm I.D.) packed with Silica Gel (Selecto Scientific, Suwannee, GA, USA) as slurry in n-hexane. Subsequently eluted with

n-hexane/ethyl acetate (4:1, v/v). The fractions were collected in test tube. TLC on *Baker-flex*® silica gel IB-F (2.5 X 7.5 cm) pre-coated flexible TLC sheets (J.T. Baker, Phillipsburg, NJ, USA) was employed to monitor the different fractions.

3.2.4.3 Purification of DHA from DHASCO using urea complexion

Preparation of DHA from DHA single cell oil (DHASCO) was conducted according to the procedures described by Wanasundara and Shahidi (1999). DHASCO (25 g, treated with 200 ppm butylated hydroxytoluene (BHT)) was saponified using potassium hydroxide (5.75 g), water (11 ml) and 66 ml 95% (v/v) ethanol by refluxing for 1 h at 60 °C under a blanket of nitrogen. Distilled water (60 ml) was added to the saponified mixture and the unsaponifiable matter was extracted with hexane (2x 100 ml) and discarded. The aqueous layer containing saponifiable matter was acidified with 3.0 M HCl to pH 1.0. The mixture was transferred to a 250 ml separatory funnel, and the liberated fatty acids were extracted into hexane (50 ml). The hexane layer containing free fatty acids was then dried over anhydrous sodium sulphate and the solvent was removed using a rotary evaporator at 45 °C. The recovered free fatty acids were then subjected to urea complexation. Free fatty acids (10 g) were mixed with urea (20%, w/v) in 150 ml 95% aqueous ethanol and heated at 60 °C with stirring until the whole mixture turned into a clear homogeneous solution. The urea-fatty acids adduct was allowed to crystallize at room temperature but was later placed in a cold room where the temperature was maintained at 4 °C for 24 h. The crystals formed were separated from the liquid by suction filtration through a filter paper (Whatman filter paper No 1). The filtrate was

diluted with an equal volume of water and acidified to pH 4-5 with 6 M HCl. An equal volume of hexane was subsequently added and the mixtures stirred thoroughly for 1 h, then transferred to a separatory funnel. The hexane layer containing liberated fatty acids was separated from the aqueous layer containing urea and washed with distilled water to remove any remaining urea and then dried over anhydrous sodium sulphate. The solvent was subsequently removed at 45 °C using a rotary evaporator (BÜCHI Labortechnik, Flawil, Switzerland).

3.2.4.4 Screening enzyme for synthesis of phytosteryl docosahexaenoates

DHA was added into a test tube together with phytosterol mixtures (0.06 g, mole ratio of DHA to phytosterol mixture, 1:2) and different lipases (8% of the total weight of substrates). Hexane (3 ml) was added and the content of test tubes was flushed with nitrogen before being screw capped. The reaction mixture was then shaken in a gyratory water bath shaker at 200 rpm and 45 °C (New Brunswick Scientific Co., Inc, New Brunswick, NJ, USA). The following 9 enzymes were used for screening the best enzyme for synthesis of phytosteryl docosahexaenoates. These were Amano lipase from *Pseudomonas fluorescens*; Amano lipase PS from *Burkholderia cepacia (Pseudomonas cepacia)*; lipase from *Candida rugosa* type VII; Novozyme 435 (lipase acrylic resin from *Candida antarctica*); lipoprotein lipase (LPL) 311; LPL 314; cholesterol esterase (COE) 301; COE 311 and COE 313. The reaction was monitored by using TLC plates on *Baker-flex*® silica gel IB-F (2.5 X 7.5 cm) pre-coated flexible TLC sheets (J.T. Baker, Phillipsburg, NJ, USA) with eluents of hexane/ethyl acetate (80:20, v/v). After 48 hours the reaction

was stopped by placing it under the running tap water. The enzyme was filtered through a filter paper (Fisherbrand filter paper P5) and the solvent evaporated. The solid residue was then subjected to column chromatography. The structure of the phytosteryl docosahexaenoates were then elucidated by HPLC-MS and FTIR analysis as explained in section 7-2 of this thesis.

3.2.4.5 Enzymatic synthesis of phytosteryl docosahexaenoates

Phytosterols (4.7 g) with DHA (3 g, at mole ratio phytosterols to DHA of 1.2) were weighted into a 250 ml conical flask. Lipase from LPL 311 (9% of the total substrate weight) was added together with 100 ml hexanes. The flask was flushed with a stream of nitrogen to remove oxygen before being screw-capped and then placed into an orbital shaking water bath at 45 °C and 200 rpm for 24 h. The reaction was stopped by cooling the flask under running tap water. Then 50 ml of n-hexane were added to the test tube, and the solvents containing reactants were passed through a filter paper (Fisherbrand filter paper P5) with a layer of sodium sulphate to remove the lipase and water generated during the esterification reaction. Two more 50 ml portion of n-hexane was used to recover all the reaction mixtures. The solvents containing sample were collected into a 500 ml round bottom flask and solvents were then completely removed using a rotary evaporator (BÜCHI Labortechnik, Flawil, Switzerland) at 45 °C. The sample was subsequently subjected to column chromatography.

3.2.4.6 Column chromatographic separation of phytosteryl docosahexaenoates

In a typical run, five grams of reaction mixtures were dissolved in a minimum amount of n-hexane and applied to a column (40 cm × 5 cm I.D.) packed with Silica Gel (Selecto Scientific, Suwannee, GA, USA) as a slurry in n-hexane, subsequently eluted with n-hexane/ethyl acetate (4:1, v/v). The fractions were collected in test tubes. TLC on *Baker-flex*® silica gel IB-F (2.5 X 7.5 cm) pre-coated flexible TLC sheets (J.T. Baker, Phillipsburg, NJ, USA) was employed to monitor the different fractions collected.

3.2.4.7 FTIR analysis of phytosteryl docosahexaenoates

The purified phytosteryl docosahexaenoates obtained were characterized using a Fourier transform infrared spectrometer. The IR spectra were recorded using a FTIR Bruker Tensor 27 spectrometer (Bruker Optik GmbH in Ettlingen, Germany). The spectrometer employed had a spectral range of 7,500 to 370 cm^{-1} and was equipped with a MIRacle ATR accessory allowing rapid and easy analysis of liquid and solid samples.

3.2.4.8 HPLC-MS analysis of phytosteryl docosahexaenoates

The structures of the phytosteryl docosahexaenoates were confirmed by both normal phase and reverse phase high performance liquid chromatography-mass spectrometry (HPLC-MS). The analysis was performed using an Agilent 1100 HPLC-MSD system (Agilent, Palo Alto, CA, USA) with an on-line solvent degasser, binary solvent delivery system, autosampler and UV-Vis diode array detector. The MS detector

has electrospray or atmospheric pressure chemical ionization capability with a mass range of *m/z* 50-3000.

Reverse phase HPLC separation was achieved on a C18 column (250 mm length, 4.6 mm i.d., 5 µm particle size, Sigma-Aldrich Canada Ltd., Oakville, ON). Phytosteryl docosahexaenoates were eluted using an isocratic solvent system containing methanol/water (95:5, v/v) at a flow rate of 1.0 ml/min. Fifty µl of each sample were injected. Phytosteryl docosahexaenoates were detected at 210 nm by UV detection. LC flow was analyzed on-line by a mass selective detector system (LC-MSD-Trap-SL, Agilent, Palo Alto, CA, USA) with positive ion mode APCI. The operating conditions used were 121V for the fragmentor voltage, 350 °C for drying temperature, 400 °C for APCI temperature, 60 psi for the nebulizer pressure, and 7 l/min for the drying gas flow.

To confirm the structure of phytosteryl docosahexaenoates, normal phase HPLC was also employed in this study. The same HPLC-MS system was employed. Separation was achieved on a Supelcosil LC-Si column (250 mm length, 4.6 mm i.d., 5 µm particle size, Sigma-Aldrich Canada Ltd., Oakville, ON) coupled with a Supelcosil LC-Si guard column. were eluted using an isocratic solvent system containing hexane/2-propanol (95:5, v/v) at a flow rate of 1.0 ml/min. Fifty µl sample were injected. Phytosteryl docosahexaenoates were detected at 210 nm by the UV detector. LC flow was analyzed on-line by a mass spectrometric detector system (LC-MSD-Trap-SL, Agilent, Palo Alto, CA, USA) with a positive ion APCI. The operating conditions used were 121V for the fragmentor voltage, 350°C for drying temperature, 400 °C for APCI temperature, 60 psi for the nebulizer pressure, and 7 l/min for the drying gas flow.

3.2.5 Evaluation of the cholesterol lowering effect of phytosteryl oleates and docosahexaenoates

3.2.5.1 Animals and diets

Twenty-one 4-week-old male C57BL/6J mice homozygous for deletion of the apo-E gene (apo-E-KO) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). After a 7-day adaptation period, they were divided into 3 groups (control, phytosteryl oleates treated, and phytosteryl docosahexaenoates treated) of 7 each, matched for their total plasma cholesterol concentrations and body weight. Most of the mice were housed in groups of two; some of them were in single housing due to male fighting. One mouse from the control group died at week 2 due to deformed a skull and another one from the same group died at week 5 due to a prolapsed penis. Overall, 19 mice completed the study. The mice had *ad libitum* access to water and food; their body weights and food consumption were measured weekly.

The mice were fed with PicoLab mouse diet 20 (Ren's Feed and Supply Ltd, Aberfoyle, ON) (1) without any supplement (control), (2) supplemented with 2% (w/w) phytosteryl oleates (phytosteryl oleates treated), or (3) supplemented with 2% (w/w) phytosteryl docosahexaenoates (phytosteryl docosahexaenoates treated). The diet was prepared as follows: PicoLab mouse diet 20 (9% fat, w/w) was finely ground by using a food processor. Cholesterol (Sigma Chemical Co, Oakville, ON) was added at 0.1% (w/w) to the diet and mixed well. Then, 2% (w/w) phytosteryl oleates or phytosteryl docosahexaenoates were added to the cholesterol-supplemented diet and used for the two treatment groups. Baking powder (10 g per kg of diet) was also added to the diet mixture

to prevent the lipid oxidation during the baking process. The dietary mixture was then re-pelleted and air-dried overnight in an oven. Animal studies were approved by the animal care committee of University of Manitoba.

3.2.5.2 Blood sampling

Mice were restrained briefly in a 50 ml plastic Falcon tube, and blood was collected from the jugular vein under light anaesthesia using isoflurane. Blood samples were centrifuged for 10 min at 4000 x g by using an Eppendorf centrifuge Model 5804 (Eppendorf Canada, Mississauga, ON). Aliquots of the plasma were used for lipid analysis. At the end of the experiment, the final blood sample was obtained from the right ventricle of euthanized animals using CO_2 (Moghadasian et al., 1997; 1999).

3.2.5.3 Lipid analysis

Plasma total cholesterol (TC) was measured at baseline, week 4 and week 7 using standard enzymatic method kits (Boehringer Mannheim, Mannheim, Germany) (Dobiasova et al., 1991; Allain et al., 1974; Moghadasian et al., 1997).

3.2.5.4 Histological evaluation of atherosclerotic lesions

At sacrifice, the heart and aortas were fixed in 10% formalin. Sections at the aortic roots were cut and stained with hematoxylin and eosin (H & E) for histological examination. These sections were used to estimate atherosclerotic lesion size using Image Pro-Plus 6.0 software (Moghadasian et al., 1997; 1999).

3.3 Statistical Analysis

One-way analysis of variance (ANOVA) with comparisons for all pairs using Turkey-Kramer HSD were used to determine differences in mean values based on data collected from replication of various experiments. Significance was determined at a 95% level of probability. All statistical analysis was carried out using software *JMP* version 6.0.0. (SAS Institute Inc. Cary, NC, USA).

Table 3-1. Three-variable and three level face-centred CCD experimental design.

Run	X_1	X_2	X_3
1	-1	-1	-1
2	-1	-1	1
3	-1	0	0
4	-1	1	-1
5	-1	1	1
6	0	-1	0
7	0	0	-1
8*	0	0	0
9*	0	0	0
10*	0	0	0
11	0	0	1
12	0	1	0
13	1	-1	-1
14	1	-1	1
15	1	0	0
16	1	1	-1
17	1	1	1

*Centre point, X_1: reaction time; X_2: enzyme load; X_3: substrate mole ratio

Table 3-2. Actual and coded range of the variables for the CCD experimental design.

Variables	Level			
	Coded level	-1	0	1
	Actual values			
X_1: reaction time (hour)		4	8	12
X_2: enzyme load (%)		2	6	10
X_3: substrate mole ratio		1	2	3

Table 3-3. Central composite design (CCD) and response for the enzymatic synthesis of phytosteryl caprylates.

RUN	Coded variables			Real variables			Response (Y) Degree of esterfication (%)
	X_1	X_2	X_3	X_1	X_2	X_3	
1	-1	-1	-1	4	2	1	9.64 ± 1.95
2	-1	-1	1	4	2	3	18.43 ± 2.88
3	-1	0	0	4	6	2	60.85 ± 1.10
4	-1	1	-1	4	10	1	65.12 ± 2.31
5	-1	1	1	4	10	3	88.84 ± 0.54
6	0	-1	0	8	2	2	31.63 ± 2.18
7	0	0	-1	8	6	1	71.45 ± 1.39
8*	0	0	0	8	6	2	89.52 ± 0.65
9*	0	0	0	8	6	2	89.99 ± 1.37
10*	0	0	0	8	6	2	90.99 ± 1.42
11	0	0	1	8	6	3	93.55 ± 1.15
12	0	1	0	8	10	2	94.70 ± 0.78
13	1	-1	-1	12	2	1	25.17 ± 3.57
14	1	-1	1	12	2	3	50.27 ± 0.70
15	1	0	0	12	6	2	92.00 ± 0.61
16	1	1	-1	12	10	1	81.62 ± 0.93
17	1	1	1	12	10	3	96.59 ±0.98

*Centre point, X_1: reaction time; X_2: enzyme load; X_3: substrate mole ratio

Results and Discussions

Chapter 4

Optimization of Enzymatic Synthesis of Phytosteryl Caprylates Using RSM

4.1 Introduction

Phytosterols are known to lower total blood cholesterol and low-density lipoprotein (LDL) cholesterol without affecting plasma high density lipoprotein (HDL) cholesterol concentration (Moreau et al., 2002; Wester, 2000). Low solubility of phytosterols in edible oils and their high melting point restrict their use in food products (Condo et al., 2001). Esterification of phytosterols with fatty acids will improve their solubility in oils and lowers their melting point (Villeneuve et al., 2005; Vu et al., 2004). A medium-chain fatty acid, caprylic acid (CPA, C8:0), was selected for the esterification process because it can be used as a rapid energy source with little or no deposition in the body and is also often recommended by nutritionists for the treatment of candidiasis due to its antimicrobial properties (Babayan, 1987; Nair et al., 2005; Vasudevan et al., 2005).

This chapter describes a method for optimizing the enzymatic synthesis of phytosteryl caprylates using response surface methodology (RSM). RSM was employed to establish and exploit the relationship between the response studied (degree of esterification) and the three selected experimental variables (reaction time, enzyme load, and substrate

mole ratio) and finally to optimize the esterification reaction conditions for the synthesis of phytosteryl caprylates.

4.2 Experimental design data

The important independent variables or factors investigated in this study were reaction time (4-12 hours; X_1), enzyme load (2-10%, weight percentage of total substrate; X_2), and substrate mole ratio (1-3, caprylic acid to total phytosterols, X_3). The selection of the three variables studied was based on the literature data and preliminary studies. The approximate conditions, namely reaction time, enzyme load, and substrate mole ratio, were determined by changing one factor at a time while keeping the others constant. Thus, a proper range for each factor was determined as mentioned earlier for RSM. Enzyme amount and reaction time are the main variables influencing the yield of phytosteryl esters during the esterification process. Moreover, reaction temperature and time can be considered important as they affect the economy of the process as well as the oxidative state of the starting material and the prepared esters.

Face-centred central composite design (CCD) was employed as it provides relatively high quality predictions over the entire design space and does not require using points outside the original factor range (Myers and Montgomery, 1995). A three-factor and three-level face-centred cube, was selected over others such as a rotatable or orthogonal design because it uses only three levels of each variable, whereas other central composite designs would need five levels of each (Mason et al., 1989). Having three levels instead of five is considered attractive because it decreases the time of preparation, and practically it is

difficult to achieve. The upper limit of a factor was coded as +1, and the lower limit was coded as –1. The actual and coded range of the three variables for the CCD face centred design is presented in Table 3-2. Table 3-3 shows the coded variables and the real experimental conditions of CCD with the corresponding measured responses.

4.3 Estimated model

The measured responses in Table 3-3 were used to compute the model coefficient using the *JMP* software. The following quadratic regression equation giving the response value as a function of three coded variables was obtained:

$$Y = 86.477759 + 10.277X_1 + 29.173X_2 + 9.468X_3 - 2.89X_1X_2 + 0.945X_1X_3 + 0.6X_2X_3 - 8.414138X_1^2 - 21.67414X_2^2 - 2.339138X_3 \quad (1)$$

Where X_1, X_2, X_3 are the coded values of the tested variables, namely reaction time, enzyme load and substrate mole ratio. The regression model (Eq.1) had a high R-square value of 0.984801 and adjusted (Adj) R-square value of 0.962003 as shown in Table 4-1. The R-square value provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. The closer the R-square value is to 1, the stronger the model is and the better it predicts the response (Mason et al., 1989). When expressed as a percentage, R-square is interpreted as the percent variability in the response explained by the statistical model. In this case, the R-square with a value of 0.984801 indicates that 98.4801% of the variability

in the response could be explained by the model. In addition, the value of the adjusted R-square (0.962003) is very high which advocates a high significance of the model. These ensured a satisfactory adjustment of the polynomial model to the experimental data.

Further, the fit quality of the estimated model is attested with the analysis of variance (ANOVA) and lack of fit analysis as shown in Table 4-2. ANOVA is a statistical technique that subdivides the total variations in a set of data into component parts associated with specific sources of variation for the model and is required to test the significance and adequacy of the model. The greater the *F*-value is from unity, the more certain it is that the factors explain adequately the variation in the data about its mean, and the estimated factor effects are real. The ANOVA of the regression model demonstrates that the model is highly significant, as is evident from the Fisher's *F*-test (*F* value = 43.197) and a very low probability value (*P* value < 0.0001). Table 4-2 shows that the regression sum of squares is statistically significant when using the *F*-test at a 95% probability level, which suggest that the variation accounted for by the model was significantly greater than the unexplained variation.

The significance of each coefficient was determined by Student's *t*-test and *p*-value, which are listed in Table 4-3. The larger the magnitude of the *t*-value and the smaller the *p*-value, the more significant the corresponding coefficient will be. This implied that reaction time, substrate mole ratio, both the first order and quadratic effect of enzyme load have a considerable influence on the response degree of esterification as these factors have a *P*-value lower than 0.003. The effect of different variables will be further illustrated graphically by contour plots.

The observed value compared with the predicted value calculated from the model is shown in Figure 4-1. The plot indicates that the model is good as it showed almost a linear distribution.

All the above considerations indicate an excellent adequacy of the regression model employed. Hence, it can be used to predict the degree of esterification for different values of tested variables and to identify the major interactions between the test variables from the circular or elliptic nature of the contours.

4.4 Canonical analysis and interpretation of the response surface model

The second-order polynomial model can be analyzed by canonical analysis. This function has a stationary point where the partial derivative of predicted response with respect to each of the variables is zero and this point could be a maximum, a minimum, or a saddle point.

In the present study, the solution from *JMP* software for canonical analysis is the three variables with critical values for reaction time (10.49 hours), enzyme load (8.5%) and substrate mole ratio (4.2) as given. It corresponds to a maximum of response Y (DE). This point is situated outside the experimental domain as one of its critical values, ie. Substrate mole ratio of 4.2 is outside the data range. The predicted value at this solution is 109% esterification; this value is higher than the theoretical value of 100%.

It is known the three dimensional response surface and two-dimensional contour plot are generally the graphical representation of the regression equation. This

representation shows the relative effects of any two variables when the remaining variables are kept constant. It can also be used to search the optimal values of the variables to reach maximum response. Therefore, in this study, three contour plots among three variables were constructed as shown in Figure 4-2 (a) (b) (c). It enables us to search for the minimum level of each variable to yield the maximum level of response for the degree of esterification theoretically set at 100%. By using arrows on each contour plot, it can be concluded that optimal conditions for synthesis of phytosteryl caprylates were: 9.25 hours, 2.15 substrate mole ratio (caprylic acid to phytosterols), and 7.93% enzyme load. To reconfirm the adequacy of the model, three additional replicate esterification reactions were conducted under the estimated optimal conditions. The degree of esterificaiton achieved was 98.03 ± 1.08%, which clearly shows that this model is sufficiently adequate.

Figure 4-2 (a) also indicated the effect of varying the reaction time (4-12 hours) and the enzyme load (2-10%) on the level of esterification at a constant substrate mole ratio 2. It can be see that, at low enzyme load and short reaction time, the degree of esterification was strongly decreased, whereas a high degree of esterification was obtained when using a high enzyme load and a long reaction time.

A three dimensional response surface was also plotted (Figure 4-3) by statistically significant model to understand the interaction of two variables, namely enzyme load and reaction time, for response, ie. the degree of esterification.

4.5 GC-MS and FTIR analysis

Phytosterols and their derivatives are conventionally analyzed by GC and most commonly detected with a flame ionization detector (FID). Due to their poor volatility and relatively labile nature at high temperatures, the analysis of phytosterols and their derivatives at high temperatures might be unreliable. To improve volatilization, stability, and resolution on GC, phytosterols and their derivatives are commonly converted to their trimethylsilyl ether (TMS) or acetate derivatives. As for the phytosteryl esters, the non-phytosterol part may be analyzed separately. For example, the fatty acid moiety in the phytosteryl esters will be analyzed as fatty acid methyl esters. Although in this approach the derivatives show less tailing in the chromatograms and render a better resolution, it prevents the recognition of the intact phytosteryl esters and thus valuable information might be lost. Advances in modern capillary column chromatography technologies have made it possible to analyze phytosterols as free alcohols or intact phytosteryl esters (Boven et al., 1997; Jekel et al., 1998; Kim and Akoh, 2007). Gas chromatography (GC) and mass spectrometry (MS) provide an effective combination for chemical analysis. GC-MS has been successfully employed for the analysis of free phytosterols (Seitz, 1989) and phytosteryl esters (Kamm et al., 2001; Kim and Akoh, 2007). Compared with FID, MS detector is suitable for structural identification and quantitation. Instead of using isothermal temperature for column, the column temperature was programmed from 180 to 325 °C within 16 min during the separation of the mixtures.

In this study, simultaneous gas chromatographic separation of the reaction mixtures was achieved using a 30 m fused silica capillary column with gradient

temperature increase. In Figure 4-4 (a), the gas chromatogram of the starting materials, that is the free phytosterols, is shown. The gas chromatogram in Figure 4-4 (b) includes signals from both the remaining reactants and the synthesized phytosteryl caprylates in the reaction mixture. The corresponding mass spectra for compounds 1-6 are shown in Figures 4-5, 4-6, 4-7. The MS data and their molecular information are summarized in Tables 4-4 and 4-5.

Under the positive electric impact (EI) mode, free phytosterols, namely sitosterol, sitostanol and campesterol, had base peak $[M]^{\cdot+}$ with *m/z* 414, 416 and 400, which exactly match their molecular mass and showed similar fragmentations. For example, their spectra showed the $[M\text{-}H_2O]^{\cdot+}$ ion. Sitosterol and sitostanol also had a characteristic ion with $[M+H_2O]^{\cdot+}$. Ions representing the four ring nucleus of sitosterol, sitostanol and campesterol were those with *m/z* 255, 257, and 255, respectively. Similar molecular ions at *m/z* 414 and 400 for sitosterol and campesterol using GC-MS have also been reported (Meszaros et al., 2007; Seitz, 1989).

The ester counterparts of the three free phytosterols, had their base peak [M-caprylic acid] with *m/z* 396, 398 and 382 for sitosteryl caprylate, sitostanyl caprylate and campesteryl caprylate, respectively. The molecular ion peaks $[M]^{\cdot+}$ of the three components (compounds 4, 5 and 6) in the reaction mixtures were, respectively, at *m/z* 540, 542, and 526 which correspond to their molecular weights, but their intensity was very low. Ions with *m/z* 255, 257, and 255 which represent the four ring nucleus of sitosteryl caprylate, sitostanyl caprylate and campesteryl caprylate were also observed.

The IR spectra of both free phytosterols and phytosteryl caprylates are shown in Figure 4-8. This provides evidence that the enzymatically synthesized products were phytosteryl esters. The esters were characterized by a strong sharp band at 1741 cm^{-1} that was assigned to an ester carbonyl group and the absorption at 1176 cm^{-1} corresponded to C-O-C asymmetric stretching vibration and symmetric vibration of the ester linkage. The free phytosterols have a strong absorption at 1053 cm^{-1} due to the C-O vibration via participation of oxygen atom of the hydroxyl group. A broad band around 3300-3600 cm^{-1} corresponds to the vibration of the hydroxyl group. These two bands disappeared in the phytosteryl caprylates products.

The information provided above proves that the products so prepared were phytosteryl caprylates.

4.6 Summary

From the experimental results, RSM was found suitable for evaluating the effective factors and building models to study the interaction, as well as for selecting optimum conditions of variables for highest degree of esterification. The results showed a satisfactory correlation between the experimental and predicted values.

In the RSM study, the effect of the reaction time, enzyme load, and substrate mole ratio on the degree of esterification of phytosteryl caprylates was evaluated. The optimization of the synthesis conditions was achieved by using RSM and a face-centred CCD determination of the optimized conditions (9.25 hours, 2.15 substrate mole ratio, and 7.93% enzyme load) which led to a high conversion yield of 98%. The success of the

esterification reaction was confirmed by using spectroscopic methods to identify the formation of the ester bond between phytosterols and caprylic acid as well as mass spectral determination of the synthesized products.

Further investigation on cholesterol lowering effect of phytosteryl caprylates and their potential antimicrobial properties may be an interesting topic for future work.

Table 4-1. Summary of the fit for CCD model for enzymatic synthesis of phytosteryl caprylates.

R Square	0.984801
R Square Adj	0.962003
Root Mean Square Error	5.886722
Mean of Response	66.21063
Observations (or Sum Wgts)	17

Table 4-2. Analysis of Variance (ANOVA) and lack of fit analysis for regression model of CCD.

Source	Sum of Squares	Degree freedom	Mean Square	F Ratio (*F*-value)	Prob > F (*P*-value)
Model	13472.343	9	1496.93	43.197	<.0001
Error	207.921	6	34.65		
Corrected Total	13680.264	16			
Lack of Fit	207.81051	5	41.5621	376.2979	0.0391
Pure Error	0.11045	1	0.1104		
Total Error	207.92096	6			

Table 4-3. Estimated regression coefficients and corresponding *t*- and *p*-values.

Term	Coefficient	Std Error	*t*-Test	*p*-Value
Intercept	86.477759	2.786963	31.03	<.0001
Linear				
Reaction time	10.277	1.861545	5.52	0.0015
Enzyme load	29.173	1.861545	15.67	<.0001
Substrate mole ratio	9.468	1.861545	5.09	0.0023
Cross-product				
Reaction time*Enzyme load	-2.89	2.08127	-1.39	0.2143
Reaction time* Substrate mole ratio	0.945	2.08127	0.45	0.6658
Enzyme load* Substrate mole ratio	0.6	2.08127	0.29	0.7828
Quadratic				
Reaction time* Reaction time	-8.414138	3.625525	-2.32	0.0594
Enzyme load*Enzyme load	-21.67414	3.625525	-5.98	0.001
Substrate mole ratio * Substrate mole ratio	-2.339138	3.625525	-0.65	0.5427

Table 4-4. Retention time, molecular mass, formula and characteristic MS data for free phytosterols.

Compound No	Compound	Retention time (min)	Molecular mass	Formula	Characteristic ions (*m/z*)			
					$[M]^+$	$[M-H_2O]^+$	$[M+H_2O]^+$	Other ions
1	Sitosterol	19.39	414	$C_{29}H_{50}O$	414	396	432	255, 145
2	Sitostanol	19.45	416	$C_{29}H_{52}O$	416	398	434	257, 147
3	Campesterol	18.06	400	$C_{28}H_{48}O$	400	382		255, 145

Table 4-5. Retention time, molecular mass, formula and characteristic MS data for phytosteryl caprylates.

Compound No	Compound	Retention time (min)	Molecular mass	Formula	Characteristic ions (*m/z*)		
					$[M]^+$	$[M\text{-}CPA]^+$	Other ions
4	Sitosteryl caprylate	28.79	540	$C_{37}H_{64}O_2$	540	396	255, 147
5	Sitostanyl caprylate	28.91	542	$C_{37}H_{66}O_2$	542	398	257, 147
6	Campesteryl caprylate	27.15	526	$C_{36}H_{62}O_2$	526	382	255, 147

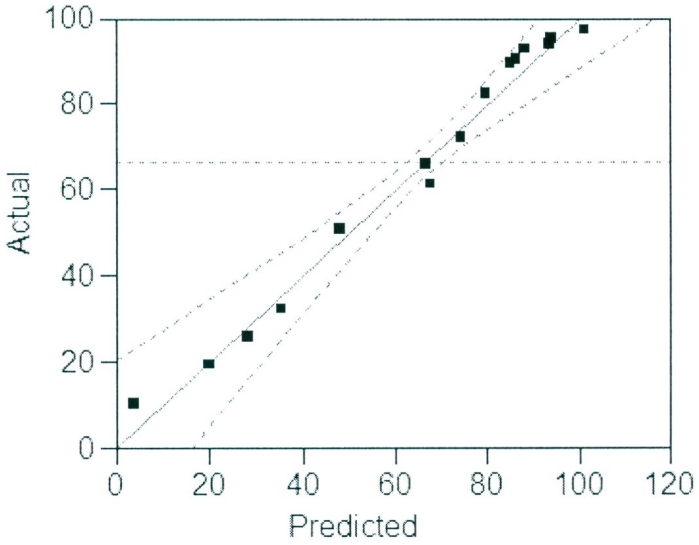
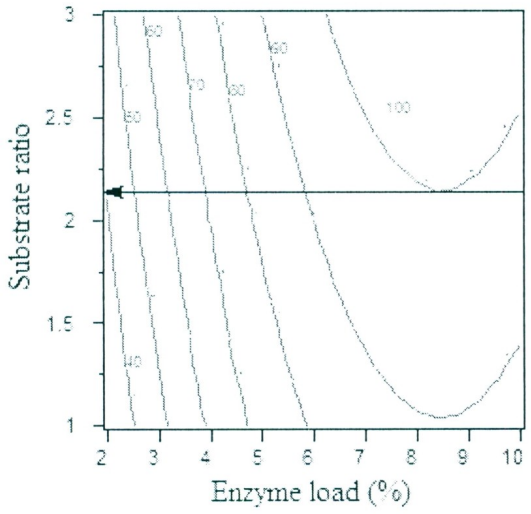
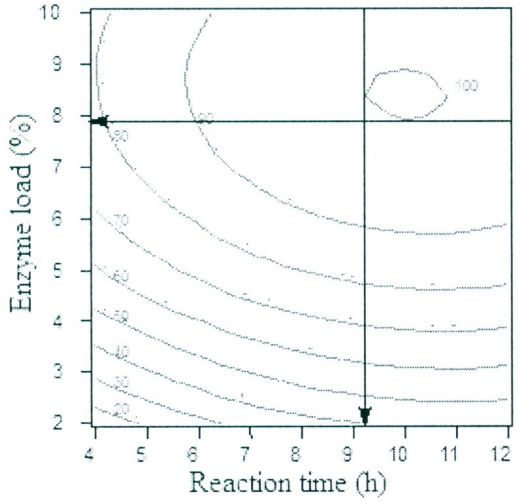
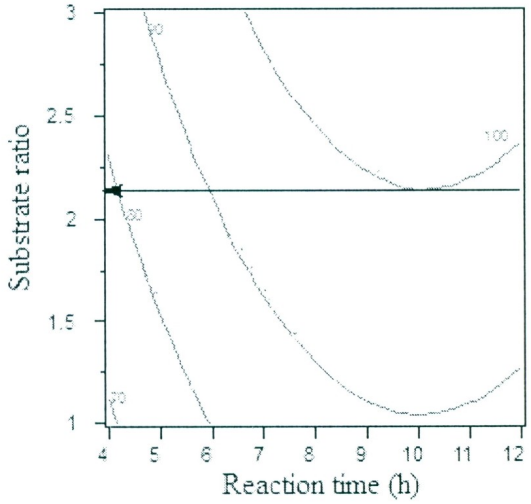


Figure 4-1. Plot of actual VS predicted response (degree of esterification for synthesis of phytosteryl caprylates) by the CCD model.



(c)





(a) (b)

Figure 4-2. Contour plots between two parameters for degree of esterification of phytosteryl caprylates: (a) reaction time and enzyme load when substrate ratio at 2; (b) reaction time and substrate ratio when enzyme load at 6 %; (c) substrate ratio and enzyme load when reaction time at 8 hour.

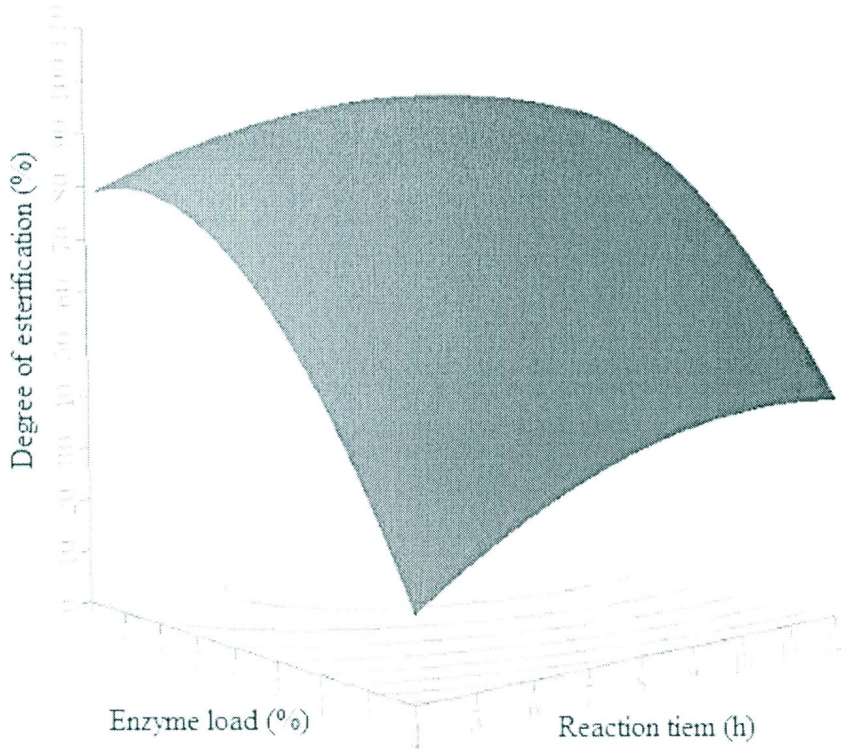


Figure 4-3. Three-dimensional response surface curve of the degree of esterification (%) showing the interaction between enzyme load (%) and reaction time (h) at various levels.

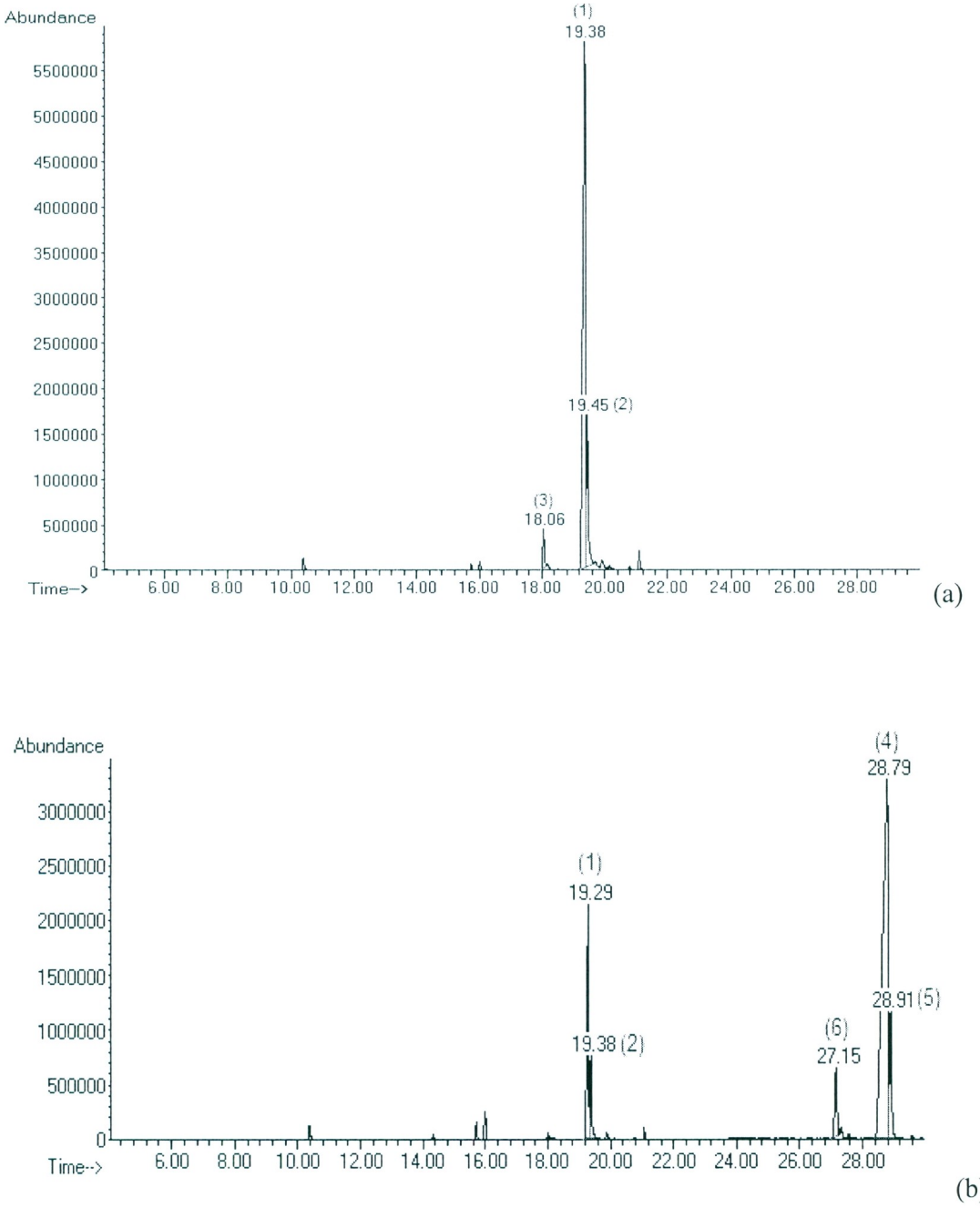


Figure 4-4. GC chromatograms for free phytosterol mixtures (a) and phytosteryl caprylates mixtures following the esterification reaction (b).

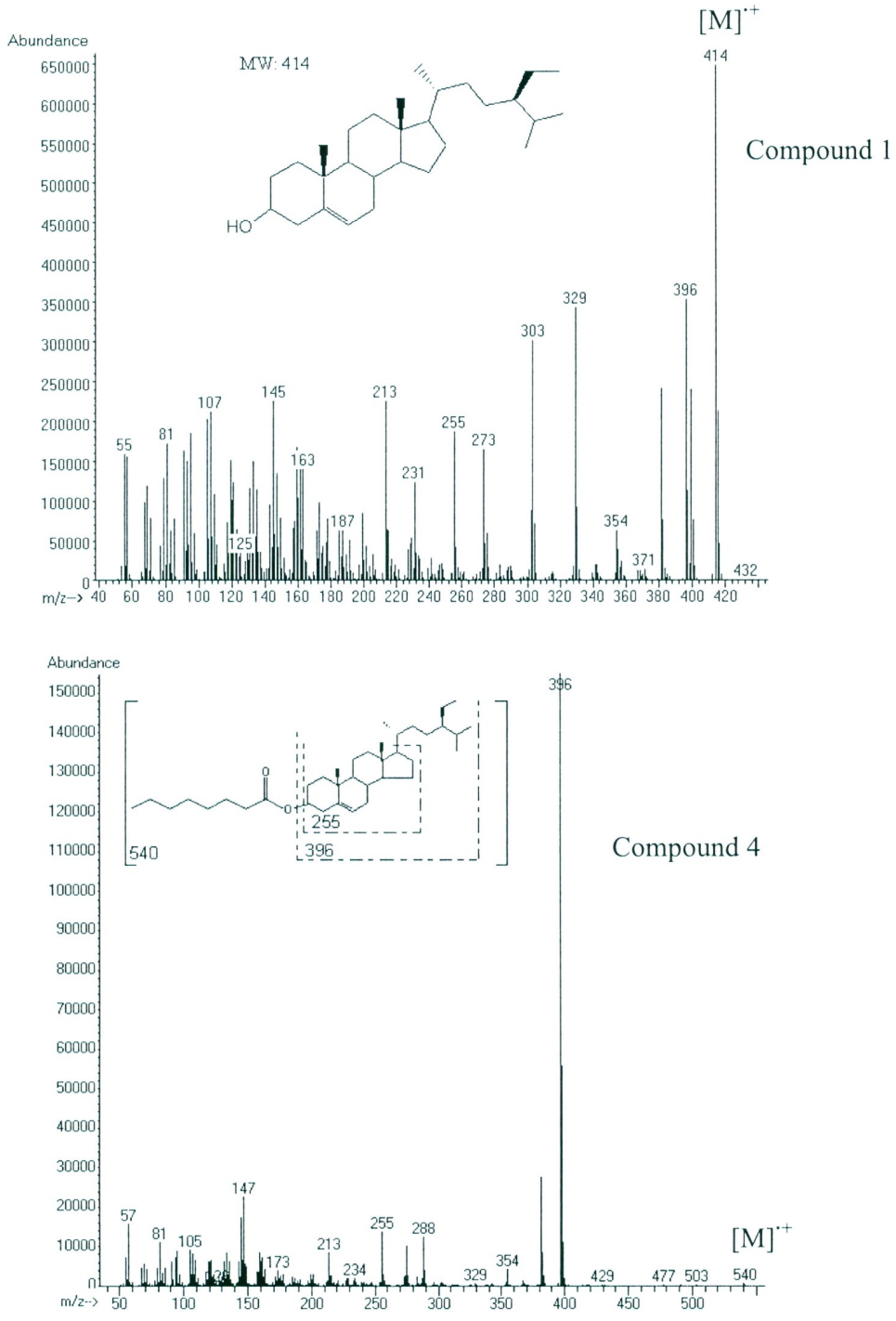


Figure 4-5. MS spectra of sitosterol (compound 1) and sitosteryl caprylate (compound 4).

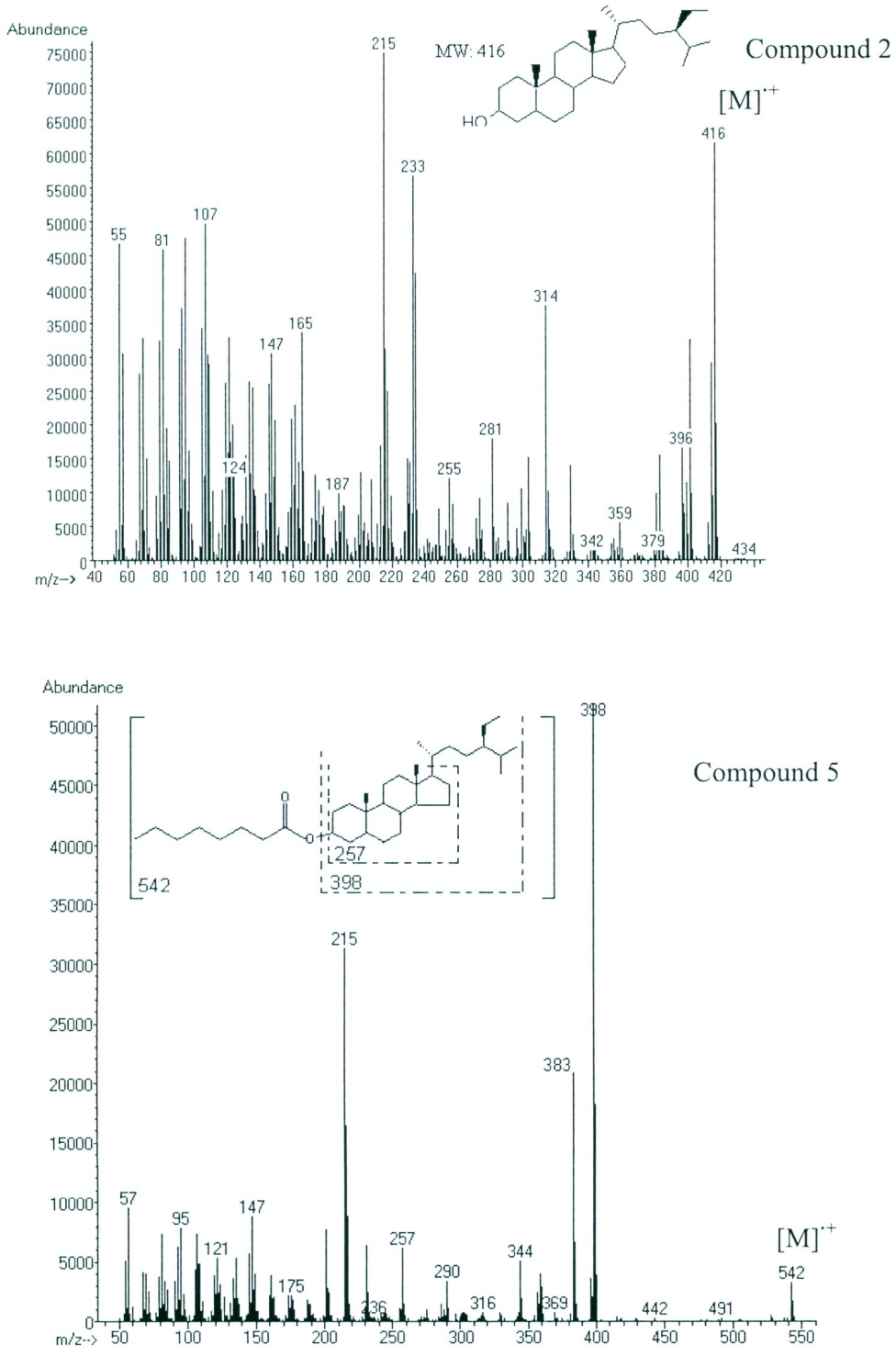


Figure 4-6. MS spectra of sitostanol (compound 2) and sitostanyl caprylate (compound 5).

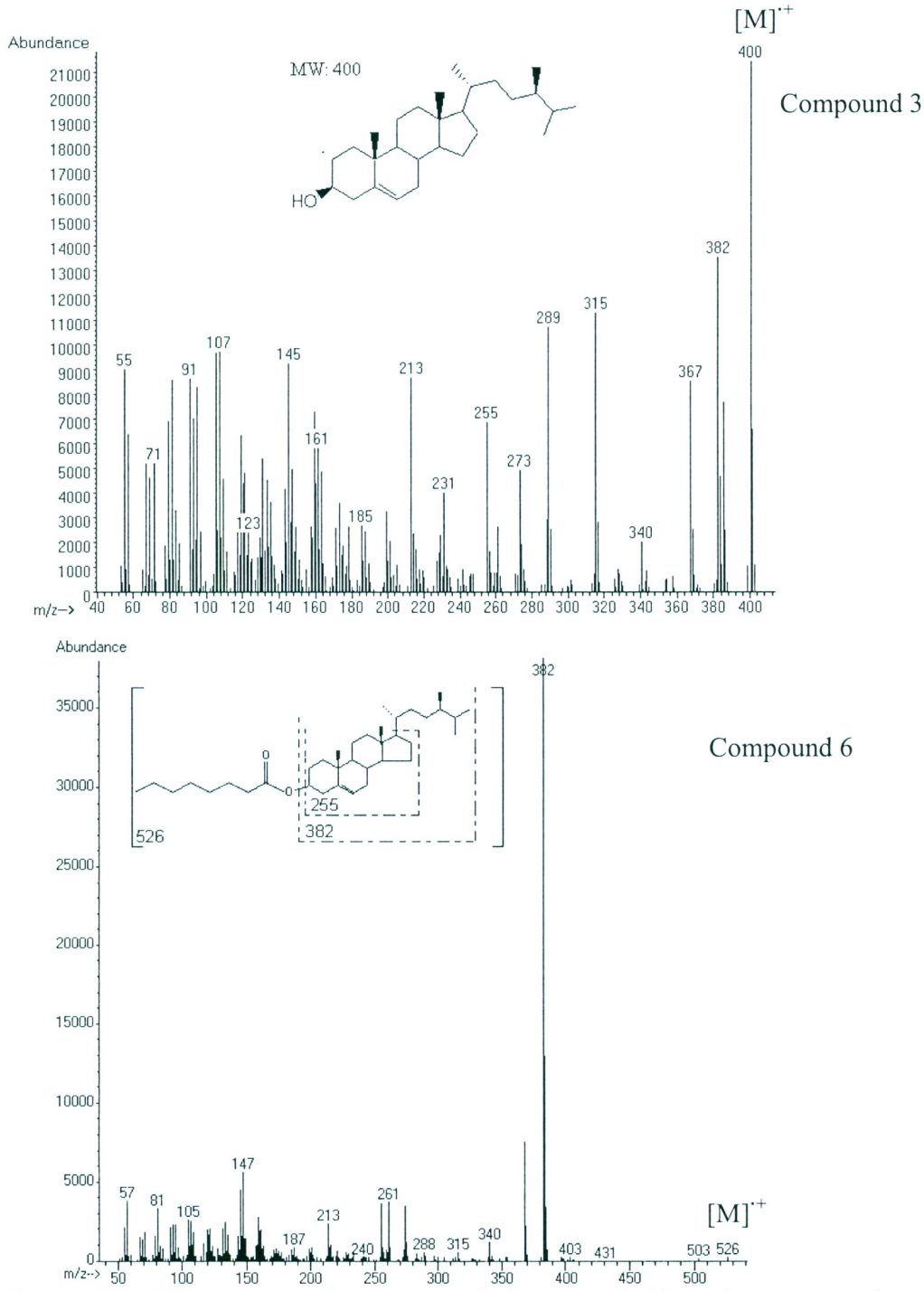


Figure 4-7. MS spectra of campesterol (compound 3) and campesteryl caprylate (compound 6).

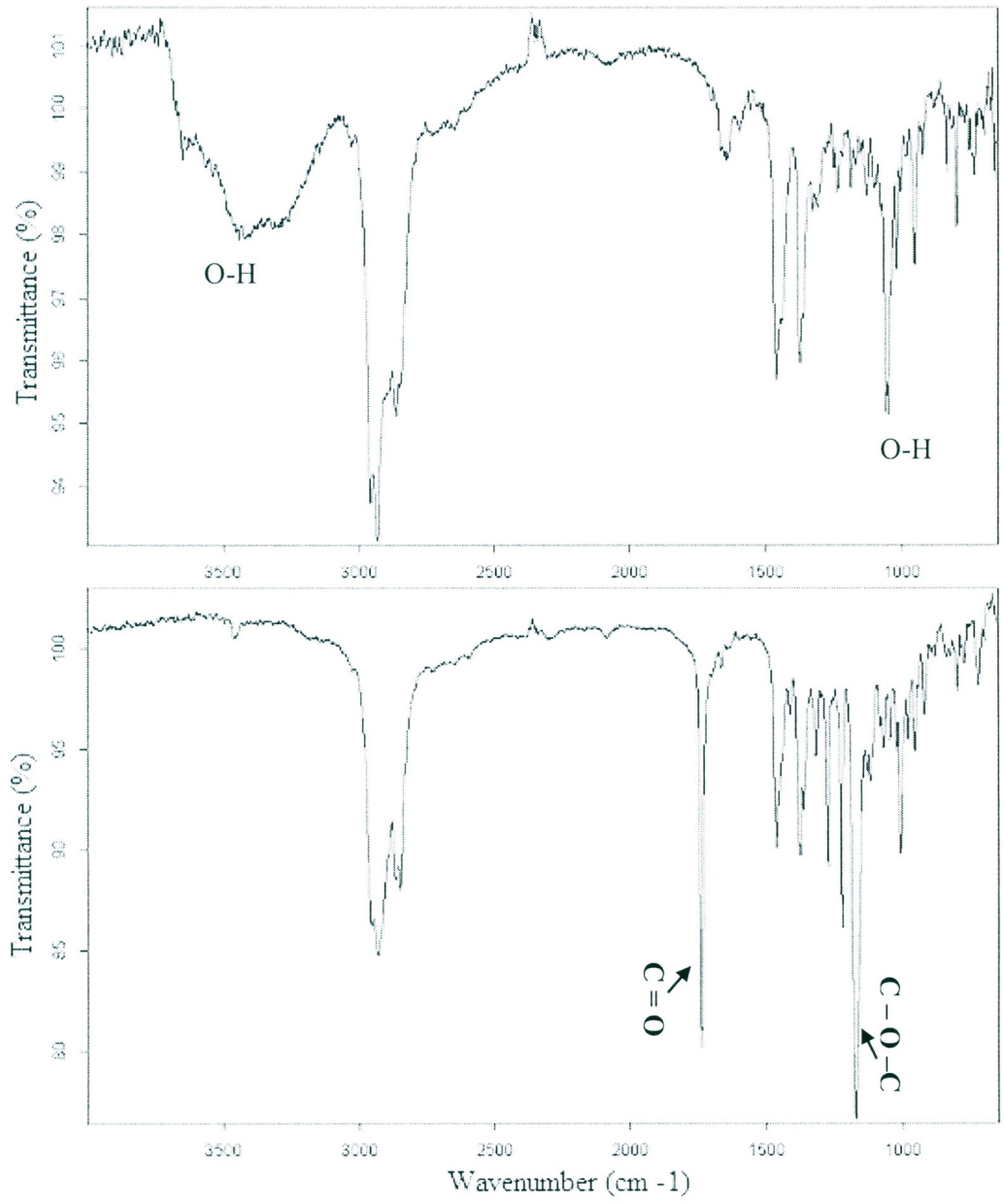


Figure 4-8. IR spectra of free phytosterols (top) and phytosteryl caprylates (bottom).

Chapter 5

Chemoenzymatic Synthesis of Phytosteryl Phenolates

5.1 Introduction

Phytosteryl phenolates such as phytosteryl ferulates, caffeates and *p*-coumarates exist naturally in plants (Fang et al., 2003; Seitz, 1989; Xu et al., 2001). Attempts to chemically synthesize them have been reported (Kondo et al., 1988 and 1991; Condo et al., 2001). These processes often use harsh chemicals and require isolation of sensitive intermediates and protection/deprotection of the functional hydroxyl group in phenolic acids. These processes often do not meet the food application requirements. To avoid these problems, enzymatic methods that use lipase may be developed to make the synthesis of this group of compounds more environmentally friendly, economical and workable, especially for future industrial production.

Hydrophobic derivatives of ferulic and other phenolic acids maybe prepared via enzyme-assisted reactions in order to improve their lipid-solubility (Buisma et al., 1998; Guyot et al., 1997; Stamatis et al., 2001; Yoshida et al., 2006). However, the formation of ferulate/other phenolate esters from ferulic acid/other phenolic acid with an alcoholic substrate follows a very low conversion rate. For example, conversion rates of only about 0.9% have been reported for enzymatic synthesis of 1-hexyl and 1-heptyl ferulates after 2 days of reaction (Yoshida et al., 2006). This may be caused by the inhibitory effect of phenolics, such as ferulic acid, which is conjugated with a carboxyl group and a bulky

noncarboxylic acid moiety (Kobayashi et al., 2003). Higher conversion rates of up to 90% were achieved in a study by Lee et al. (2006) for enzymatic synthesis of ethyl ferulate and octyl methoxycinnamate when higher temperatures of up to 90 °C were employed. The high temperature employed may denature and inactivate the enzyme and degrade phenolic acids during long time heating in alcohols as they are heat sensitive (Liu et al., 2006).

The preliminary study carried out in this work (data not shown here) indicated that direct enzymatic synthesis of phytosteryl phenolates from the reaction of phytosterols and phenolic acids was not possible. Inhibition of lipase activity by phenolic acids (Kobayashi et al., 2003), also reported by other researchers, may be contemplated. Therefore, a two-step chemoenzymatic synthesis of phytosteryl phenolates was attempted. In this method, an intermediate was first chemically produced and subsequently esterified with phytosterols via lipase-assisted alcoholysis, using a mixed solvent system. Vinyl ester of phenolic acids was chosen as acyl donors in this study because the vinyl alcohol formed during the transesterification reaction tautomerizes to acetaldehyde, thus making the process irreversible (Santaniello et al., 1993). Vinyl esters have previously been shown to be useful for enzymatic synthesis (Akoh et al., 1998; Wang et al., 1989; Wu et al., 1996).

5.2 Preparation of vinyl phenolates

The enzymatic synthesis of vinyl phenolates using phenolic acid and vinyl acetate as substrates and employing different lipases as catalysts was also attempted in this study (data not shown). Ten commercially available lipases were used, none of them led to

successful transesterification between phenolic acids and vinyl acetate. This may be due to the inhibition of enzyme activity by both phenolic acids and vinyl acetate. Therefore, synthesis of vinyl phenolates was achieved through a chemical pathway. The reactions for the synthesis of vinyl phenolates are shown in Figure 5-1 (caffeic acid to vinyl caffeate is shown as an example). The yield of the product after column chromatographic separation of the four vinyl phenolates, namely vinyl caffeate, vinyl ferulate, vinyl sinapate and vinyl vanillate, was 30, 46, 38 and 32%, respectively. No further data was presented on the other four vinyl phenolates, namely vinyl *p*-coumarate, vinyl gallate, vinyl syringate and vinyl gentisate. Among them, vinyl gallate and vinyl gentisate were not synthesized successfully. Although vinyl *p*-coumarate and vinyl syringate were synthesized successfully as proved by their NMR spectra (Appendix 5-4 and 5-5), their further reactions with phytosterols failed. The low yield of vinyl phenolates may be due to the chemical synthesis pathway, formation of by-products and loss of the synthesized product during the flash column chromatographic separation. A similar yield of vinyl ferulate, at 45-61%, was reported by Gao et al. (2001) and Nyaradzo et al. (2009). The yield of the above four vinyl phenolates may be enhanced by optimization of the reaction and purification conditions. This was not attempted as the primary objective of this work was to assess the feasibility of the enzymatic synthesis method rather than to maximize the yield of this particular group of compounds. The polarity of the phenolic acids and their derivatives was estimated using R_f values obtained by TLC, as presented in Table 5-1. According to the R_f values, the relative increasing order of the polarity for vinyl phenolates was vinyl vanillate < vinyl ferulate < vinyl sinapate < vinyl caffeate. All

synthesized vinyl phenolates had a relatively higher hydrophobicity compared with their phenolic acids counterparts as indicated by their higher R_f values.

It has been reported that electron donating effect of *p*-hydroxyl group (s) leads to a reduction of the reactivity of the electrophilic carbon centre of carboxylic group, especially when the side chain on the aromatic ring was unsaturated (Buisman et al., 1998; Safari et al., 2006; Karboune et al., 2008). Modification of phenolic acids into their vinyl esters was favoured in alcoholysis reactions with phytosterols. This may be explained by facile access of vinyl esters to the active site of the enzyme and formation of the intermediate adduct which favours alcoholysis with phytosterols. In contrast, the carboxylic acid group in the original phenolic acid together with the hydroxyl groups on the aromatic ring may inhibit enzyme activity for their reaction with phytosterols. The modification may also render them different chemical and biological properties, for example, antioxidant activity. This will be tested and discussed in Chapter 6.

The structures of the synthesized vinyl phenolates were confirmed by ^{1}H-NMR spectroscopy after their purification by flash column chromatography. DMSO-d_6 was used as a solvent for NMR studies as it is an excellent solvent for phenolic acids and vinyl phenolates and the chemical shift of phenolic hydroxyl protons in this solvent is characteristic and proton exchange is slow. In order to observe the phenolic hydroxyl proton signals by ^{1}H-NMR it is required that their rate of exchange with the solvent be sufficiently slow. This can be achieved by using an organic solvent such as DMSO-d_6 (Leeflang et al., 1992). The strong hydrogen bonds formed between DMSO-d_6 and phenolic hydroxyl protons can retard the exchange and thus give isolated and narrow

NMR signals for phenolic hydroxyl protons (Skrunts et al., 1970). On studies on lignin, the chemical shift for phenolic hydroxyl proton was mainly at δ 8.0-9.4 when DMSO-d_6 was used as a solvent (Li and Lundquist, 1994). NMR data for phenolic acids and their corresponding vinyl phenolates are presented below.

Caffeic acid: ^{1}H-NMR (500 MHz, DMSO-d_6) δ 12.11 (1H, COO<u>H</u>), 9.51 (1H, O<u>H</u>), 9.12 (1H, O<u>H</u>), 7.40 (1H, O=C-CH=C<u>H</u>-), 7.03 (1H, caffeic acid Ar), 6.95 (1H, caffeic acid Ar), 6.75 (1H, caffeic acid Ar), and 6.18 (1H, O=C-C<u>H</u>=CH-).

Vinyl caffeate: ^{1}H-NMR (500 MHz, DMSO-d_6) δ 9.69 (1H, O<u>H</u>), 9.22 (1H, O<u>H</u>), 7.61 (1H, O=C-CH=C<u>H</u>-), 7.34 (1H, C-O-C<u>H</u>=CH_2), 7.11 (1H, caffeate Ar), 7.01 (1H, caffeate Ar), 6.78 (1H, caffeate Ar), 6.32 (1H, O=C-C<u>H</u>=CH-), 4.92 (1H, C-O-CH=C<u>H</u>$_2$), and 4.68 (1H, C-O-CH=C<u>H</u>$_2$).

The assignment of the chemical shit δ to the proton in caffeic acid and vinyl caffeate is given in Figure 5-2. The predicted chemical shift values using *ChemDraw Ultra* 11.0 software are also shown and compared with the experimental values (in bracket). Most of the predicted values were very close to those of their experimental counterparts except two protons from two phenolic hydroxyl groups. The peak with δ 12.11 in the caffeic acid represents the proton from the carboxylic acid group (COOH) which disappeared in the vinyl caffeate. Instead three new peaks with δ 4.61, 4.92 and 7.34 which appeared signified the presence of the three vinyl protons in the synthesized vinyl caffeate. The ^{1}H-NMR data for caffeoyl moiety are in agreement with those reported by Cabanillas et al. (2010). In their study, the ^{1}H-NMR spectrum of 1-methylbutyl caffeate exhibited three aromatic proton resonances (δ 6.88, 6.98 and 7.11),

indicating a 1,3,4-trisubstituted aromatic ring. The ^{1}H-NMR also showed signals of two *trans* double-bond protons (δ 7.56 and 6.23). There is no report of the chemical shift for the two phenolic hydroxyl protons from caffeic acid esters in their study when $CDCl_3$ was used as a solvent for their NMR study. In this study, two broad singlets (δ 9.22 and δ 9.65) indicated that two hydroxyl groups in caffeic acid remained intact in vinyl caffeate, in agreement with the data reported by Silva et al. (2000). They used DMSO-d_6 in their NMR study of the synthesized methyl caffeate and propyl caffeate. The two phenolic hydroxyl groups gave two broad signals at δ 9.19-9.56. The structural data of vinyl caffeate were also in accordance with those reported by Teda et al. (1996). Thus the above discussions provide solid proof that the synthesized product was vinyl caffeate.

The ^{1}H-NMR data for ferulic acid and vinyl ferulate are presented below.

Ferulic acid: ^{1}H-NMR (500 MHz, DMSO-d_6) δ 12.11 (1H, COO<u>H</u>), 9.53(1H, O<u>H</u>), 7.50 (1H, O=C-CH=C<u>H</u>-), 7.28 (1H, ferulic acid Ar), 7.08 (1H, ferulic acid Ar), 6.80 (1H, ferulic acid Ar), 6.38(1H, O=C-C<u>H</u>=CH-), and 3.82(3H, -O-C<u>H</u>$_3$).

Vinyl Ferulate: ^{1}H-NMR (500 MHz, DMSO-d_6) δ 9.71 (1H, O<u>H</u>), 7.72 (1H, O=C-CH=C<u>H</u>-), 7.38 (1H, ferulate Ar), 7.34 (1H, C-O-C<u>H</u>=CH$_2$), 7.17, 6.81 (1H, ferulate Ar), 6.54 (1H, ferulate Ar), 6.46 (1H, O=C-C<u>H</u>=CH-), 4.95 (1H, C-O-CH=C<u>H</u>$_2$), 4.69 (1H, C-O-CH=C<u>H</u>$_2$), and 3.83 (3H, -O-C<u>H</u>$_3$). The NMR data for vinyl ferulate are in agreement with those reported by Gao et al. (2001).

The structure of vinyl ferulate was confirmed by comparing its ^{1}H-NMR spectrum with that of ferulic acid (Figure 5-3). The experimental values of the proton chemical shifts (in bracket) were compared with the predicted ones using *ChemDraw Ultra* 11.0

software. As indicated in Figure 5-3, most of the predicted values were very close to the experimental values except for the protons in the phenolic hydroxyl groups. The ^{1}H-NMR spectrum of vinyl ferulate showed three vinyl proton signals, two of which were with δ 4.69 and δ 4.95, the chemical shift value for the third one was δ 7.34. The peak with δ 12.11 in the ferulic acid represents the proton from the carboxylic acid group (COOH) which disappeared in vinyl ferulate. A broad peak with δ 9.71 indicated that the hydroxyl group in ferulic acid was not derivatized during the chemical synthesis of vinyl ferulate. All proofs indicated that vinyl ferulate was successfully synthesized.

The ^{1}H-NMR data for sinapic acid and vinyl sinapate are presented and discussed below.

Sinapic acid: ^{1}H-NMR (500 MHz, DMSO-d_6) δ 12.11 (1H, COO<u>H</u>), 8.89 (1H, O<u>H</u>), 7.48 (1H, O=C-CH=C<u>H</u>-), 6.99 (2H, sinapic acid Ar), 6.40 (1H, O=C-C<u>H</u>=CH-), and 3.80 (6H, -O-C<u>H</u>$_3$).

Vinyl sinapate: ^{1}H-NMR (500 MHz, DMSO-d_6) δ 9.07 (1H, O<u>H</u>), 7.72 (1H, O=C-CH=C<u>H</u>-), 7.36 (1H, C-O-C<u>H</u>=CH$_2$), 7.10 (2H, sinapate Ar), 6.63 (1H, O=C-C<u>H</u>=CH-), 4.95 (1H, C-O-CH=C<u>H</u>$_2$), 4.69 (1H, C-O-CH=C<u>H</u>$_2$), and 3.78 (6H, -O-C<u>H</u>$_3$).

The ^{1}H-NMR spectra of sinapic acid and vinyl sinapate (Figure 5-4) with assignments of proton chemical shifts were used to confirm the structure of the synthesized vinyl sinapate. The experimental values of the proton chemical shifts (in bracket) were also compared with the predicted ones using *ChemDraw Ultra* 11.0 software. Most of the experimental values were very close to the predicted ones, except for protons from the phenolic hydroxyl group as indicated in Figure 5-4. The ^{1}H-NMR

spectrum of vinyl sinapate showed three vinyl proton signals, two with δ 4.95 and δ 4.69, while the chemical shift for the third vinyl proton was δ 7.49. The peak with δ 12.47 in the sinapic acid represented the carboxylic acid (COOH) proton which disappeared in vinyl sinapate. The ^{1}H-NMR spectrum exhibited signals of the phenolic hydroxyl group from sinapic acid (δ 8.89) and vinyl sinapate (δ 9.07), which meant that the phenolic hydroxyl group remained intact following the chemical reaction. The above NMR data indicated that vinyl sinapate was successfully synthesized.

Similar results as other for vinyl phenolates were also obtained for vanillic acid and vinyl vanillate, the corresponding NMR data, presented also in Figure 5-5, as given below.

Vanillic acid: ^{1}H-NMR (500 MHz, DMSO-d_6) δ 12.47 (1H, COO<u>H</u>), 9.82 (1H, O<u>H</u>), 7.45 (1H, vanillic acid Ar), 7.44 (1H, vanillic acid Ar), 6.84 (1H, vanillic acid Ar), and 3.81 (3H, -O-C<u>H</u>$_3$).

Vinyl vanillate: ^{1}H-NMR (500 MHz, DMSO-d_6) δ 10.17 (1H, O<u>H</u>), 7.56 (1H, vanillate Ar), 7.50 (1H, vanillate Ar), 7.40 (1H, C-O-C<u>H</u>=CH$_2$), 6.91 (1H, vanillate Ar), 5.09 (1H, C-O-CH=C<u>H</u>$_2$), 4.69 (1H, C-O-CH=C<u>H</u>$_2$), and 3.84 (3H, -O-C<u>H</u>$_3$).

The presence of three vinyl proton signals (δ 7.40, 5.09 and 4.69), disappearance of the carboxylic acid proton (δ 12.47) and the retention of the hydroxyl proton (δ 10.17) indicated that vinyl vanillate was successfully synthesized.

5.3 Preparation of phytosteryl phenolates

Nine enzymes, namely the Amano lipase *AK* from *Pseudomanos fluorescens*, Amano lipase *PS* from *Burkholderia cepacia (Pseudomanos cepacia)*, lipase from *Candida rugosa*, Novozyme 435 (lipase acrylic resin from *Candida antarctica*), Lipoprotein lipase (LPL) 311, LPL 314, cholesterol esterase (COE) 301, COE 311 and COE 313, were initially screened for their ability in catalyzing the alcoholysis reaction between phytosterols and vinyl phenolates in mg scale in test tubes. Vinyl ferulate was chosen as the model vinyl phenolates for the screening process based on its relatively higher yield for the reaction between ferulic acid and vinyl acetate. Lipase from *Candida rugosa* was the only enzyme that successfully catalyzed the alcoholysis reaction of the esters formed between phytosterols and vinyl ferulate. This enzyme was then employed for the reaction between phytosterols with other three vinyl phenolates (vinyl caffeate, vinyl sinapate and vinyl vanillate); no enzyme activity inhibition was observed in these reactions. Lipase from *Candida rugosa* was then used for all the remaining alcoholysis reactions for phytosteryl phenolates in this research work.

Hydrophobic solvents generally support higher lipase activity than the hydrophilic ones (Dordick, 1989; Kvittingen, 1994), however, the solubility of phenolic acids in hydrophobic solvents was limited. To solve this problem, a binary organic solvent consisting of hexane and 2-butanone has been used. This mixture is a non-reactive and non-toxic solvent and has been successfully employed in other enzymatic reactions (Lue et al., 2004). Furthermore, the ratio of these two solvents is an important factor for biosynthesis reactions as it may affect the rate of enzymatic reactions, the bioconversion

yield as well as reaction selectivity (Sabally et al., 2006a). The hexane/2-butanone solvent mixture at a ratio of 85:15 (v/v) was reported as the appropriate reaction medium for the biosynthesis of phenolic lipids with a high bioconversion yield (Sabally et al., 2006b). Therefore, the hexane/2-butanone mixture at a ratio of 85:15 (v/v) was used as an effective solvent in this study.

The R_f values of the synthesized phytosteryl phenolates are presented in Table 5-2. The higher R_f values of phytosteryl phenolates compared with their corresponding intermediate products and phenolic acids indicated the higher hydrophobicity of the synthesized compounds.

The synthesis of phytosteryl phenolates has also been successfully scaled up from milligram quantities in test tubes to grams quantities in flasks. The yield of phytosteryl ferulates, sinapates and vanillates after 10 days of reaction were 90, 80 and 88%, respectively, however, the yield for phytosteryl caffeates was relatively low (50%), partially to the low conversion rate during the reaction or high loss of products during the column chromatographic separation. Lower yield of vinyl caffeate compared with other vinyl phenolates was reported in Section 5.1. Similar findings on the lower reactivity of caffeic acid compared with other phenolic acids have also been reported by others (Buisman et al., 1998; Karboune et al., 2008; Safari et al., 2006).

5.4 Structural analysis of phytosteryl phenolates

The HPLC chromatogram of the starting materials, free phytosterols, is presented in Figure 5-6. Of the three components, sitosterol and campesterol were detected by UV

detector at 210 nm, but sitostanol exhibited no absorption at several wavelengths used. Quantitative analysis of the composition of the starting materials was achieved by GC-MS as discussed in Chapter 4. The mass spectra under positive-ion atmospheric pressure chemical ionization (APCI) mode of the starting materials are shown in Figure 5-11. The spectra were characterized by low abundance of protonated molecular ions of the three analytes and an abundant signal corresponding to fragment ion due to the loss of a water molecule $[M+H-H_2O]^+$. These base peak ions were present at *m/z* 397.3, 399.3 and 383.3 for sitosterol, sitostanol and campesterol, respectively. This unique ionization pathway for phytosterols as dehydration form was also observed in other studies with phytosterols using HPLC-APCI-MS (Careri et al., 2001; Rozenberg et al., 2003; Palmgren et al., 2005).

The phytosteryl phenolates were purified by flash column chromatography and then subjected to different chemical analysis to confirm their structures. The retention time for three components in phytosteryl ferulates were 18.7, 20.45 and 22.88 min for campesteryl ferulate, sitosteryl ferulate and sitostanyl ferulate, respectively. The same elution order and very close retention times under similar HPLC conditions have also been reported for the HPLC separation of rye bran extract (Hakala et al., 2002). In their study, the first eluting compound was campesteryl ferulate (17.5 min), after which sitosteryl ferulate (19.5 min), followed by sitostanyl ferulate (22 min) were observed.

The analysis of phytosteryl ferulates, phytosteryl sinapates, and phytosteryl vanillates in this study was carried out under the same HPLC conditions. As indicated by the chromatograms in Figures 5-8, 5-9, 5-10, the retention times for the same group of

compounds in these three mixtures were very close. For example, the retention time differences between campesteryl ferulate, sinapate and vanillate was 0.3-0.7 min; the retention time differences for the three sitosteryl phenolates and sitostanyl phenolates were 0.5-0.8 and 0.3-0.9 min, respectively. If these compounds coexisted naturally in plants or foodstuffs, they may be coeluted since their polarities are very similar to each other if their separation is not optimized. Therefore, only the predominating one will be interpreted from the data and the minor phytosteryl phenolates may be missed. This might be one of the reasons for the abundance reports on the existing of phytosteryl ferulates in cereals such as corn, rice, rye and wheat while much less research has been reported for other phytosteryl phenolates.

Except phytosteryl ferulates, other naturally existing phytosteryl phenolates include phytosteryl *p*-coumarates, phytosteryl caffeates (Fang et al., 2003; Seitz, 1989). Cycloartenyl caffeate and campesteryl caffeate were first reported by Fang et al. (2003). In their study, gradient mobile phase with acetonitrile in water from 20 to 100% within 80 min was used for HPLC separation of rice bran oil mixtures. Under this mobile phase, most phytosteryl ferulates yielded very sharp peaks except those assigned to cycloartenyl and campesteryl caffeates. The two peaks for cycloartenyl and campesteryl caffeates were quite broad. The ionization reagents, ammonium acetate in the negative ion mode and formic acid in the positive-ion mode, were added for optimum MS analysis as reported by Fang et al. (2003). Fragments at *m/z* 179 in negative ion mode MS spectra and cations $[M+H-180]^+$ for intact phytosterol moieties in positive mass spectra provided strong evidence for caffeoyl moieties and established that these compounds were caffeate esters.

Our results lend further support to the above findings of Fang et al. (2003). The isocratic solvent system containing methanol/water (95:5, v/v) has successfully been employed in the analysis of three other phytosteryl phenolates that included phytosteryl ferulates, phytosteryl sinapates and phytosteryl vanillates, however, the separation of phytosteryl caffeate mixtures with the same isocratic solvent system, it caused big tailing as well as poor resolution of the MS spectrum. Therefore, 1% formic acid was added to the mobile phase which then gave nice and sharp peaks for the three components in the mixture of phytosteryl caffeates as shown in Figure 5-7.

Detection of phytosteryl ferulates, caffeates and sinapates was at 325 nm, while that for phytosteryl vanillates was at 280 nm. This is because the three cinnamic acid derivatives had carbon-carbon double bond adjacent to the aromatic ring. This allows for a continuous, conjugated π-system throughout the molecule. An electron can be delocalized throughout the π-system by photoexcitation with energy corresponding to ~305 nm. For hydroxybenzoic acid derivatives, the carbon-carbon double bond is not present in the side chain.

Because phytosterols are relatively lipophilic with few polar functional groups, they are difficult to ionize through conventional electrospray methods (Rozenberg et al., 2003; Trosken et al., 2004). Although atmospheric pressure chemical ionization (APCI) is not the most sensitive method, it is most widely used for the analysis of phytosterols. APCI instruments are commonly available, and APCI ionization can be easily coupled with HPLC system. Thus, APCI has successfully been employed for identification of phytosterols in soybean oil (Careri et al., 2001), for characterization of phytosterols in

spelt (Rozenberg et al., 2003), for determination of ergosterol levels in bulrush (Headley et al., 2002), and for measurement of cholesterol oxides in different food supplies (Razzazi-Fazeli et al., 2000). To illustrate the structures of the synthesized phytosteryl phenolates, both negative-ion and positive-ion APCI-MS modes have been employed. The test compounds showed different ionization pathways under these two ionization modes and the MS data are presented below.

Using negative-ion APCI-MS, compounds 7, 8, 9 yielded base peaks for the deprtonated negative molecular ions $[M-H]^-$, and indeed there were no other significant peaks in their mass spectra (Figures 5-12, 5-13 and 5-14). The deprtonated negative molecular ions of compounds 7, 8, 9 were at *m/z* 561.5, 575.6 and 577.6, respectively, in accordance with the calculated molecular weights of campesteryl, sitosteryl and sitostanyl caffeates, respectively. The MS/MS is known to be specific and selective, so the molecular ions were then chosen as a precursor to run a tandem MS/MS. The corresponding spectra are shown in Figures 5-12 (b), 5-13 (b) and 5-14 (b). The deprotonated caffeic acid at *m/z* 178.9 indicated the presence of the caffeoyl moiety in the sitosteryl, sitostanyl and campesteryl caffeates. No fragment corresponding to phytosterols means that phytosterols were not ionized using the negative-ion mode APCI-MS. The positive ion mode was further used to confirm the identification of peaks. In contrast to the abundant yield of molecular ion, positive-ion mode APCI-MS generated the base peaks corresponding to cations of phytosterol moieties $[M+H\text{-caffeic acid}]^+$ from loss of caffeic acid moiety. Peaks at *m/z* 397.7, 399.6 and 383.6 are for sitosteryl caffeate, sitostanyl caffeate and campesteryl caffeate, respectively. These characteristic

fragmentation pathways for phytosteryl caffeates have also been reported from the positive ion mode electrospray ionization-mass spectroscopy (ESI-MS) (Fang et al., 2003). Due to the structural difference between sitostanyl caffeate and sitosteryl caffeate resulting from the absence of a double bond between C5 and C6, sitostanyl caffeate showed slightly different fragmentation pattern as shown in Figure 5-14. It yielded more ion peaks in both negative ion mode APCI-MS and positive-ion mode APCI-MS. A similar fragmentation pathway for campesteryl caffeate was reported from electrospray ionization-mass spectroscopy (ESI-MS) (Fang et al., 2003). For example, base peak at $[M-H]^-$ under the negative-ion ESI-MS and cations $[M+H\text{-caffeic acid}]^+$ under the positive-ion ESI-MS were observed (Fang et al., 2003). The above APCI-MS data support the successful synthesis of phytosteryl caffeates in this study.

APCI-MS spectra of phytosteryl ferulates are shown in Figures 5-15, 5-16 and 5-17. Under the NI-APCI-MS, compounds 10, 11 and 12 yielded base peaks at *m/z* 575.6, 589.7 and 591.7, respectively, which match the molecular weight of campesteryl ferulate, sitosteryl ferulate and sitostanyl ferulate as illustrated in the same figures. These base peaks represented the deprotonated negative molecular ion $[M-H]^-$ of the three components of interest. Lack of fragments corresponding to phytosterols means that phytosterol moieties were not ionized when the negative-ion mode APCI-MS was used. As the MS/MS is known to be specific and selective, the molecular ions were then chosen as parent ions to run the second MS. As shown in Figure 5-16 (b), sitosteryl ferulate yielded a peak at *m/z* 193 which represented the feruloyl moiety in sitosteryl ferulate. Sitostanyl ferulate yielded a base peak at *m/z* 577.5, which represents the $[M-CH_4]^-$.

Peaks with very low intensity for the feruloyl moiety in sitostanyl ferulate were shown in the enlarged spectrum in Figure 5-17 (b). The characteristic ions for the feruloyl moiety include ferulic acid at *m/z* 194.2, deprotonated ferulic acid at *m/z* 193.3, as well as fragments at *m/z* 178, 177, and 175, which are derived from deprotonated ferulic acid. The positive ion mode was further used to confirm the identification of compounds. In PI-APCI-MS, the base peaks of compounds 11, 12 and 13 were the typical fragments for the intact moieties of phytosterols as shown in Figures 5-15 (b), 5-16 (b) and 5-17 (b). These ions were $[M+H\text{-ferulic acid}]^+$ with *m/z* at 383.9, 399.3 and 397.9 for campesteryl ferulate, sitosteryl ferulate and sitostanyl ferulate, respectively. This characteristic fragmentation pathway for campesteryl ferulate and sitosteryl ferulate has also been reported from positive-ion chemical ionization-mass spectroscopy (CI-MS) (Rogers et al., 1993), positive-ion electrospray ionization-mass spectroscopy (ESI-MS) (Fang et al., 2003) and positive-ion APCI-MS (Stoggl et al., 2005; Hakala et al., 2002). Sitostanyl ferulate also showed a characteristic ion of the protonated molecular ion $[M+H]^+$ as well as protonated feruloyl moiety at *m/z* 194.9. These MS data confirmed that the synthesized products were campesteryl ferulate (compound 11), sitosteryl ferulate (compound 12) and sitostanyl ferulate (compound 13).

MS data for synthesized phytosteryl sinapates are shown in Figures 5-18, 5-19 and 5-20. A similar ionization pattern was also observed for phytosteryl sinapates as those for phytosteryl caffeates and phytosteryl ferulates. The deprotonated negative molecular ions $[M-H]^-$ were the base peaks in NI-APCI-MS for compounds 13, 14, 15 with *m/z* at 605.8, 619.9 and 621.8, respectively. Lack of fragments corresponding to phytosterols indicates

that the phytosterol moieties are not ionized when negative-ion mode APCI-MS was employed. Thus, a second MS was carried out using these molecular ions as precursors. Peaks indicated that the characteristic ions were $[M-CH_4]^-$, $[M-H-HCHO]^-$ as well as the deprotonated sinapic acid ion at *m/z* 223, which also represented the presence of the sinapoyl moiety in the synthesized phytosteryl sinapates. The positive ion mode was also further used for confirmation of the identification process. These three components (compounds 13, 14 and 15) yielded base peaks with *m/z* 383.6, 397.7 and 399.6, respectively, under the positive ion mode APCI-MS. These ions $[M+H\text{-sinapic acid}]^+$ represent the intact phytosteryl moiety after cleavage of the ester bond during the ionization process. More fragments are shown for compound 14. One of the characteristic ions is the protonated sinapic acid at *m/z* 225.5. All the evidences indicated that these three compounds (compound 13, 14 and 15) are campesteryl sinapate, sitosteryl sinapate and sitostanyl sinapate, respectively. Since this is the first time to report the synthesis of phytosteryl sinapates and there is no literature report on its natural presence in plants, no comparison of the MS data can be made here. We hypothesize that phytosteryl sinapates might be naturally present in canola seeds as it contains a relatively high content of both sinapic acid and phytosterols. More research is needed to demonstrate this hypothesis.

The mass spectra of compounds 16, 17, and 18 in the APCI-MS revealed the same fragmentation behaviour as the three groups of phytosteryl phenolates discussed above. The MS spectra for compounds 16, 17, and 18, as eluted in the HPLC chromatogram of Figure 5-10, are presented in Figures 5-21, 5-22 and 5-23, respectively. Base peaks for those three compounds under NI-APCI-MS are the deprotanted negative molecular ions

$[M-H]^-$ alone at *m/z* 549.6, 563.6 and 565.6 for compounds 16, 17, and 18, respectively. Lack of fragments corresponding to phytosterol moieties indicate that they are not ionized using the negative-ion mode APCI-MS. Further fragmentation of these molecular ions using MS/MS are shown in Figures 5-21 (b), 5-22 (b) and 5-23 (b), respectively. The characteristic ions include $[M-CH_4]^-$ and the deprotonated sinapic acid ion at *m/z* 223. The PI-APCI-MS was also carried out for further confirmation of the structures of these three compounds (16, 17, and 18). The base peaks under positive ion mode APCI-MS were the ions $[M+H\text{-vanillic acid}]^+$ which represent the intact phytosteryl moiety after cleavage of the ester bond. In this case, compound 16 showed a base peak at *m/z* 383.6, which corresponds to the campesteryl moiety. Compound 17 displayed a base peak at *m/z* 397.7, which corresponds to the sitosteryl moiety. In addition to the base peak at *m/z* 399.7 representing the sitostanyl moiety, compound 18 also showed other fragments. One of them was the protonated vanillic acid at *m/z* 168.2.

The free phytosterols and the four synthesized phytosteryl phenolates were compared using their corresponding IR spectra as shown in Figure 5-24 to Figure 5-27. These spectra could provide evidence that the chemoenzymatically synthesized products were phytosteryl esters.

The free phytosterols had a strong absorption at 1053 cm^{-1} that was assigned to the C-O vibration via participation of oxygen atom of the hydroxyl group. A broad band around 3300-3600 cm^{-1} corresponds to the O-H vibration of the hydroxyl group of phytosterols.

The IR spectrum of phytosteryl caffeates (Figure 5-24 buttom) shows a strong sharp band at 1673 cm^{-1} that is due the ester carbonyl group (C=O) and the absorption at 1186 cm^{-1} corresponds to the C-O-C symmetric vibration of the ester linkage. The sharp band at 3445 cm^{-1} and a broader band around 3100-3300 cm^{-1} were assigned to the two hydroxyl groups on the benzene ring of caffeic acid moiety of phytosteryl caffeates.

The IR spectra of free phytosterols and phytosteryl ferulates are shown in Figure 5-25. The ester bond of phytosteryl ferulates was confirmed by the sharp band at 1689 cm^{-1} and 1159 cm^{-1}, which indicates the functional groups C=O and C-O-C, respectively. A sharp band at 3531 cm^{-1} in the phytosteryl caffeates also indicates that the presence of hydroxyl group in ferulic acid moiety.

Similar IR results were also observed for phytosteryl sinapates and phytosteryl vanillates. For example, the disappearance of the sharp band at 1053 cm^{-1} from their phytosterol moiety, the band for the ester carbonyl group (1706 cm^{-1} for phytosteryl sinapates and 1708 cm^{-1} for phytosteryl vanillates), the band for the ester linkage C-O-C (1171 cm^{-1} for phytosteryl sinapates and 1220 cm^{-1} for phytosteryl vanillates), as well as a broad band around 3300-3500 cm^{-1} represent the remaining hydroxyl group in the phenolic acid moiety.

UV spectroscopic study of neutral methanolic solutions of sitostanyl ferulate extracted from corn fibre oil and ferulic acid showed UV maxima at 325 nm and 321 nm, respectively (Seitz, 1989). Ultraviolet spectra of the synthesized phytosteryl phenolates are displayed in Figures 5-28, 5-28, 5-30 and 5-31. Our results are in agreement with the above findings. All phytosteryl esters with hydroxycinnamic acids (namely caffeic,

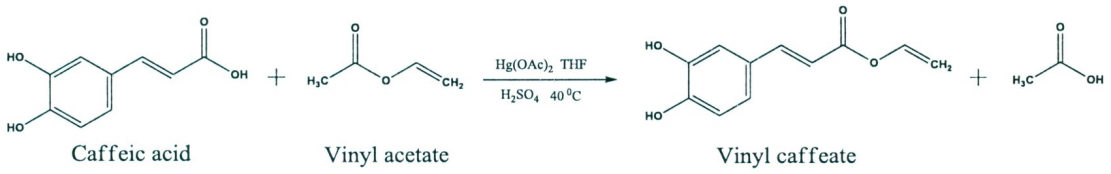
ferulic and sinapic acid) have maximum UV absorption at 325 nm, while phytosteryl esters with hydroxybezoic acids showed two maxima around 260 nm and 290 nm.

The above information collectively demonstrated that the synthesized products were phytosteryl phenolates.

Table 5-1. R_f values of phenolic acids and their corresponding derivatives on TLC plates.

Phenolic acids	R_f value	Vinyl phenolates	R_f value	Phytosteryl phenolates	R_f value
Caffeic acid	0.03	Vinyl caffeate	0.16	Phytosteryl caffeates	0.21
Ferulic acid	0.07	Vinyl ferulate	0.27	Phytosteryl ferulates	0.38
Sinapic acid	0.04	Vinyl sinapate	0.20	Phytosteryl sinapates	0.25
Vanillic acid	0.05	Vinyl vanillate	0.29	Phytosteryl vanillates	0.39

Step one



Step two

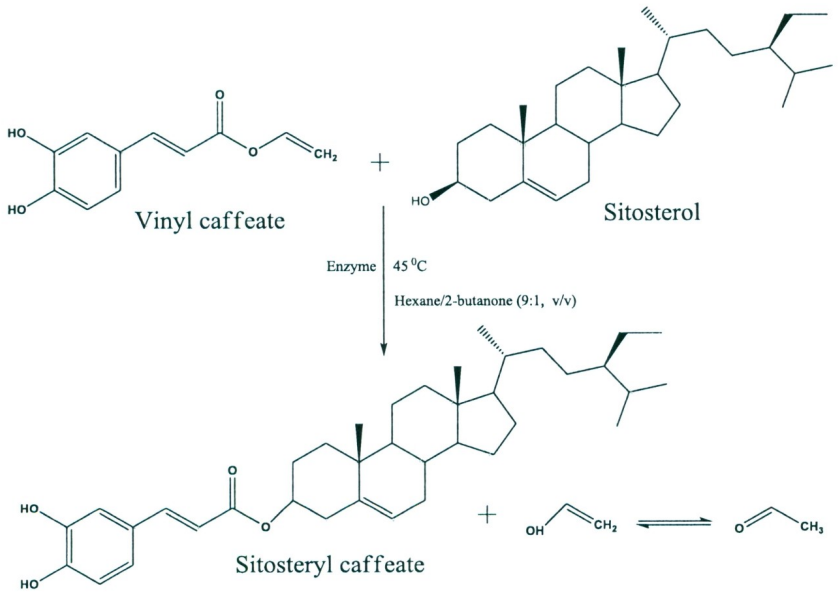


Figure 5-1. Two step chemoenzymatic syntheses of phytosteryl phenolates (sitosteryl caffeate is shown as an example; other phenolic acids, namely ferulic, sinapic and vanillic acids have also been successfully subjected to the same synthesis procedure. Figures were presented in appendix. Figures are attached in the Appendix).

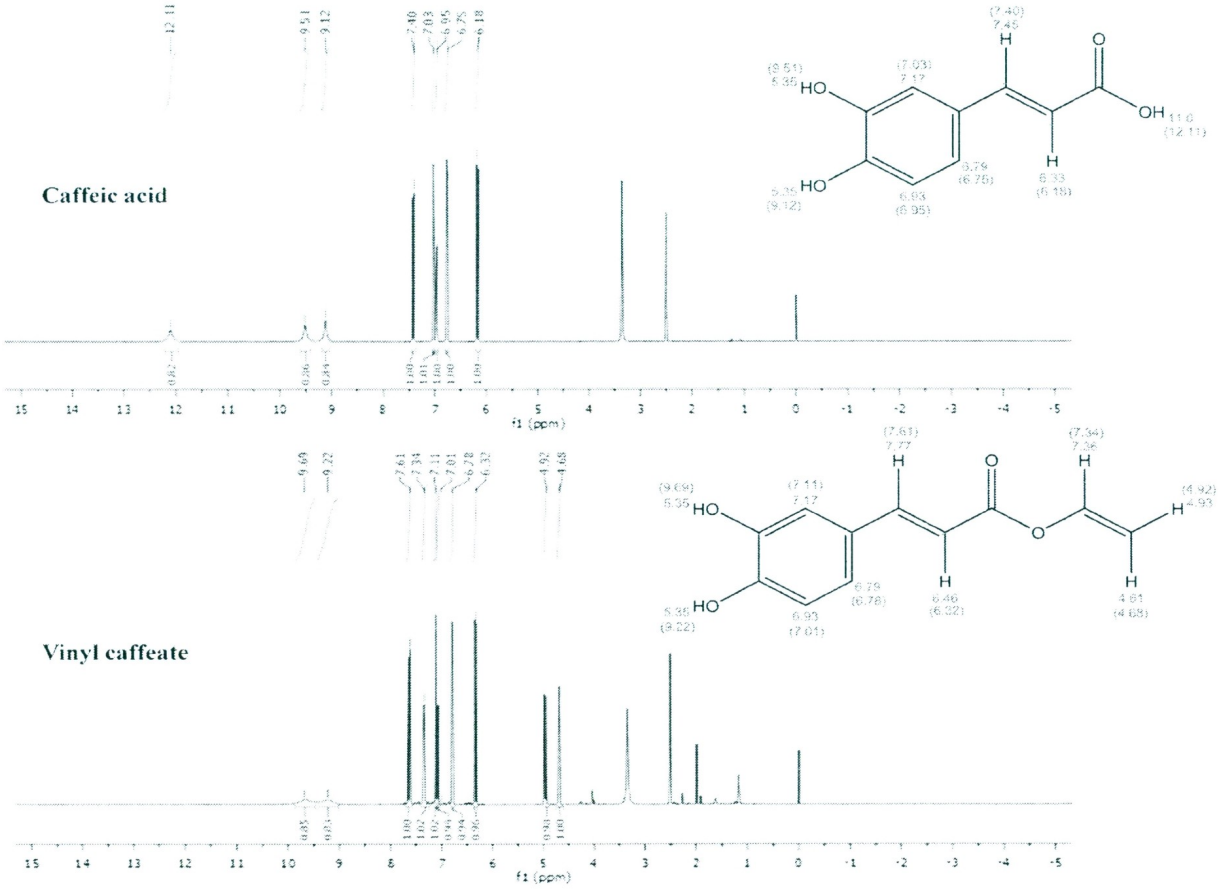


Figure 5-2. The ^{1}H-NMR spectra of caffeic acid and vinyl caffeate. Structures provide the assignment for the predicted and experimental (in bracket) chemical shifts.

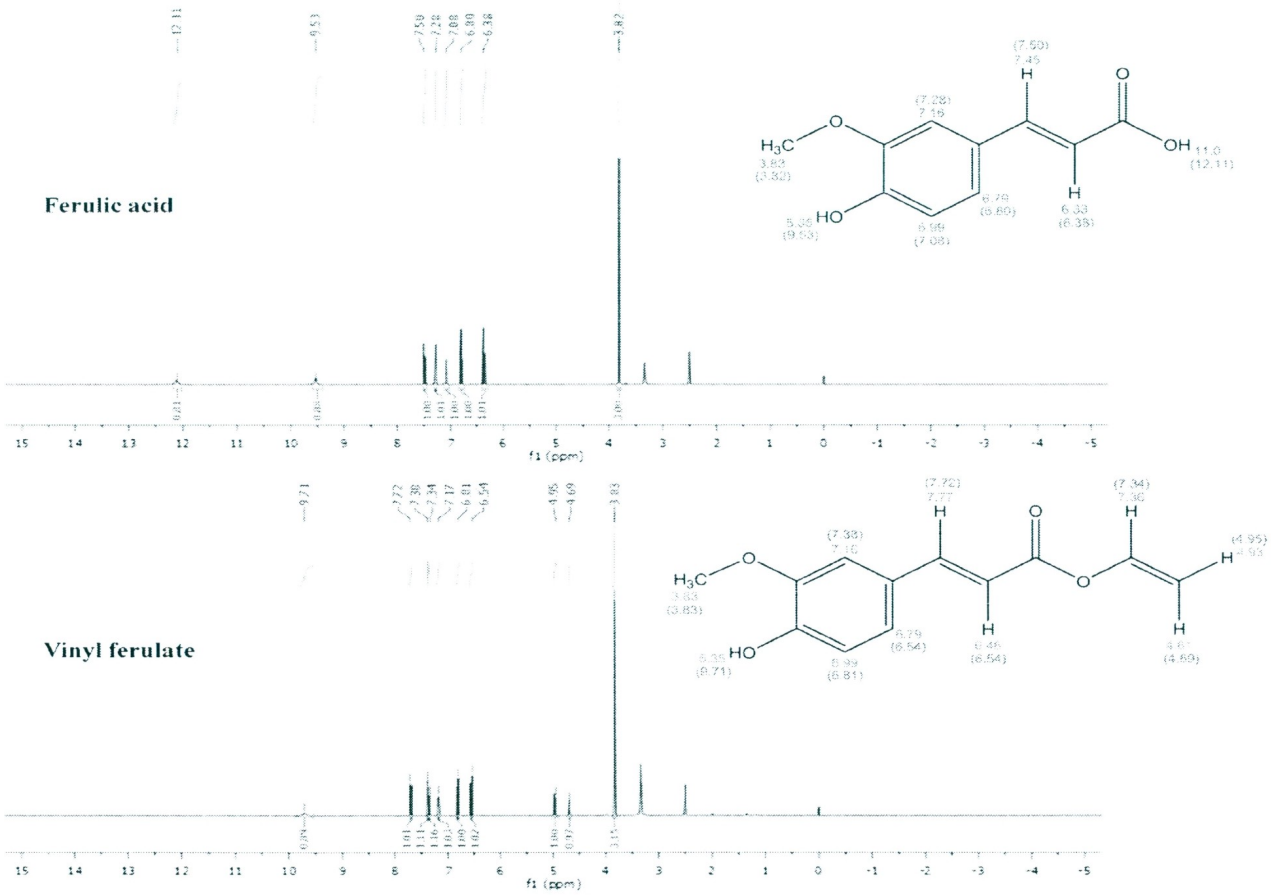


Figure 5-3. The ^{1}H-NMR spectra of ferulic acid and vinyl ferulate. Structures provide the assignment for the predicted and experimental (in bracket) chemical shifts.

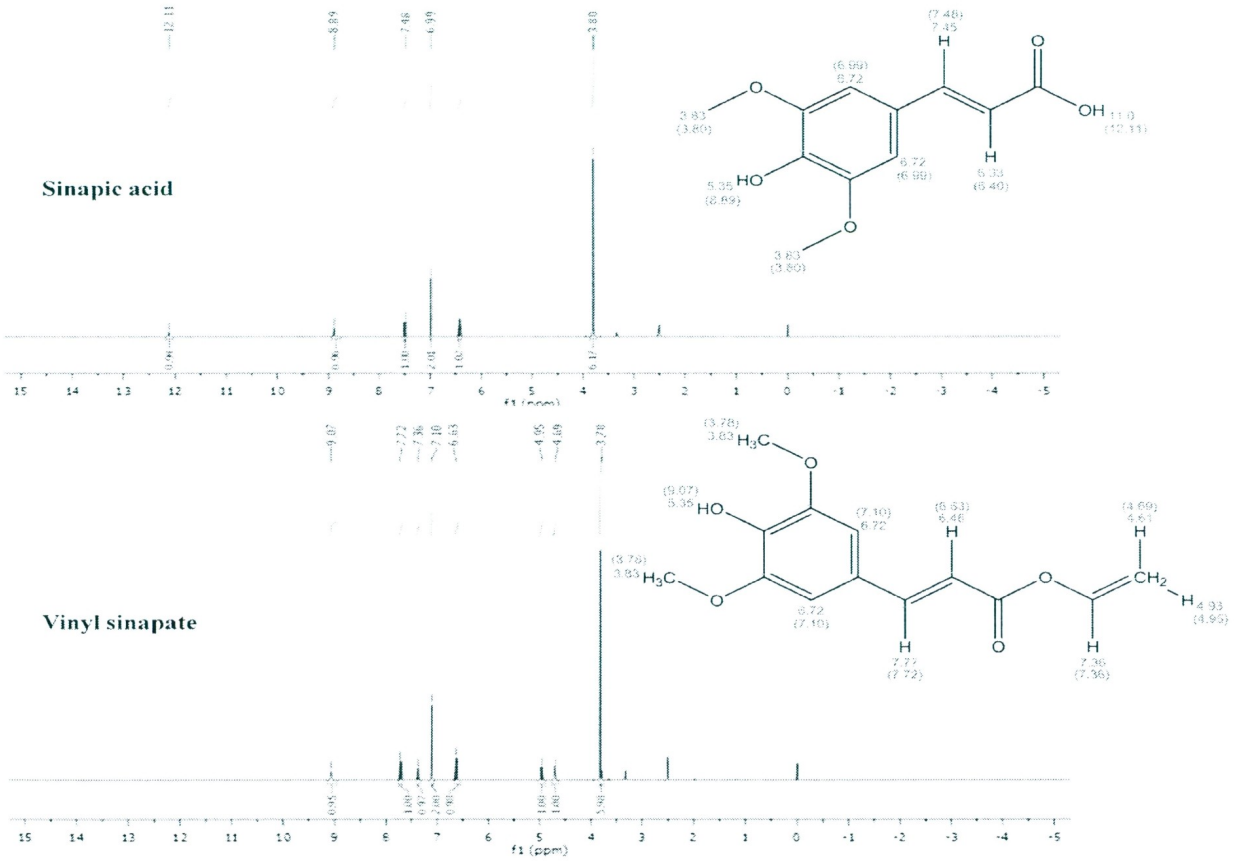


Figure 5-4. The ^{1}H-NMR spectra of sinapic acid and vinyl sinapate. Structures provide the assignment for the predicted and experimental (in bracket) chemical shifts.

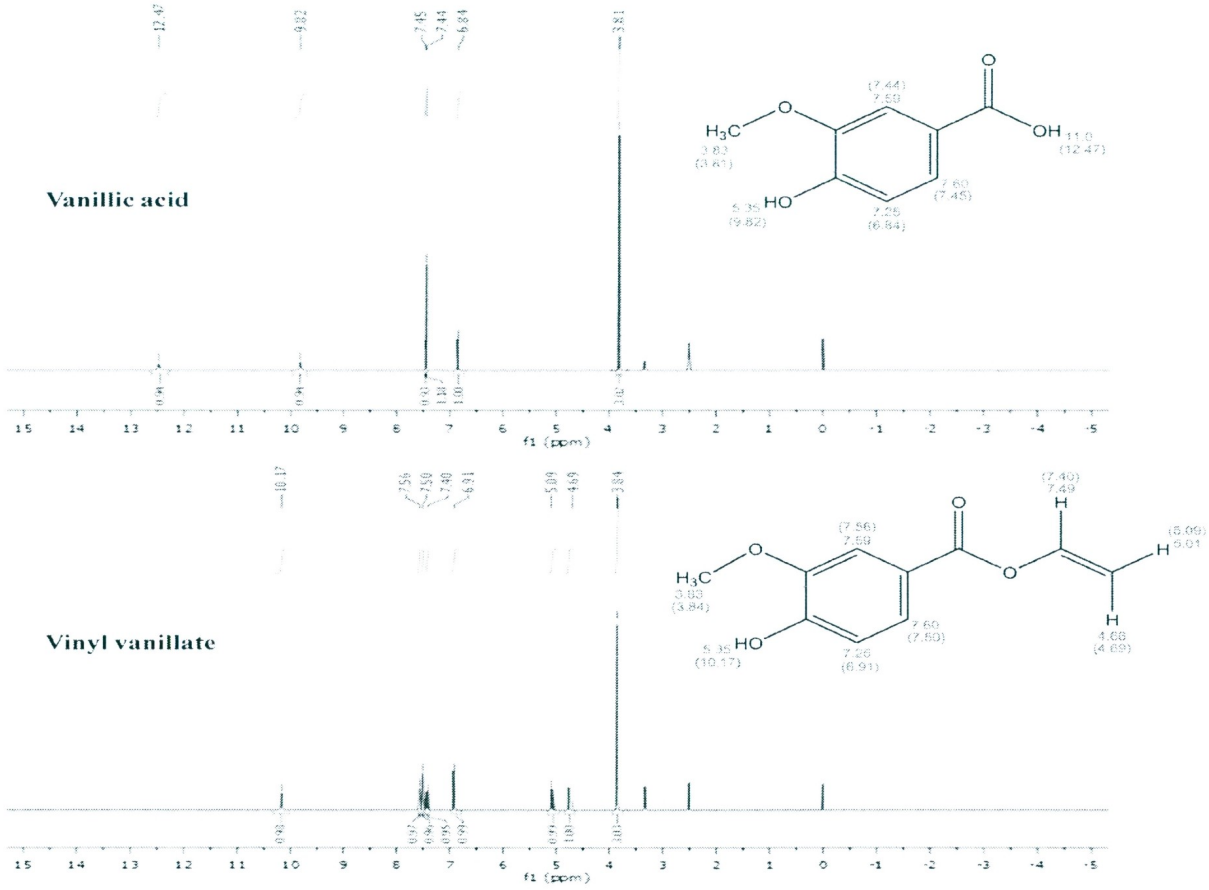


Figure 5-5. The ^{1}H-NMR spectra of vanillic acid and vinyl vanillate. Structures provide the assignment for the predicted and experimental (in bracket) chemical shifts.

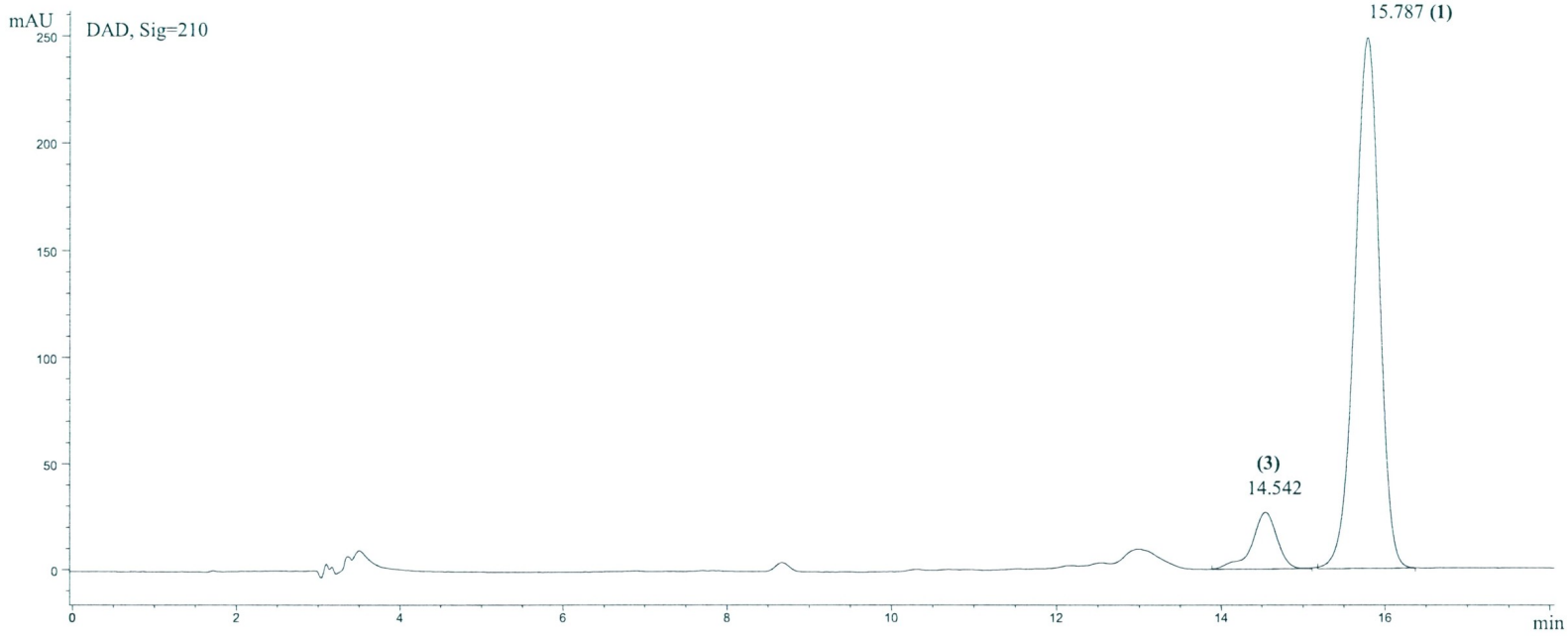


Figure 5-6. The HPLC chromatogram of free phytosterols (compounds 1 and 3 are sitosterol and campesterol, respectively).

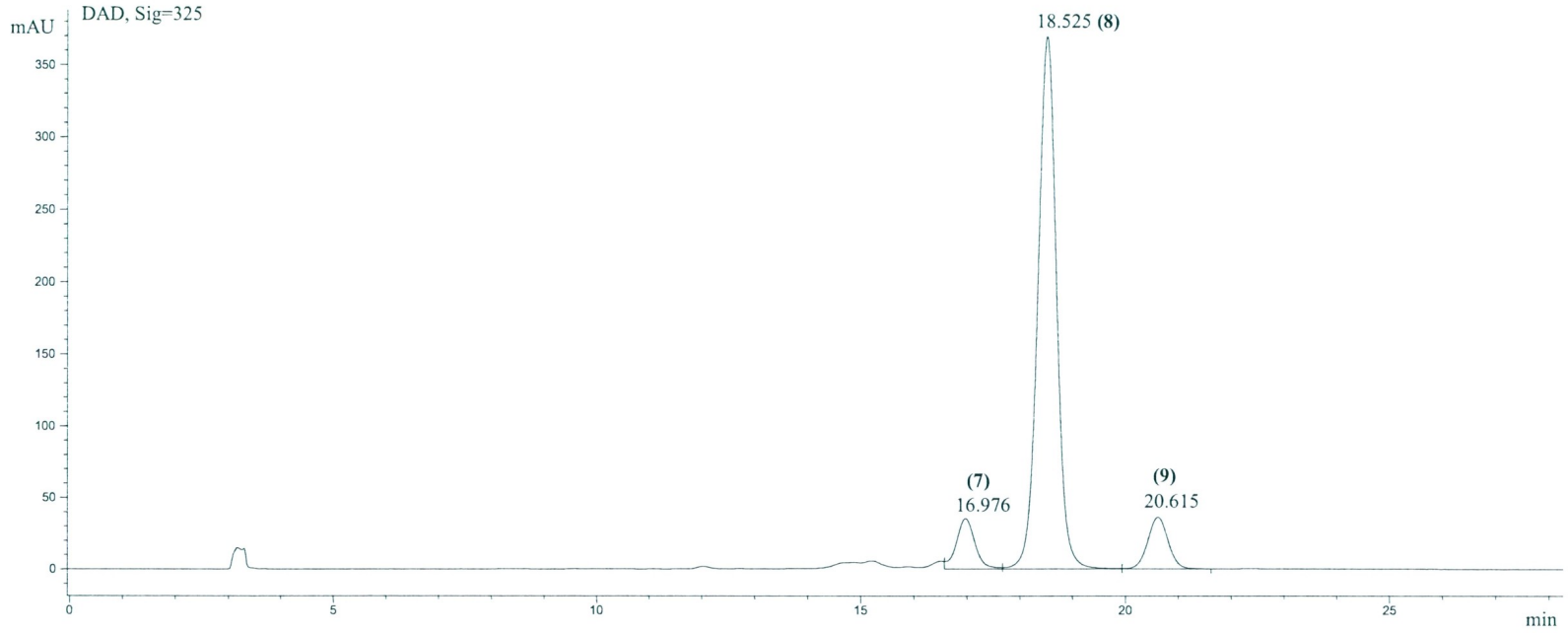


Figure 5-7. The HPLC chromatogram of phytosteryl caffeate mixtures (compounds 7, 8, 9 are campesteryl caffeate, sitosteryl caffeate, and sitostanyl caffeate, respectively).

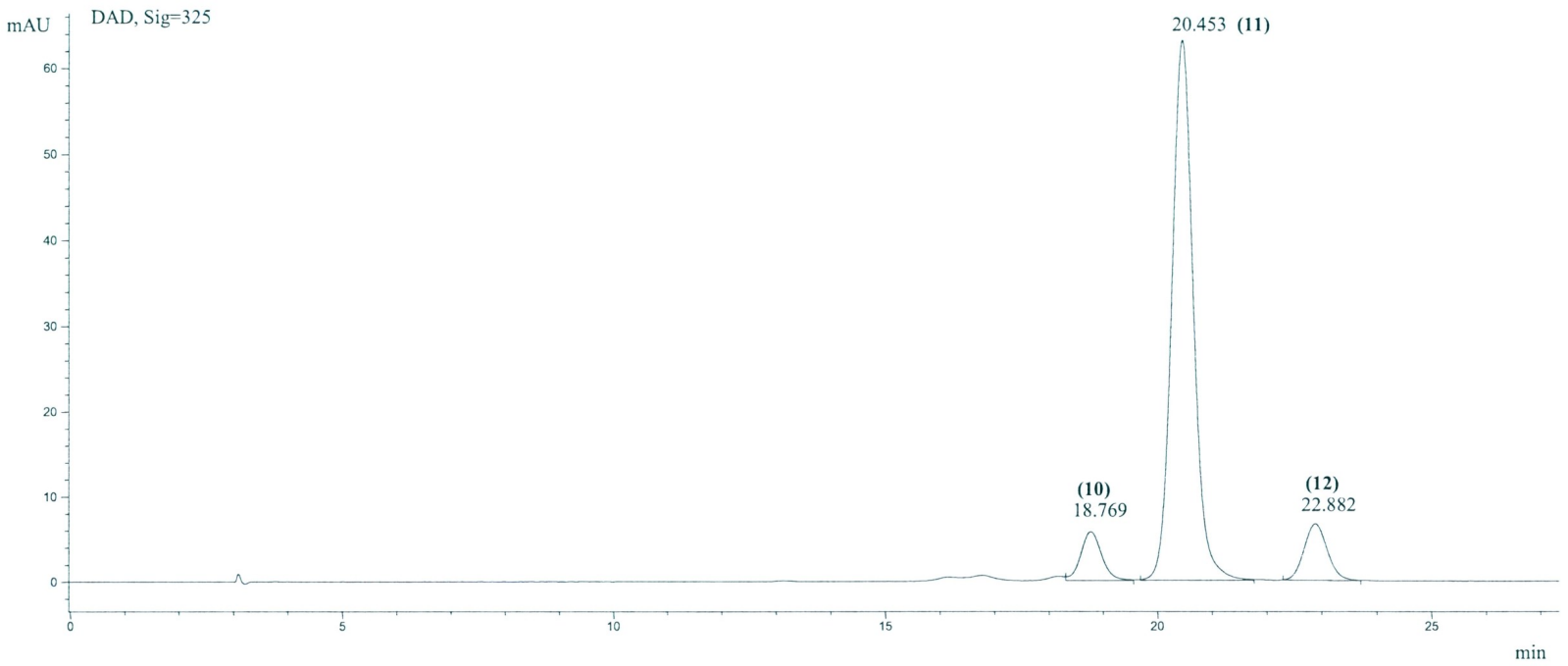


Figure 5-8. The HPLC chromatogram of phytosteryl ferulate mixtures (compounds 10, 11, 12 are campesteryl ferulate, sitosteryl ferulate, and sitostanyl ferulate, respectively).

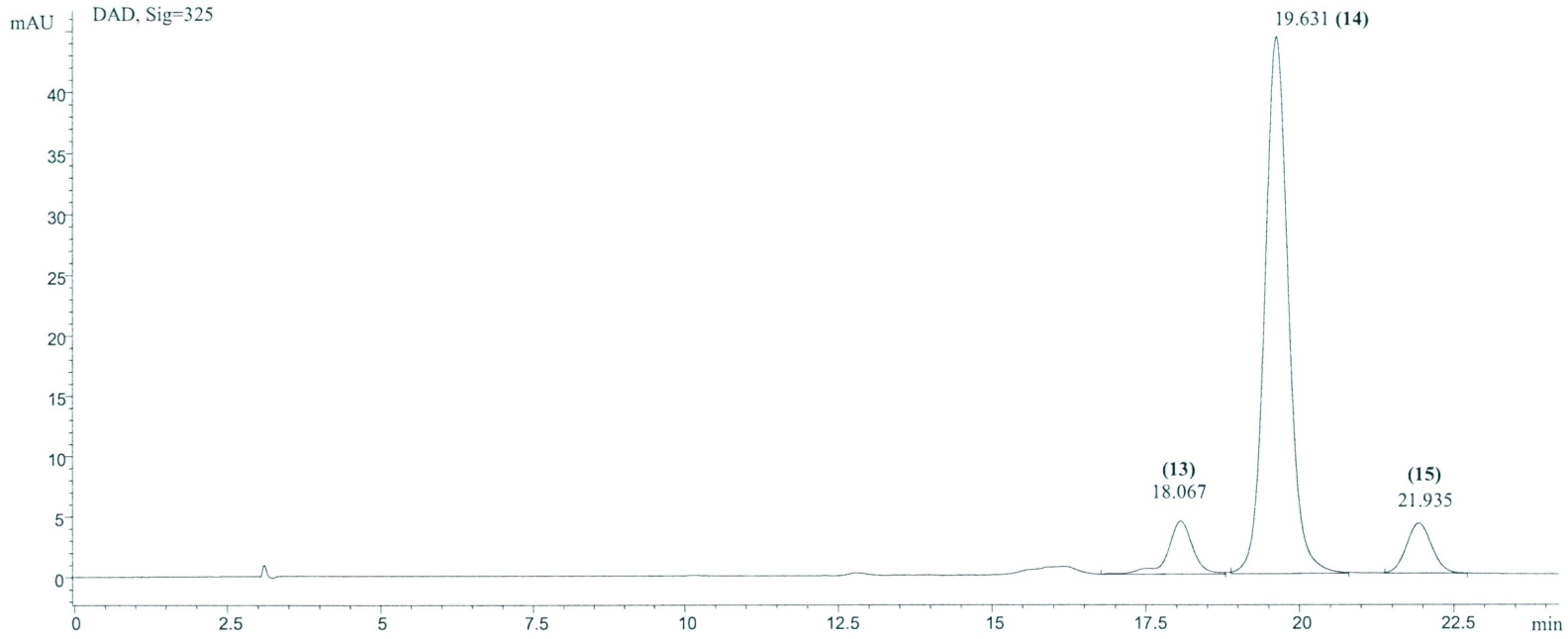


Figure 5-9. The HPLC chromatogram of phytosteryl sinapate mixtures (compounds 13, 14, 15 are campesteryl sinapate, sitosteryl sinapate, and sitostanyl sinapate, respectively).

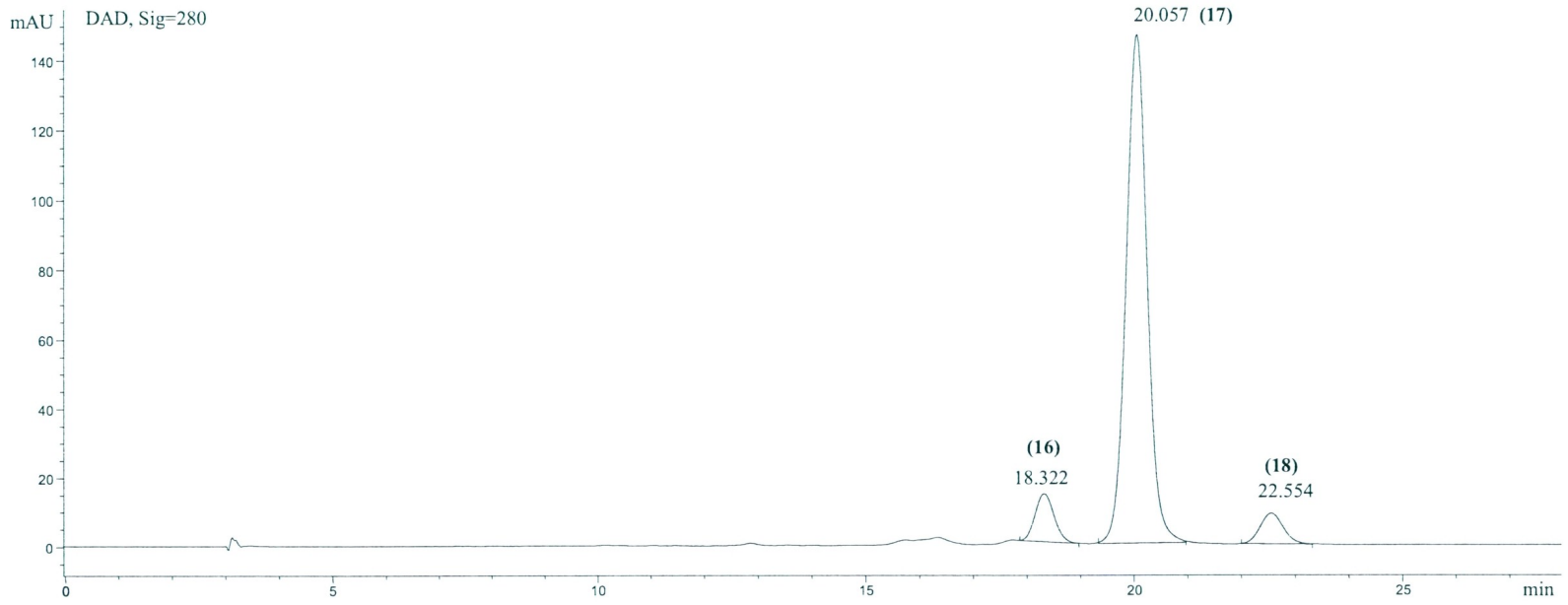


Figure 5-10. The HPLC chromatogram of phytosteryl vanillate mixtures (compounds 16, 17, 18 are campesteryl vanillate, sitosteryl vanillate, and sitostanyl vanillate, respectively).

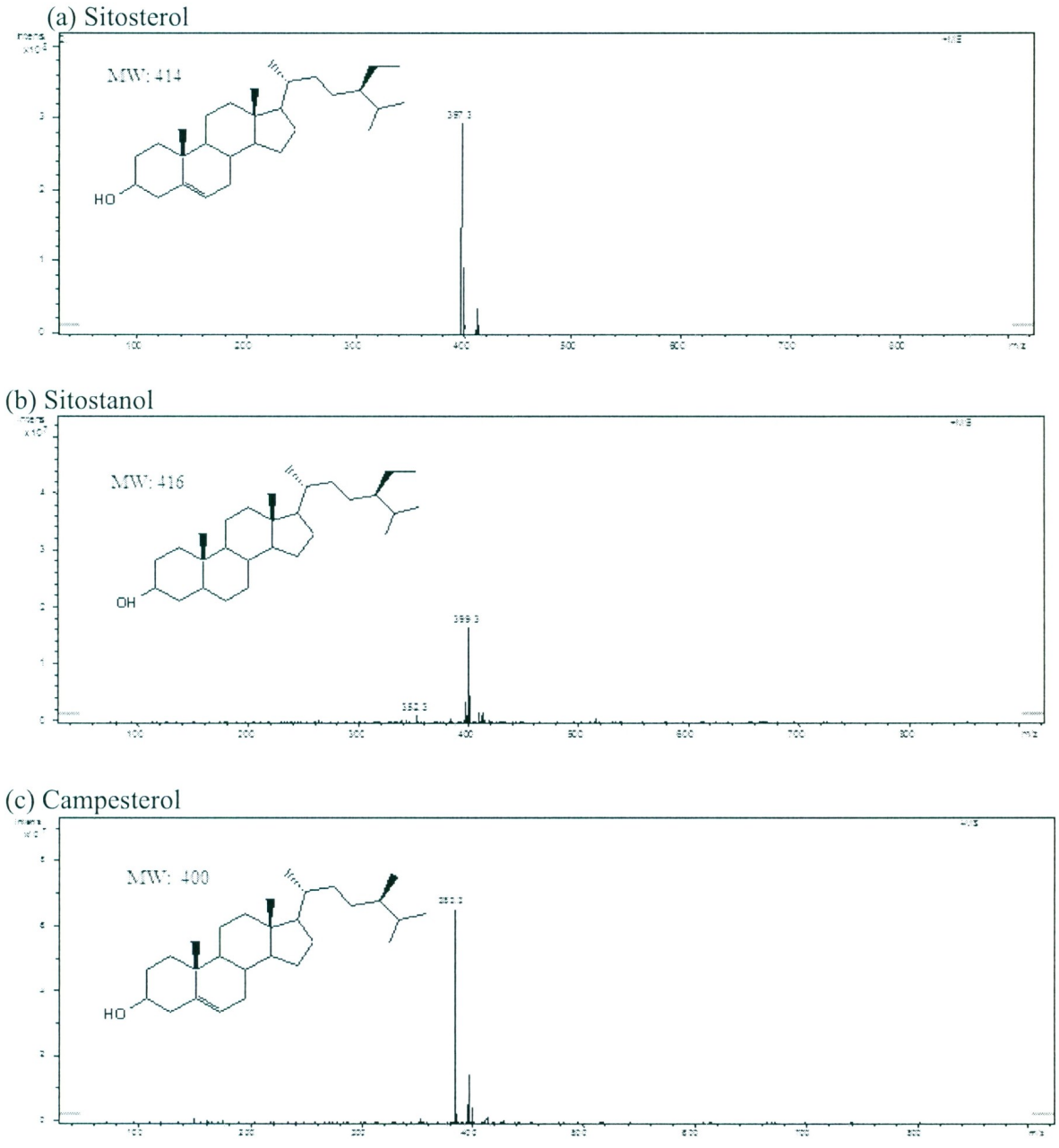
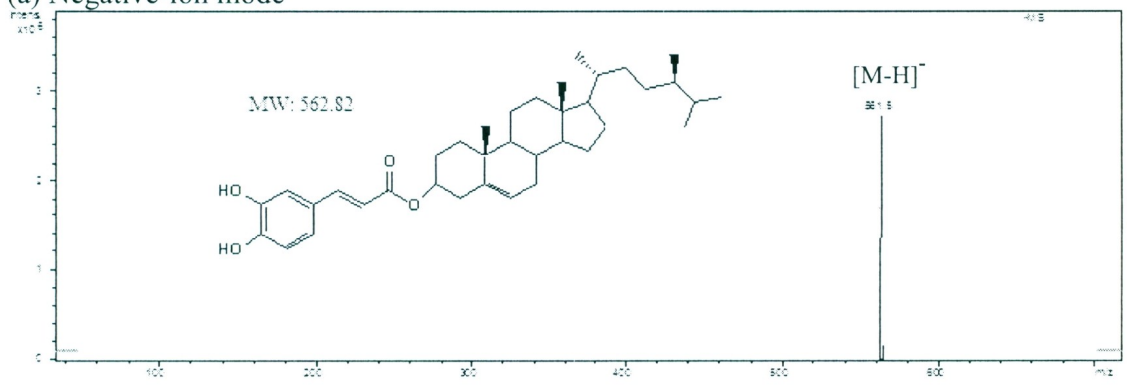
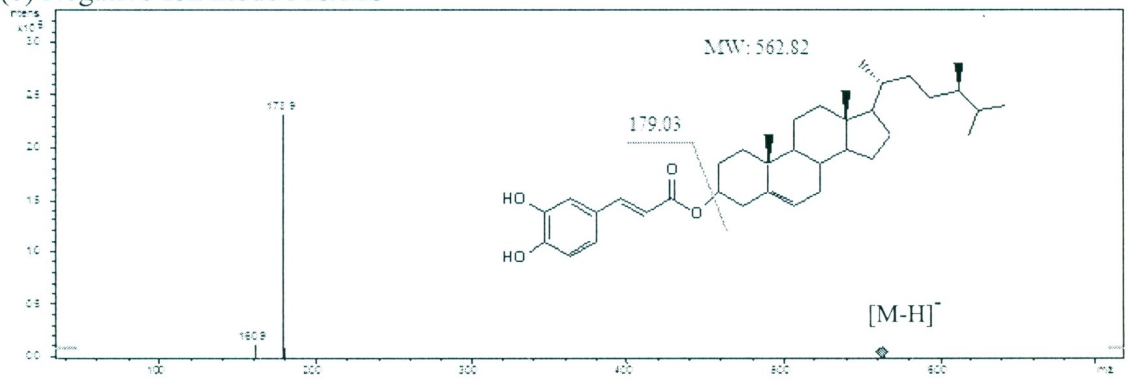


Figure 5-11. Mass spectra under positive-ion mode APCI-MS for free phytosterols (a) sitosterol, (b) sitostanol and (c) campesterol.

(a) Negative-ion mode



(b) Negative-ion mode MS/MS



(c) Positive-ion mode

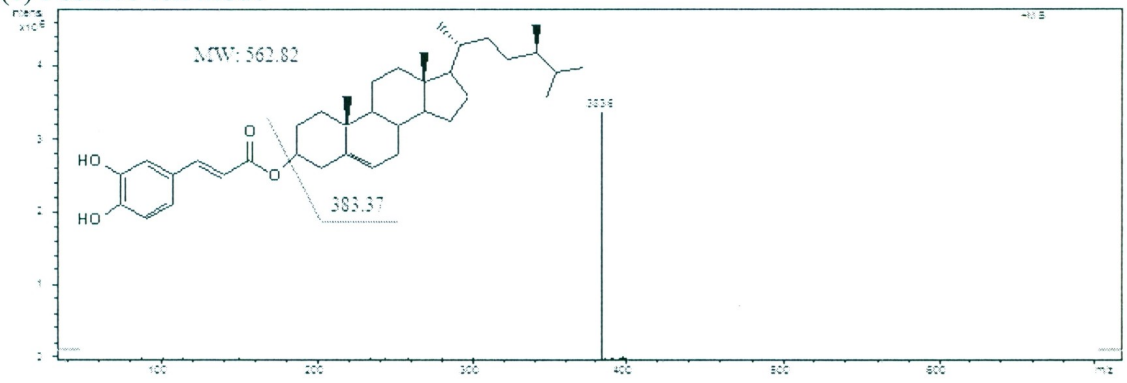
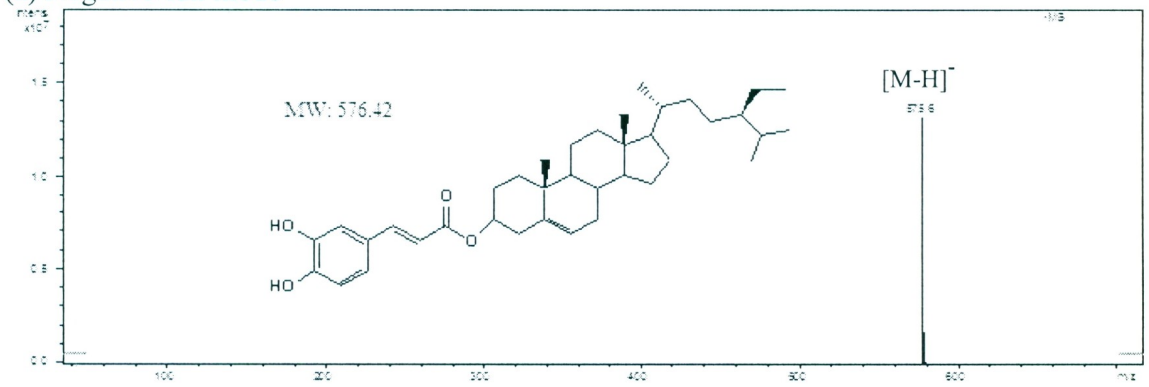
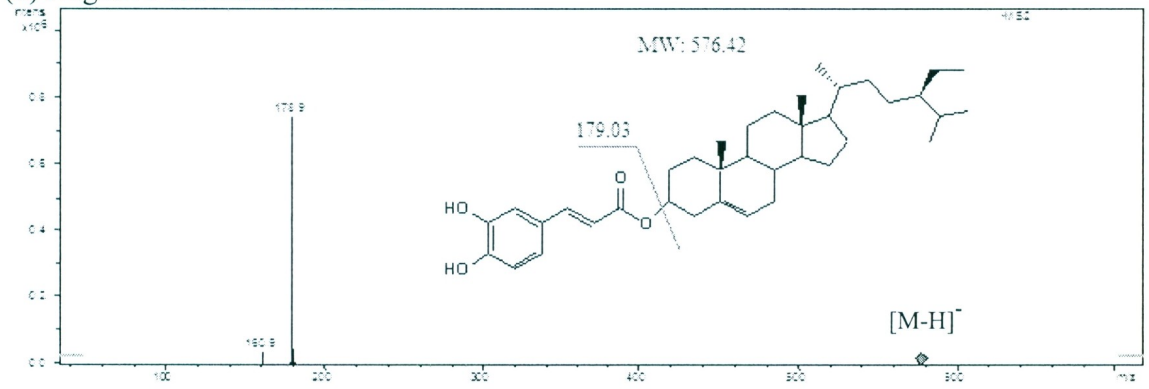


Figure 5-12. Mass spectra of campesteryl caffeate (compound 7), (a) negative-ion mode; (b) negative-ion mode MS/MS; (c) positive-ion mode.

(a) Negative-ion mode



(b) Negative-ion mode MS/MS



(c) Positive-ion mode

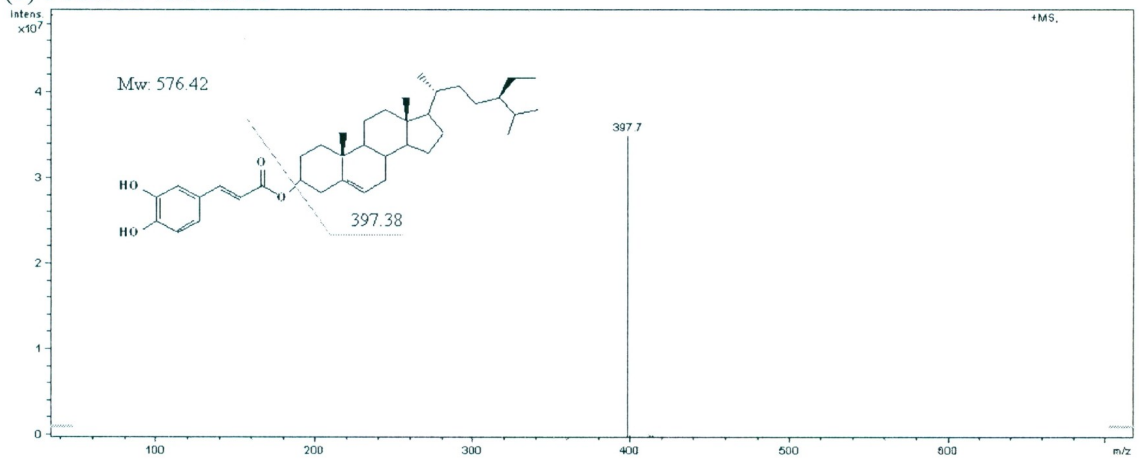
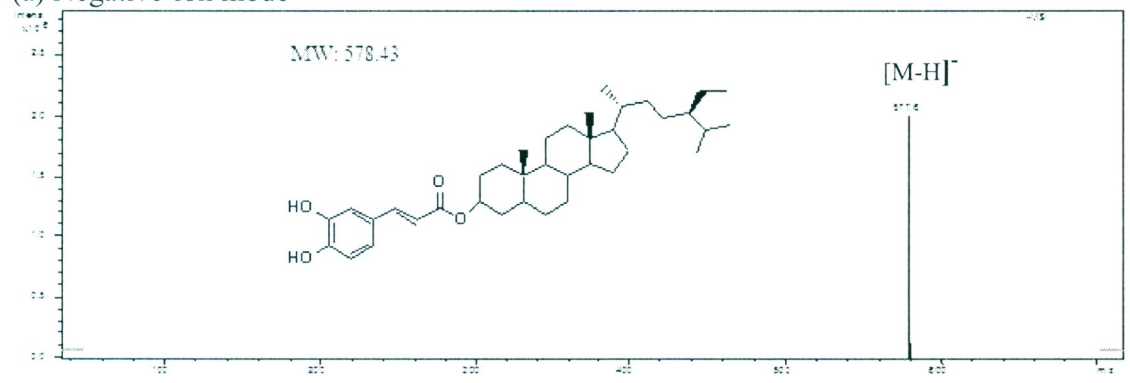
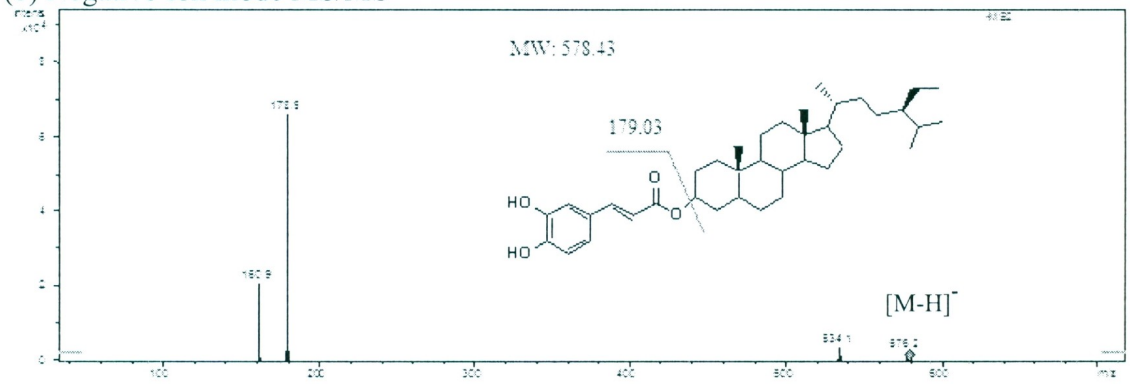


Figure 5-13. Mass spectra of sitosteryl caffeate (compound 8), (a) negative-ion mode; (b) negative-ion mode MS/MS; (c) positive-ion mode.

(a) Negative-ion mode



(b) Negative-ion mode MS/MS



(c) Positive-ion mode

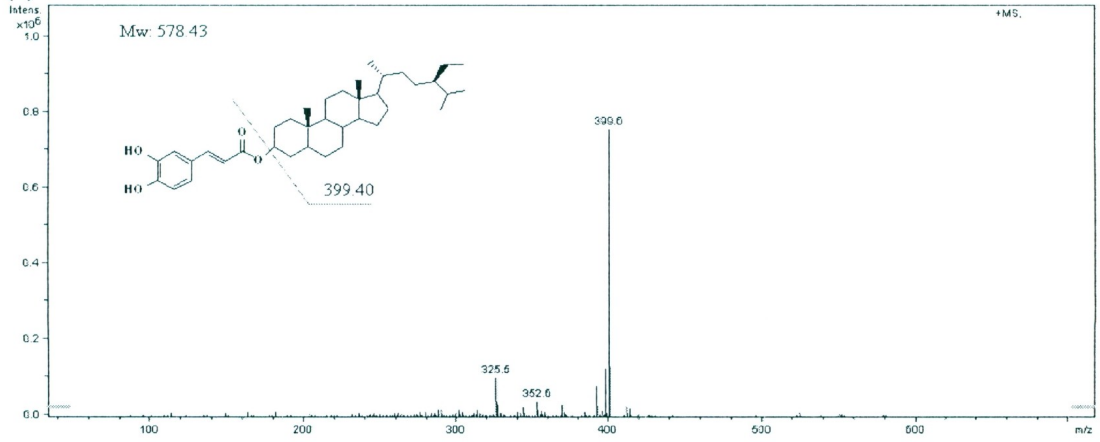
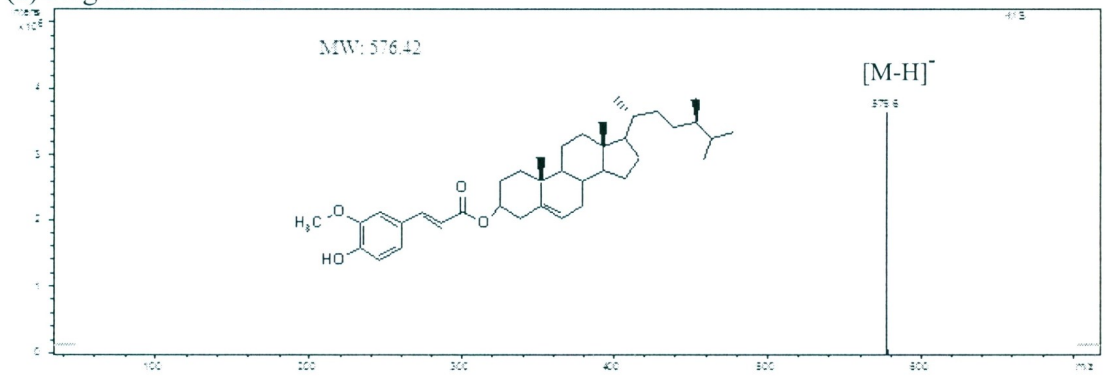


Figure 5-14. Mass spectra of sitostanyl caffeate (compound 9), (a) negative-ion mode; (b) negative-ion mode MS/MS; (c) positive-ion mode.

(a) Negative-ion mode



(b) Positive-ion mode

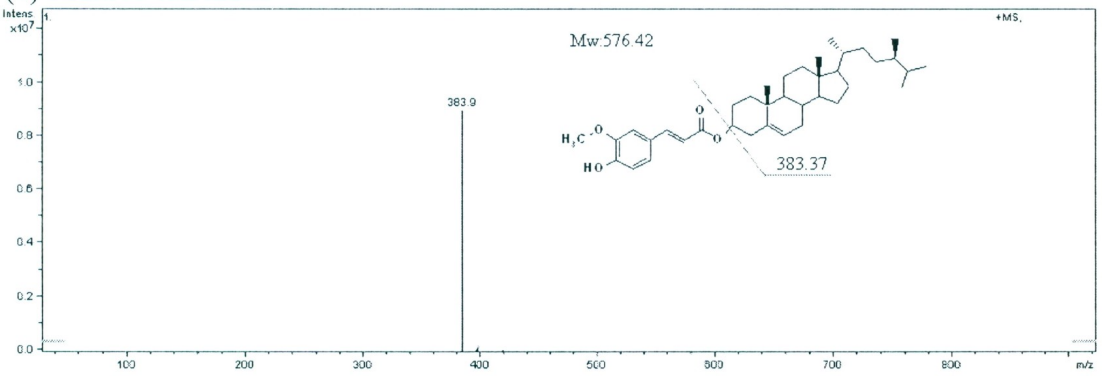
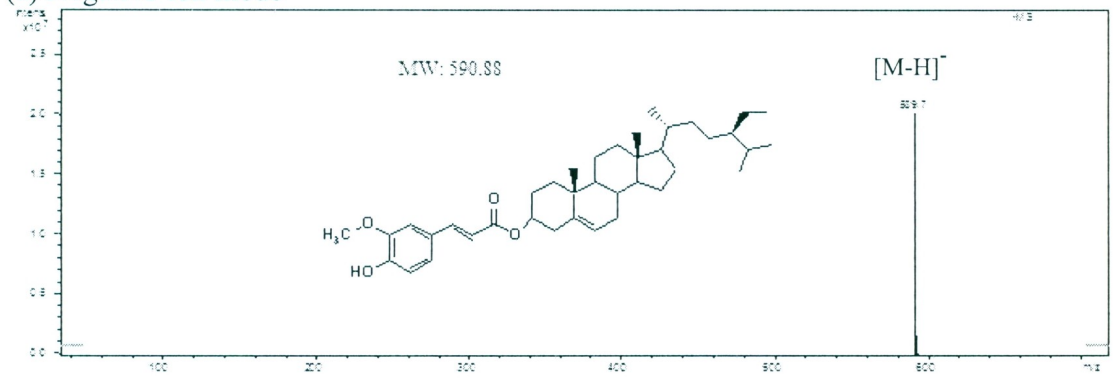
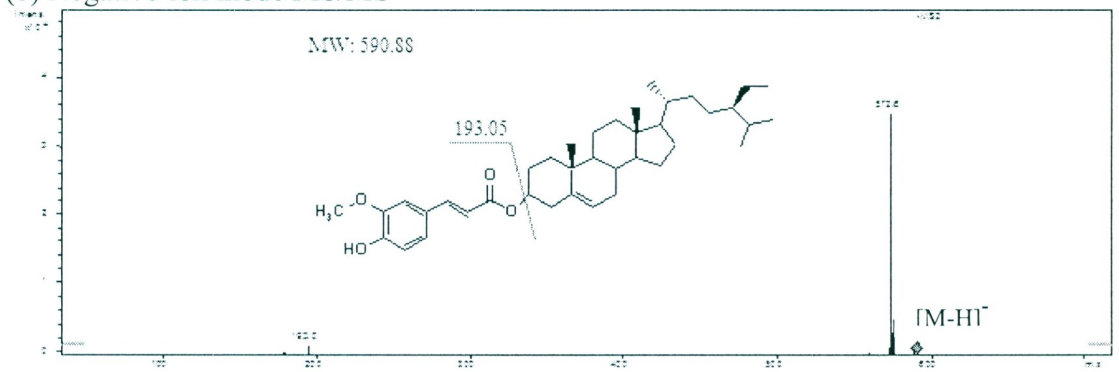


Figure 5-15. Mass spectra of campesteryl ferulate (compound 10), (a) negative-ion mode; (b) positive-ion mode.

(a) Negative-ion mode



(b) Negative-ion mode MS/MS



(c) Positive-ion mode

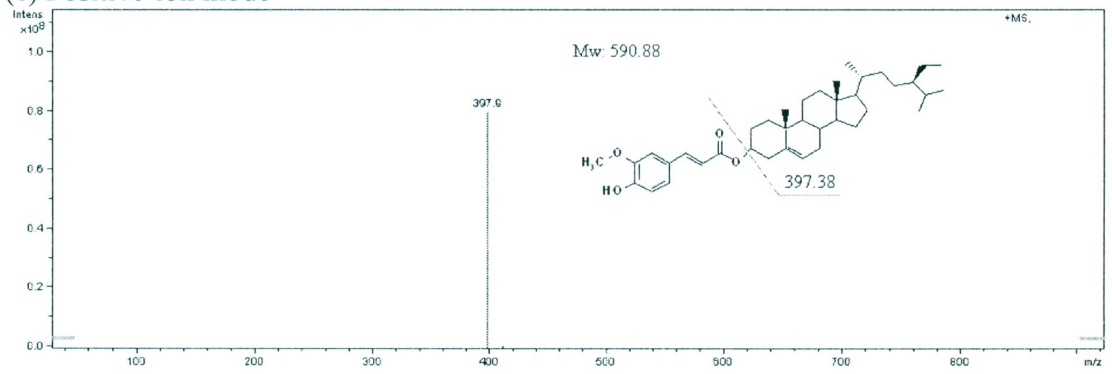
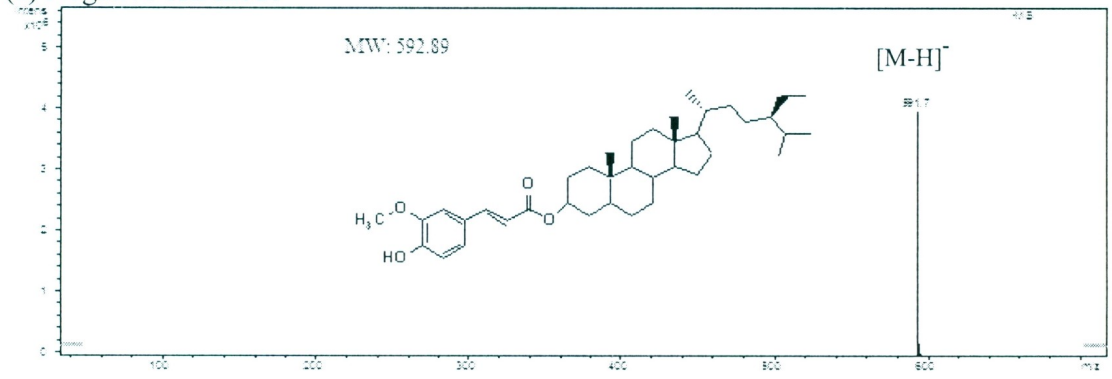


Figure 5-16. Mass spectra of sitosteryl ferulate (compound 11), (a) negative-ion mode; (b) negative-ion mode MS/MS; (c) positive-ion mode.

(a) Negative-ion mode



(b) Positive-ion mode

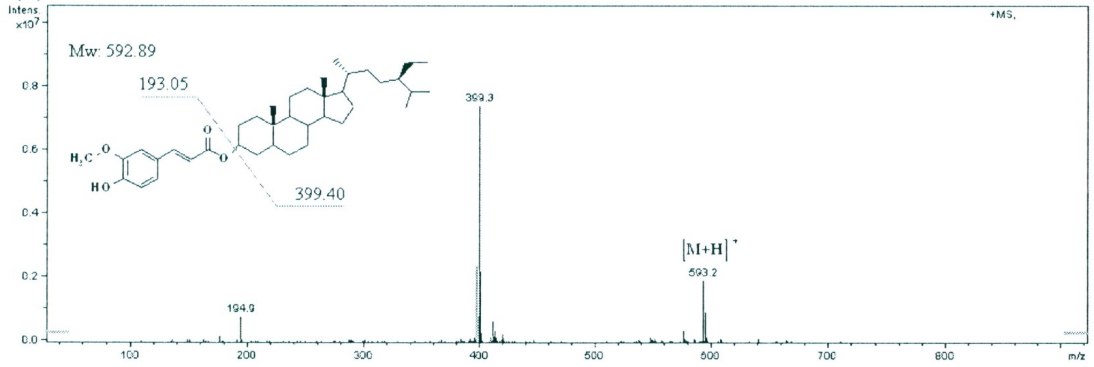
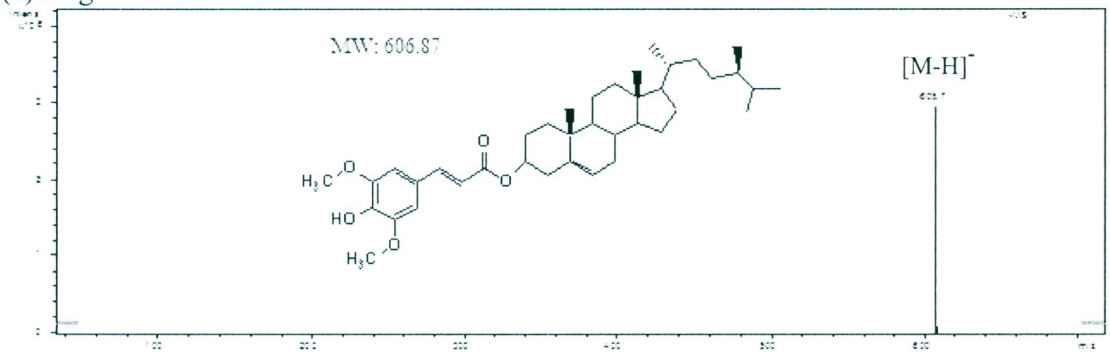
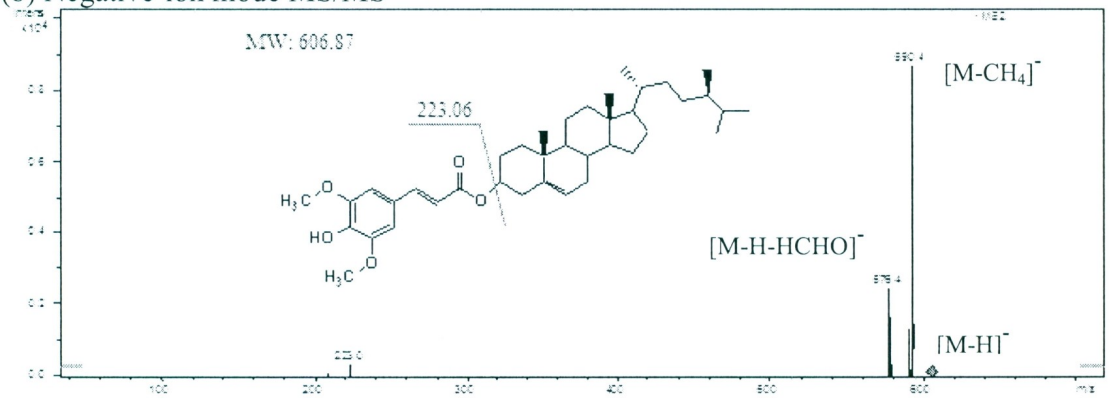


Figure 5-17. Mass spectra of sitostanyl ferulate (compound 12), (a) negative-ion mode; (b) positive-ion mode.

(a) Negative-ion mode



(b) Negative-ion mode MS/MS



(c) Positive-ion mode

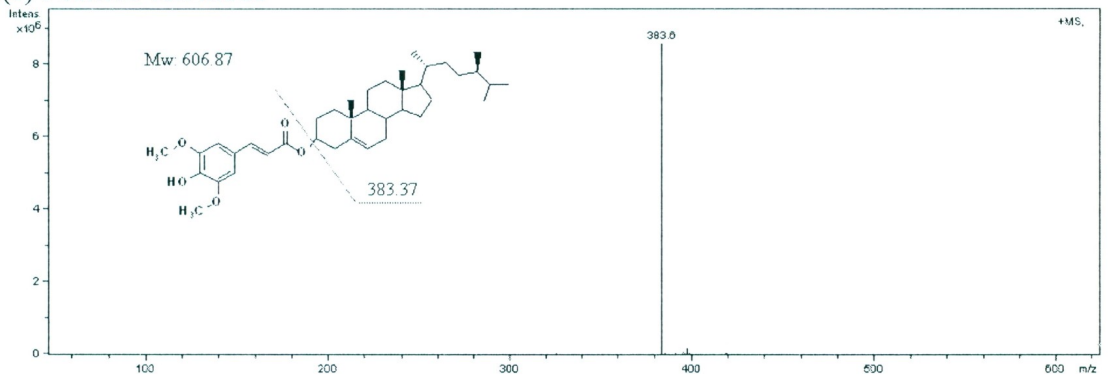
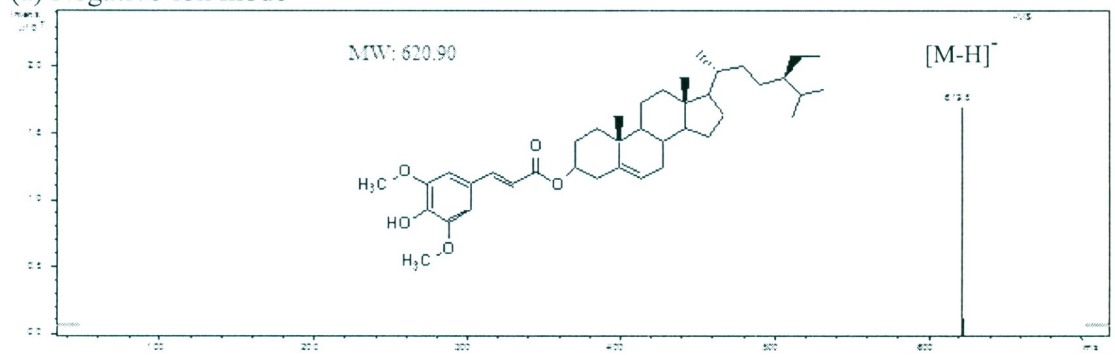
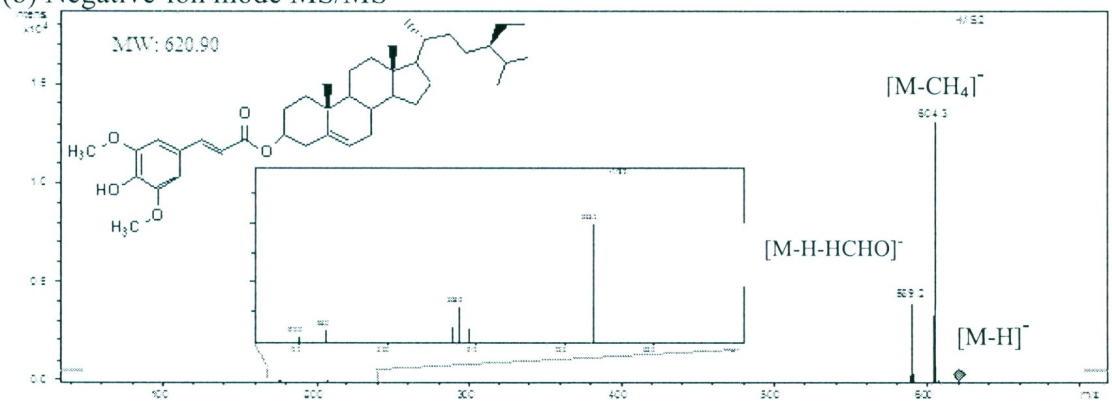


Figure 5-18. Mass spectra of campesteryl sinapate (compound 13), (a) negative-ion mode; (b) negative-ion mode MS/MS; (c) positive-ion mode.

(a) Negative-ion mode



(b) Negative-ion mode MS/MS



(c) Positive-ion mode

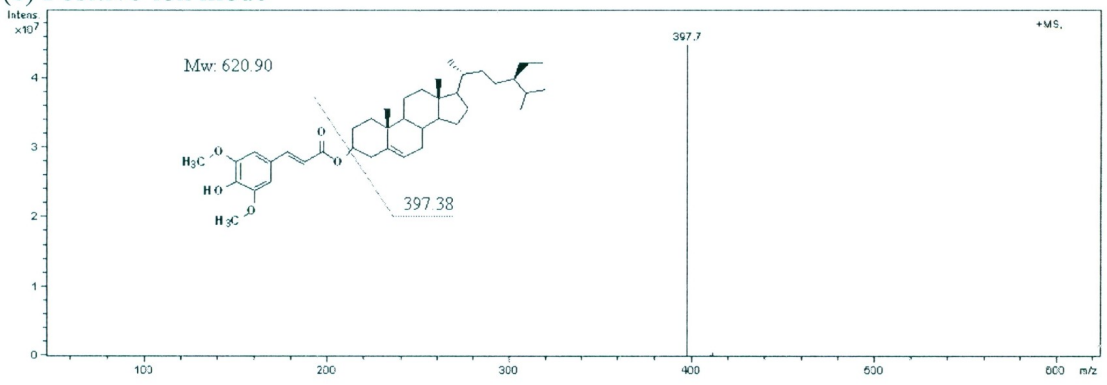
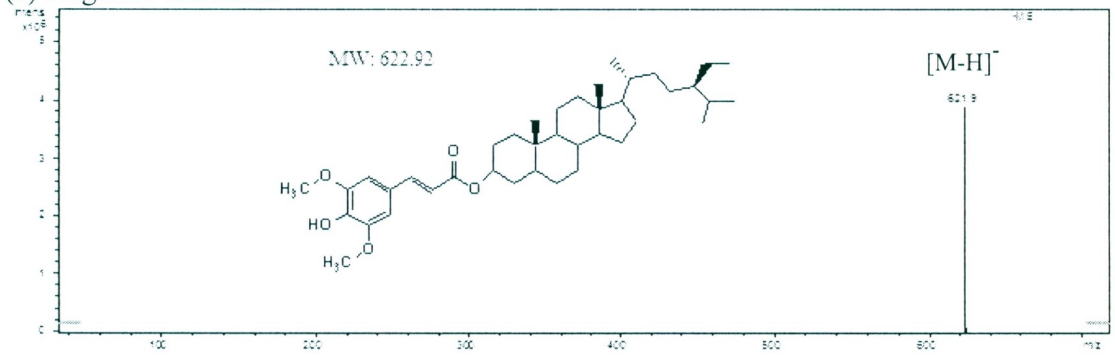
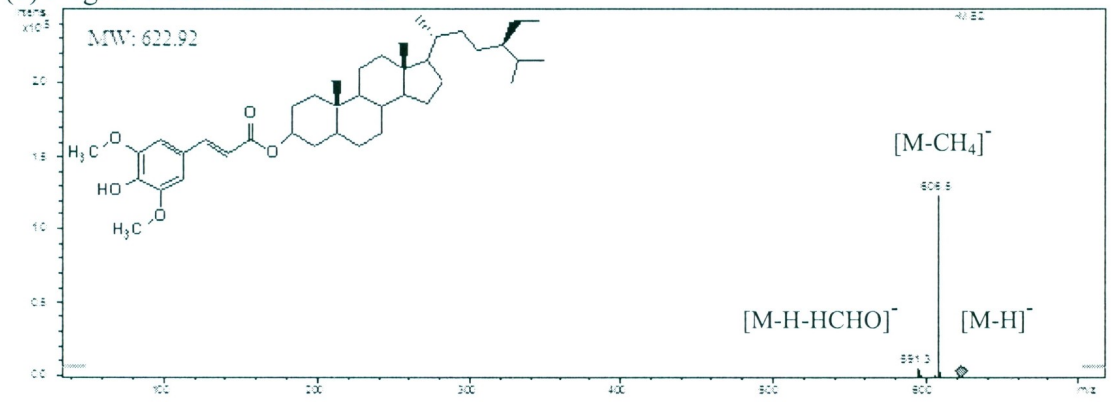


Figure 5-19. Mass spectra of sitosteryl sinapate (compound 14), (a) negative-ion mode; (b) negative-ion mode MS/MS; (c) positive-ion mode.

(a) Negative-ion mode



(b) Negative-ion mode MS/MS



(c) Positive-ion mode

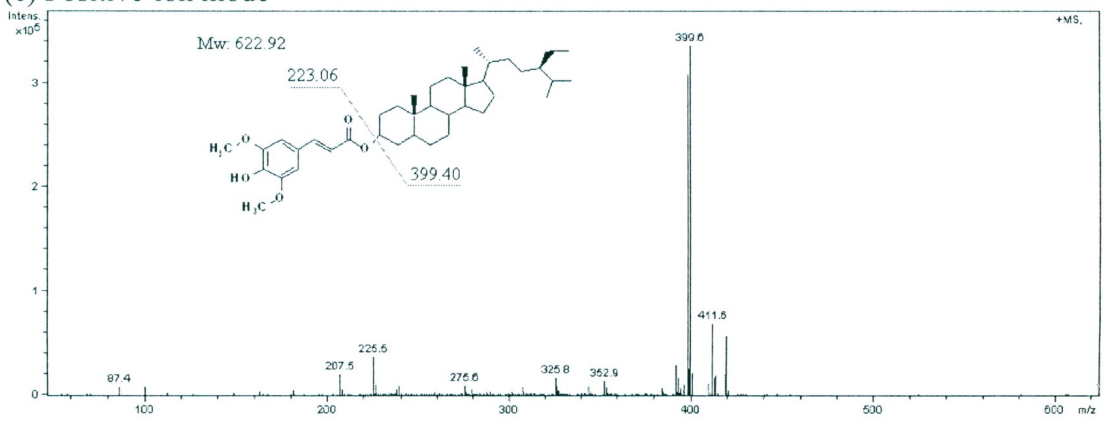
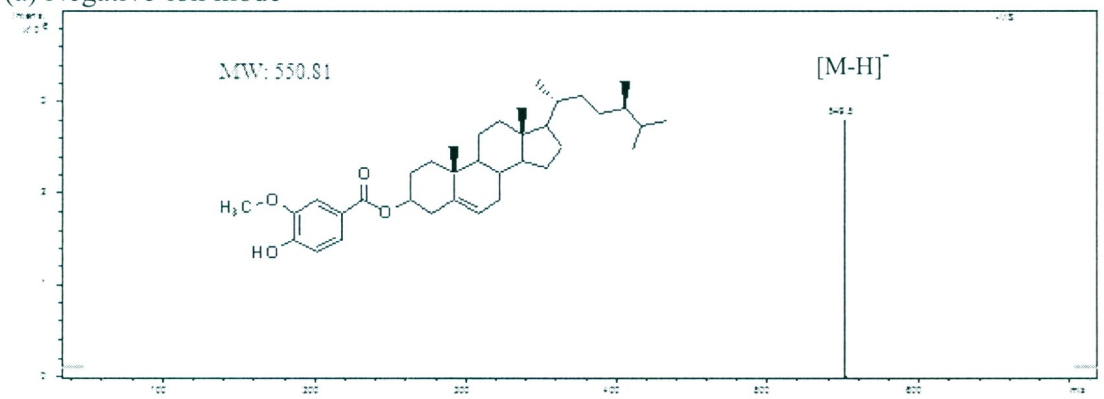
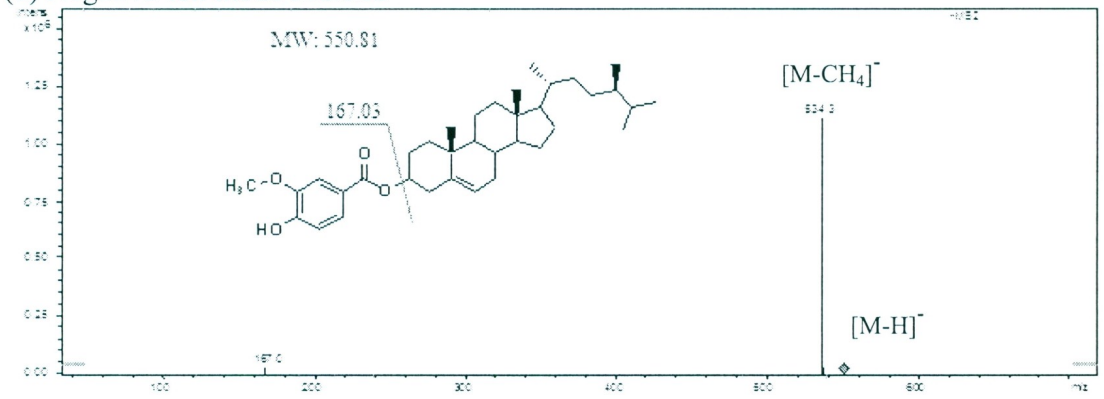


Figure 5-20. Mass spectra of sitostanyl sinapate (compound 15), (a) negative-ion mode; (b) negative-ion mode MS/MS; (c) positive-ion mode.

(a) Negative-ion mode



(b) Negative-ion mode MS/MS



(c) Positive-ion mode

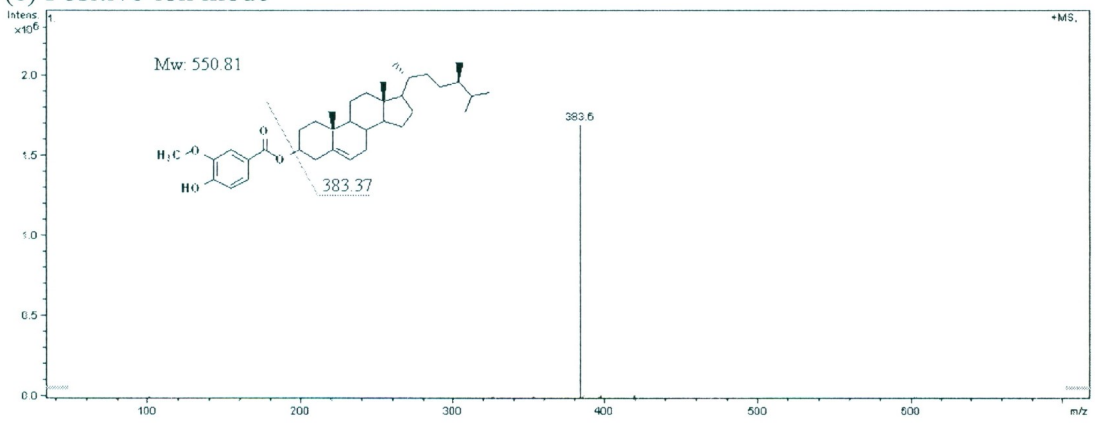
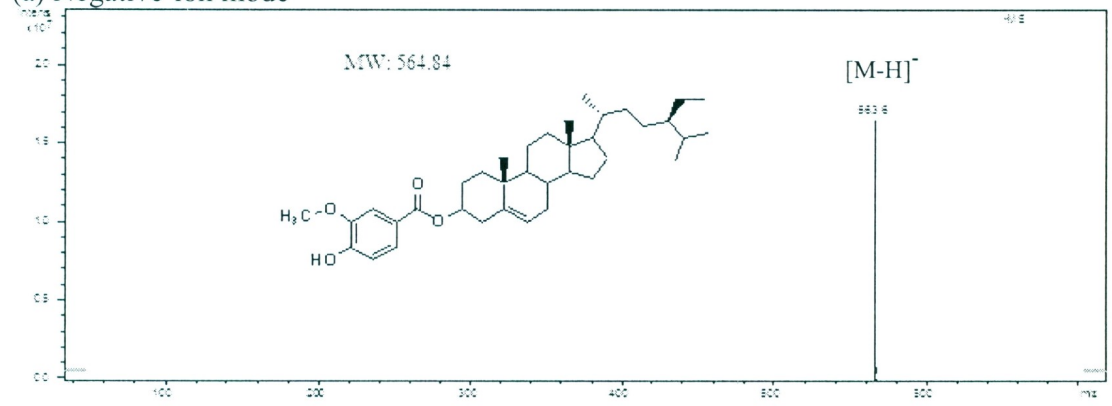
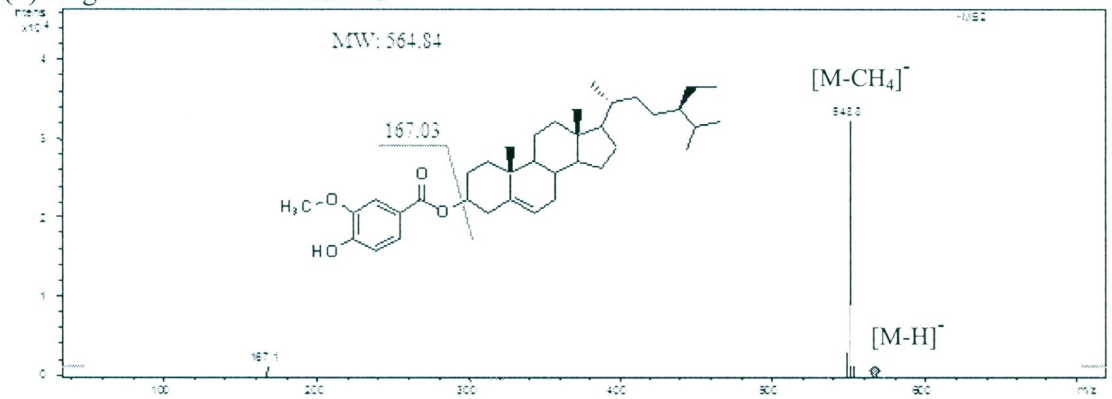


Figure 5-21. Mass spectra of campesteryl vanillate (compound 16), (a) negative-ion mode; (b) negative-ion mode MS/MS; (c) positive-ion mode.

(a) Negative-ion mode



(b) Negative-ion mode MS/MS



(c) Positive-ion mode

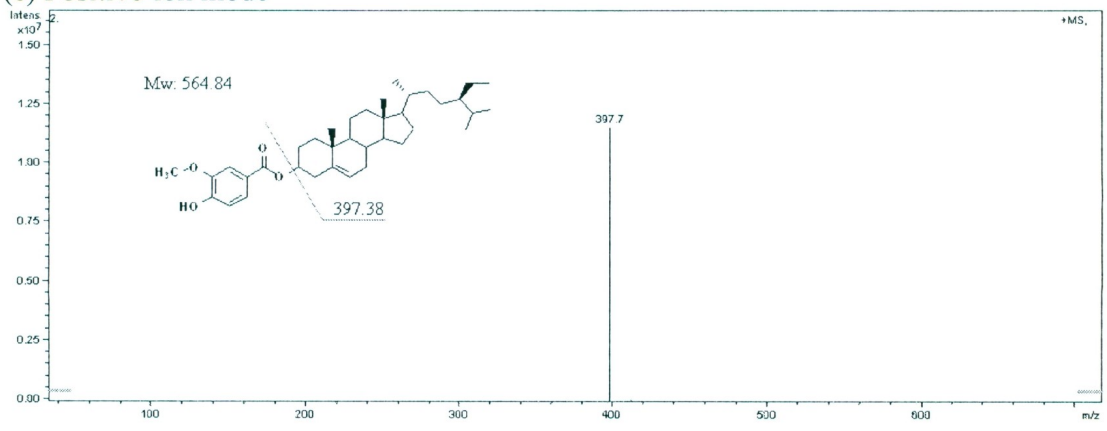
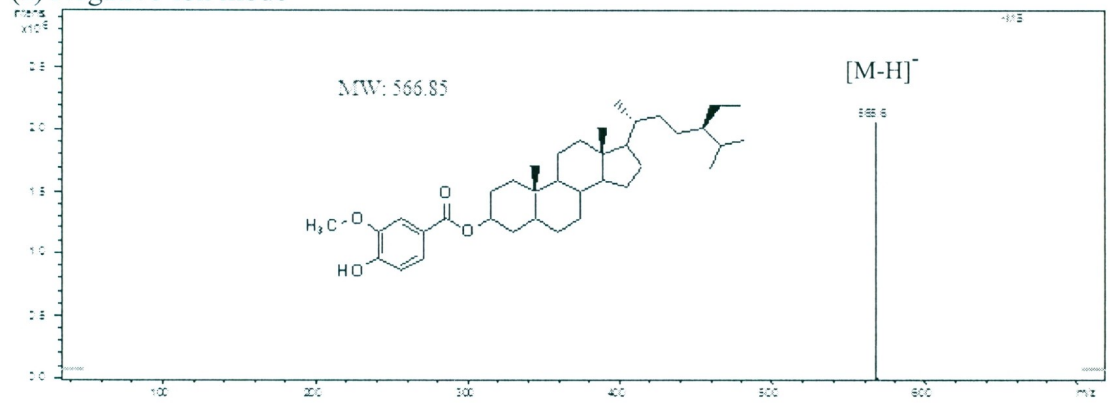
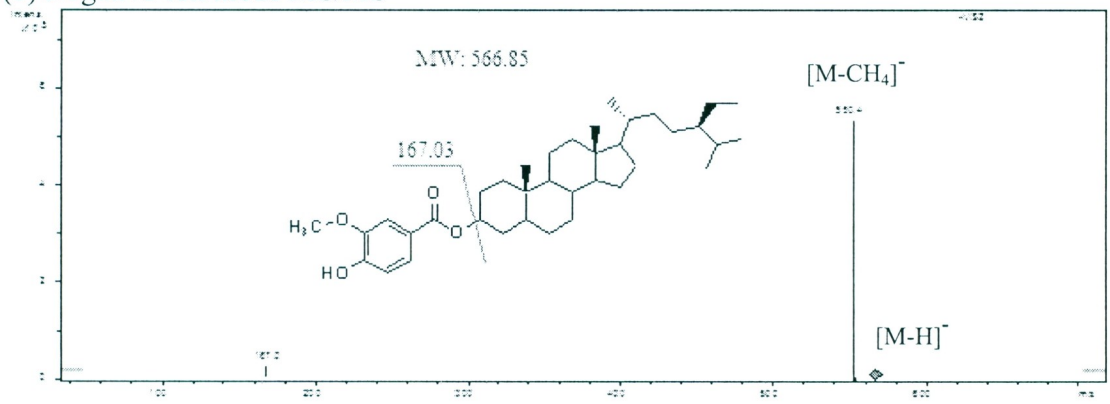


Figure 5-22. Mass spectra of sitosteryl vanillate (compound 17), (a) negative-ion mode; (b) negative-ion mode MS/MS; (c) positive-ion mode.

(a) Negative-ion mode



(b) Negative-ion mode MS/MS



(c) Positive-ion mode

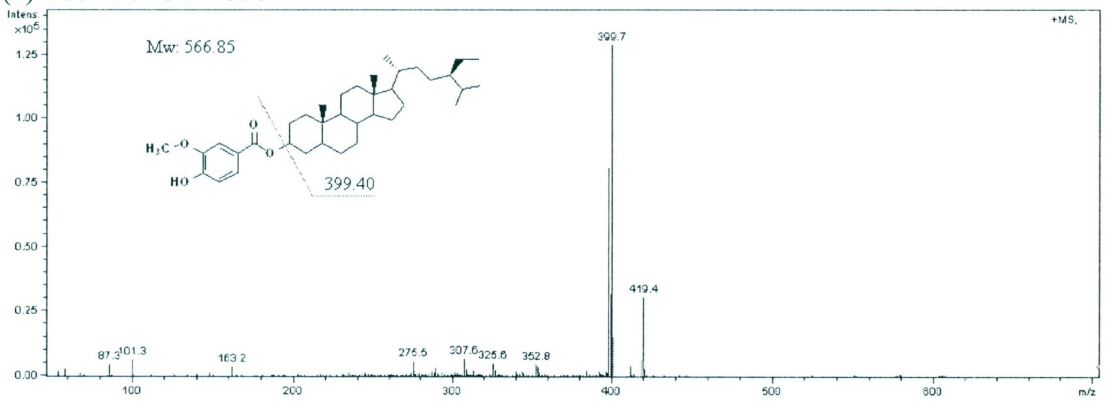


Figure 5-23. Mass spectra of sitostanyl vanillate (compound 18), (a) negative-ion mode; (b) negative-ion mode MS/MS; (c) positive-ion mode.

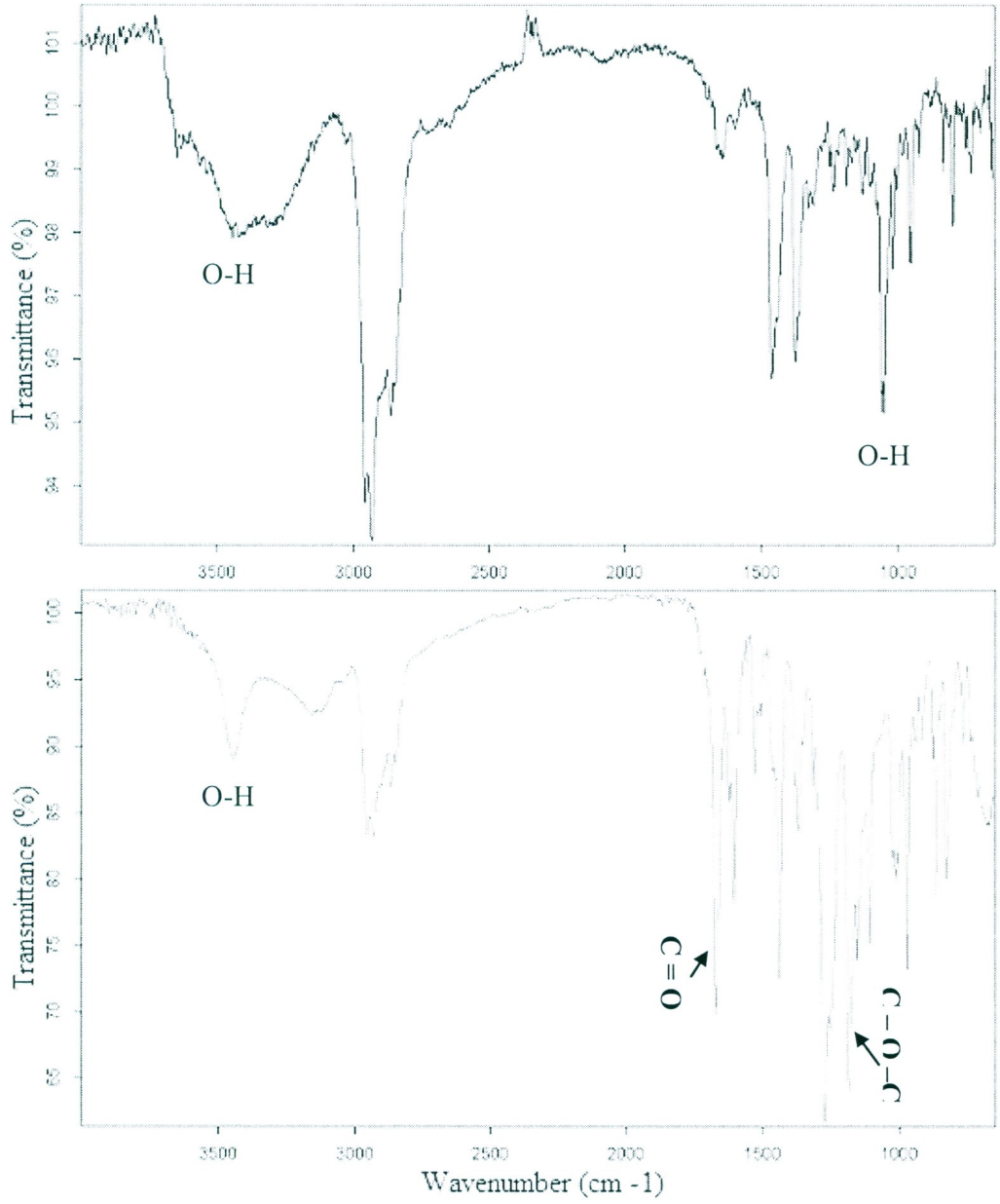


Figure 5-24. IR spectra of free phytosterols (top) and phytosteryl caffeates (bottom).

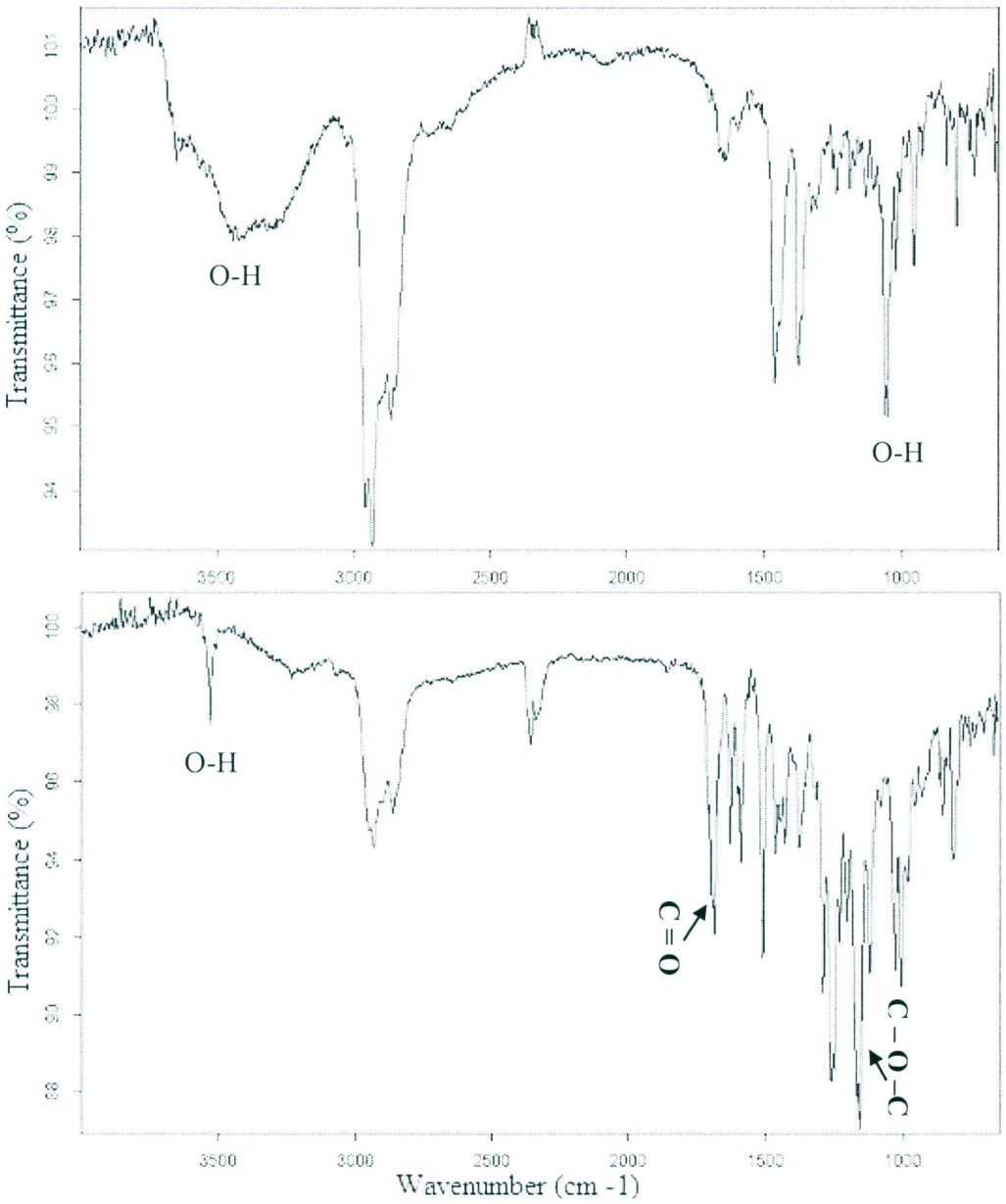


Figure 5-25. IR spectra of free phytosterols (top) and phytosteryl ferulates (bottom).

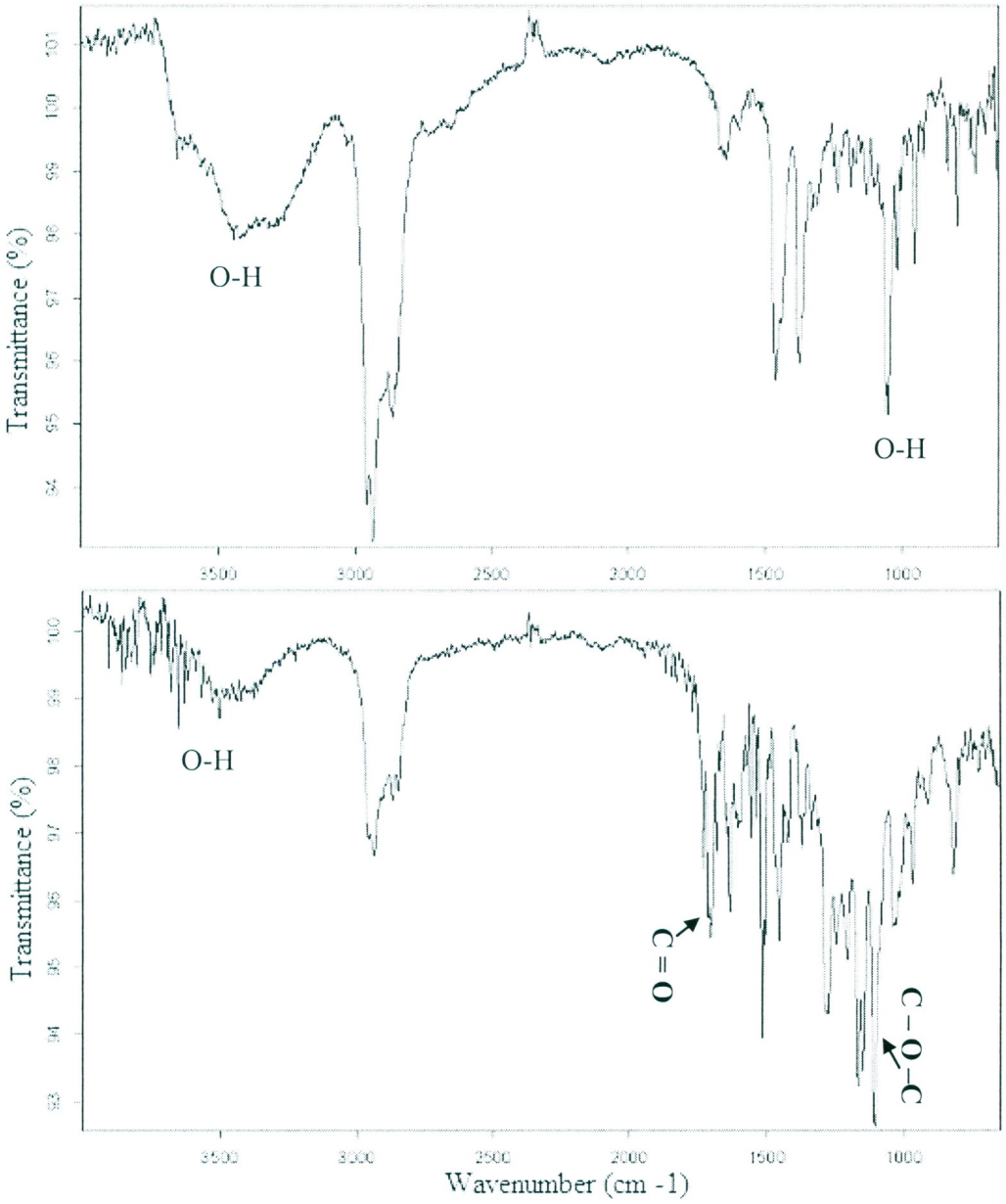


Figure 5-26. IR spectra of free phytosterols (top) and phytosteryl sinapates (bottom).

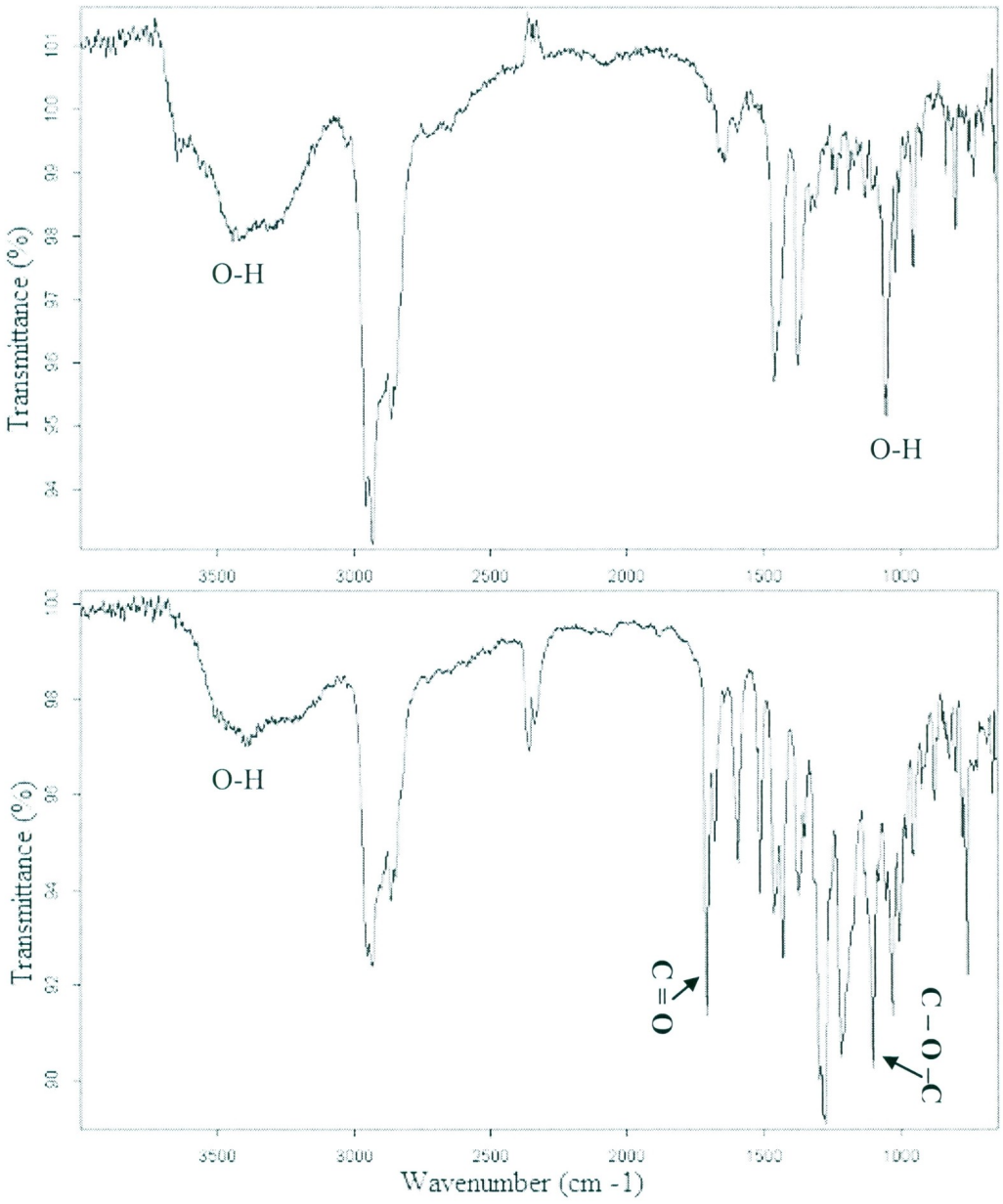


Figure 5-27. IR spectra of free phytosterols (top) and phytosteryl vanillates (bottom).

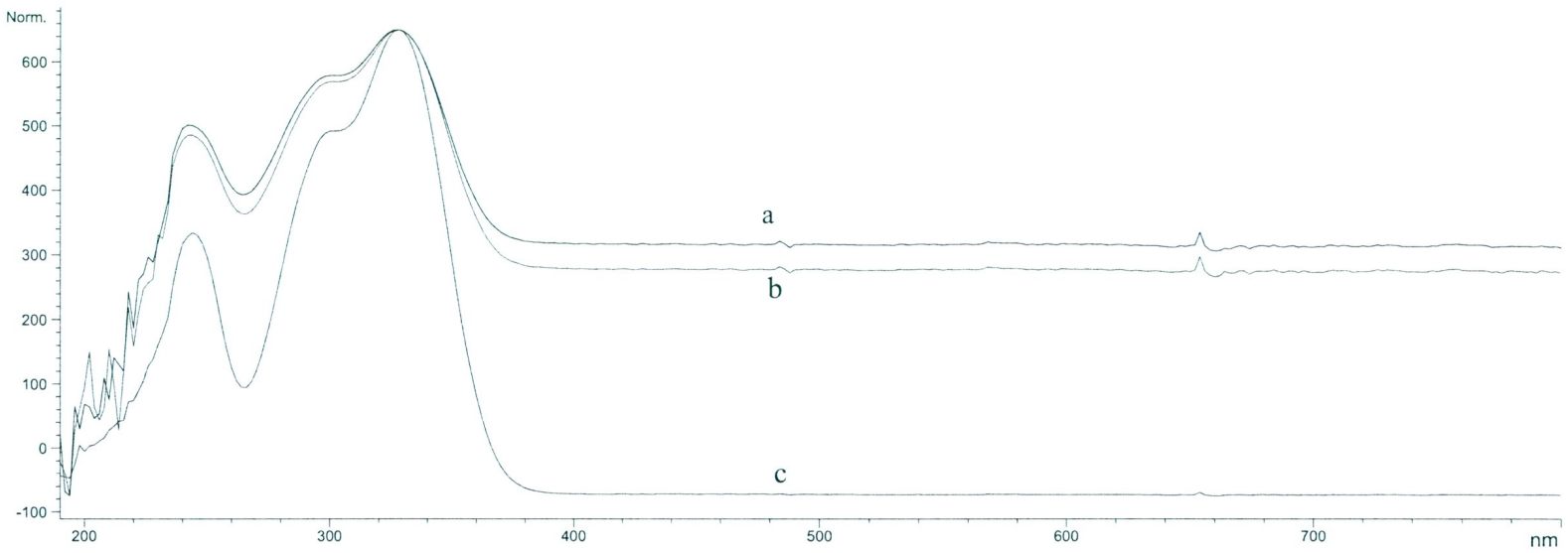


Figure 5-28. Ultraviolet spectra of HPLC peaks for phytosteryl caffeates (a: sitostanyl caffeate; b: campesteryl caffeate; c: sitosteryl caffeate). Spectra taken by diode array detector (DAD) during HPLC separation.

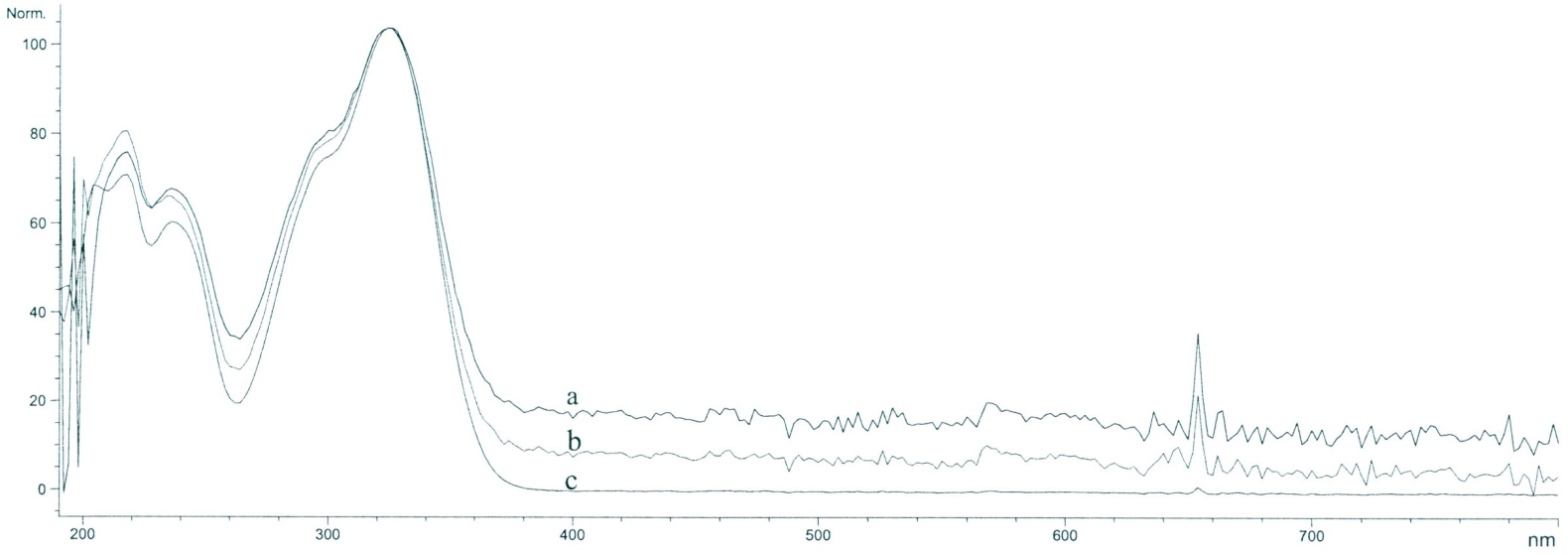


Figure 5-29. Ultraviolet spectra of HPLC peaks for the phytosteryl ferulates (a: campesteryl ferulate; b: sitostanyl ferulate; c: sitosteryl ferulate). Spectra taken by diode array detector (DAD) during HPLC separation.

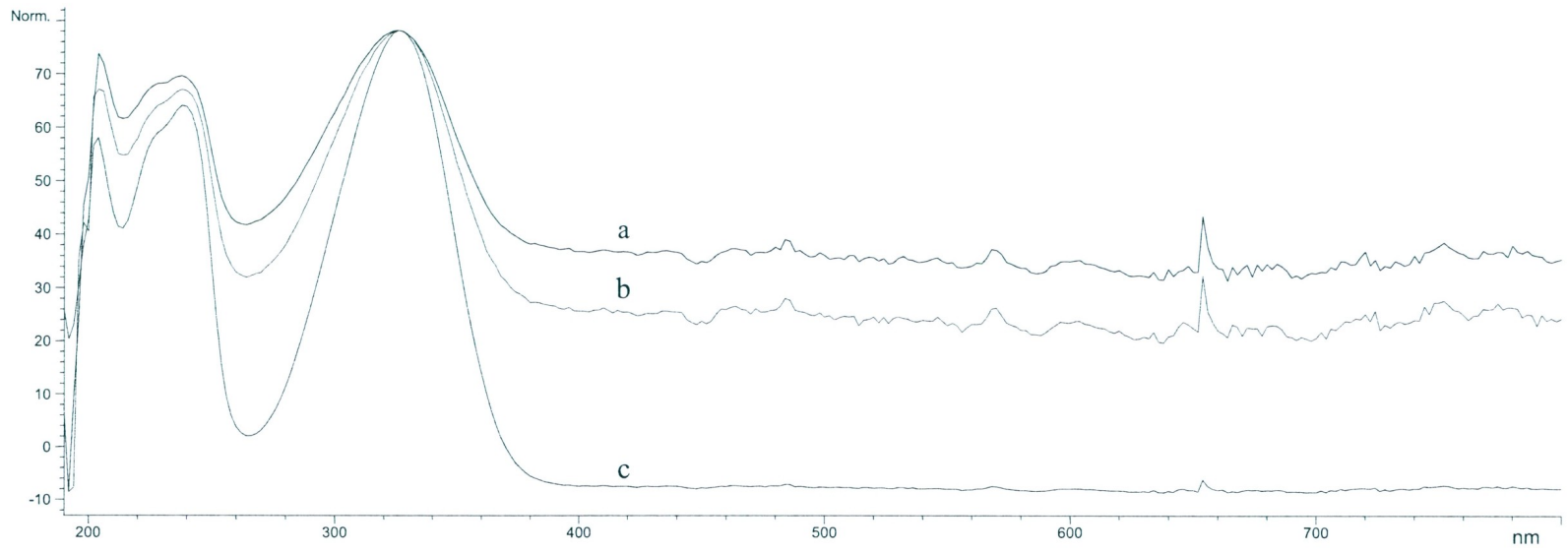


Figure 5-30. Ultraviolet spectra of HPLC peaks for the phytosteryl sinapates (a: sitostanyl sinapate: b: campesteryl sinapate; c: sitosteryl sinapate). Spectra taken by diode array detector (DAD) during HPLC separation.

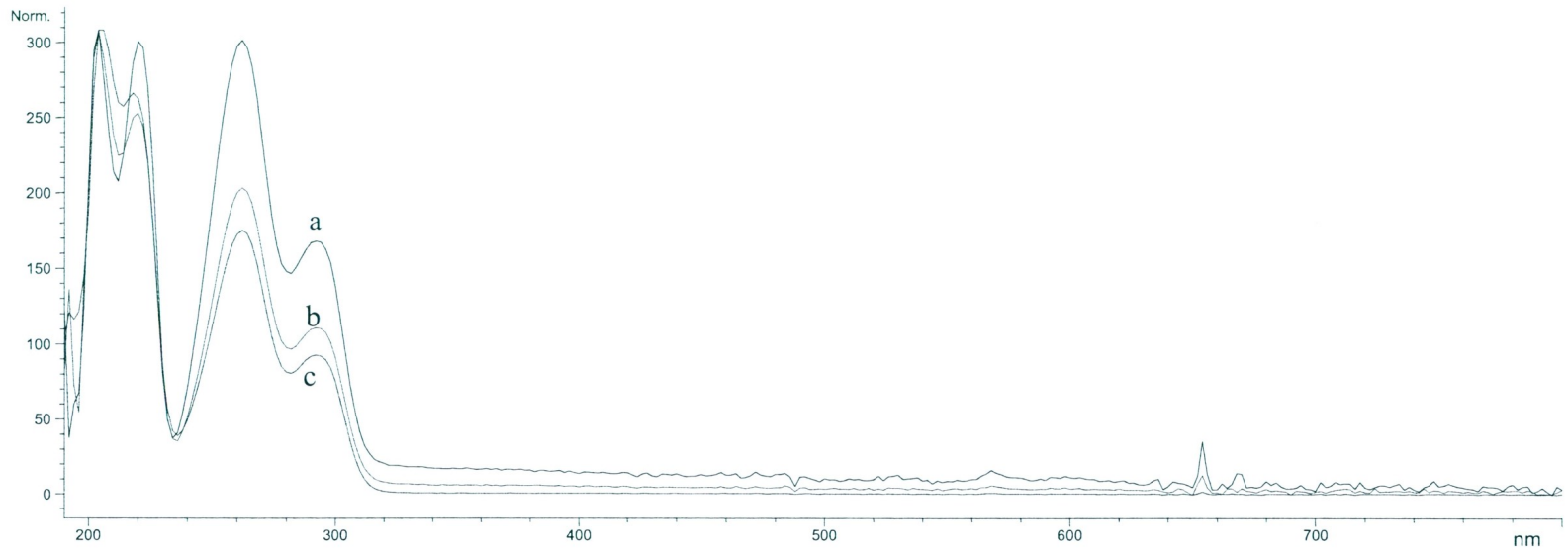


Figure 5-31. Ultraviolet spectra of HPLC peaks for the phytosteryl vanillates (a: sitostanyl vanillate; b: campesteryl vanillate; c: sitosteryl vanillate). Spectra taken by diode array detector (DAD) during HPLC separation.

Chapter 6

Evaluation the Antioxidant Activity of Phytosteryl Phenolates

6.1 Introduction

The effectiveness of antioxidants is influenced by a number of factors, including their structural features, composition, concentration, temperature, oxygen pressure, type of oxidation substrate, solubility, and presence of pro-oxidants and synergists (Burton and Ingold, 1986; Yanishlieva-Maslarova, 2001). As far as specific molecular properties are concerned, the bond dissociation energy (BDE) and the ionization potential (IP) are of particular importance (Wright et al., 2001). Other factors may also play a role in determining the effectiveness of an antioxidant including the presence of bulky groups in the *ortho* postion of the OH group of phenolic compounds (Pedulli et al., 1997) and hydrogen bonding characteristics of the solvent (Valgimigli et al., 1996; Barclay et al., 1999).

In this study, the lipophilicity of the water-soluble phenolic acids was increased by esterification with free phytosterols. The remaining hydroxyl groups on the aromatic rings of the phenolic acids as hydrogen donors may contribute to the antioxidant properties of these moderately lipophilic derivatives, although alterations in their antioxidant efficacy are expected due to changes in their physical and chemical characteristics. To evaluate the antioxidant activity of phytosteryl phenolates so produced,

they were subjected to different antioxidant activity assays. These assays were also used for evaluation of the antioxidant activity of the starting phenolic acids and intermediate vinyl phenolates. The assays employed included DPPH radical scavenging capacity test, $ORAC_{FL}$ assay, bulk oil model system using the Rancimat test, β-carotene-linoleate model system, cooked pork model system, reducing power test, LDL cholesterol oxidation and DNA scission assays.

6.2 DPPH radical scavenging capacity test using electron paramagnetic resonance (EPR) spectrometry

As the main mechanism of action of phenolic antioxidants is considered to be the scavenging of free radicals (Frankel, 1998), antiradical capacity of the synthesized phytosteryl phenolates was initially evaluated as scavenging capacity against DPPH radical, a stable hydrophobic radical frequently used in antioxidant activity assessments. The phytosteryl phenolates synthesized in this study had one or two hydroxyl groups on the aromatic ring of phenolic acids. These hydroxyl groups may contribute to the antiradical property of the phytosteryl phenolates. The sample EPR spectra of DPPH radical as affected by caffeic acid and their derivatives are shown in Figure 6-1. The tested compounds significantly decreased the signal intensity, indicating the scavenging effect of phytosteryl caffeates against DPPH radical. Compounds so tested exhibited antiradical properties to varying degrees as indicated in Figure 6-1.

When comparing the radical scavenging capacity of phenolic acids, caffeic acid showed the highest capacity as trolox equivalents, followed by sinapic acid, ferulic acid

and vanillic acid derivatives (Figure 6-1). The same trend of DPPH radical scavenging capacity for three phenolic acids with the order of sinapic acid > ferulic acid > vanillic acid, was also reported by Shimoji et al. (2002). Studies on cinnamic acids and derivatives have pointed out the importance of the catechol moiety to the antiradical efficacy of the compound of interest (Moon and Terao, 1998). The antiradical property of phenolic acids and their esters depended on the number of hydroxyl groups in the molecule (Rice-Evans et al., 1996). The two hydroxyl groups in the caffeic acid may attribute to their higher antiradical capacity for scavenging DPPH radicals when compared with other phenolic acids. The higher antiradical capacity of sinapic acid compared with ferulic acid may be due to one more *ortho* substituted methoxy group with electron-donating capability, which increases the stability of the aryloxyl radical and thus its antioxidant potential (Chimi et al., 1991). Cuvelier et al. (1992) reported that the presence of the -CH=CH-COOH group in cinnamic acid ensures greater H-donating ability and subsequent radical stabilization than the carboxylic acid group in benzoic acids. This explains why vanillic acid had the lowest antiradical capacity in this assay. The same trend of the antiradical capacity for their corresponding vinyl esters and phytosteryl esters was observed, for example both vinyl caffeate and phytosteryl caffeates had the highest antioxidant activity while their vanillate counterparts had the lowest efficacies.

The structure modification of the carboxyl group of phenolic acids through esterification with phytosterols affected their antiradical activity in a different way. Esterification of caffeic acid and ferulic acid with phytosterols significantly improved

their radical scavenging capacity. However, there was no significant difference (α=0.05) between the antiradical capacity of vanillic acid and its corresponding vinyl ester and phytosteryl ester; however, when the same process was applied to sinapic acid, the antiradical capacity of its corresponding vinyl ester and phytosteryl esters decreased. The different effect of the esterification process on the antiradical capacity of phytosteryl phenolates may be due to the different influence of the bond dissociation energy (BDE) and the ionization potential (IP) of the synthesized phytosteryl phenolates, which in turn will alter their hydrogen atom donation capacity by changing its electron distribution and density on the aromatic rings. The enhanced antiradical capacity of phytosteryl caffeates and phytosteryl ferulates may also be explained by their increased hydrophobicity which enables them to be better accessible to the hydrophobic DPPH radical than their hydrophilic parent phenolic acids.

The maintaining or enhancing of the antiradical capacity of phytosteryl phenolates suggests that these derivatives may be used as antioxidants in more liphophilic systems. As one of the main constituents of γ-oryzanol, phytosteryl phenolates, including sitosteryl ferulate and campesteryl ferulate, have been reported to possess high free radical scavenging capacity (Akiyama et al., 2001). In our study, vinyl caffeate and phytosteryl caffeates are the only two groups of compounds that showed higher radical scavenging capacity than the reference trolox. Table 6-1 also shows that the antiradical capacity of phytosteryl caffeates was five times higher than that of phytosteryl ferulates, which indicates that they may potentially be used as functional food ingredients or nutraceuticals due to their improved antiradical capacity.

Further investigation is needed to understand the mechanisms of action of different antiradical activity of the synthesized phytosteryl phenolates and explore their potential application in functional food and nutraceutical industries. For example, it is important to monitor changes in the bond dissociation energy (BDE) and the ionization potential (IP) for phenolic acids and their corresponding phytosteryl esters as well as to determine of the antioxidant efficacy of phytosteryl phenolates in other model systems.

6.3 Oxygen radical absorbance capacity (ORAC) assay

As discussed above, some of the synthesized phytosteryl phenolates were effective in scavenging DDPH radicals, however, the antioxidant activity obtained from DPPH scavenging assay may not necessarily reflect the real situation in food and biological systems as DPPH radical is a stable artificial radical. In order to have better understanding of their free radical scavenging capacity in biological environments, phytosteryl phenolates together with the starting materials and intermediates were evaluated for their ability to scavenge peroxyl radicals, measured as oxygen radical absorbance capacity (ORAC). The ORAC assay utilizes a biologically relevant radical source and has been established as a standard method for assessing the activity of hydrophilic antioxidants. In this study, a modified ORAC method for lipophilic antioxidants, proposed by Huang et al. (2002), was adapted, which introduces acetone/water (1:1, v/v) as the solvent and randomly methylated β-cyclodextrin (RMCD) as a solubility enhancer.

ORAC values, expressed as μmol trolox equivalents/ μmol of sample, are given in Figure 6-2. Sinapic acid and ferulic acid showed the highest ORAC values, followed by caffeic acid and vanillic acid. As for the phytosteryl phenolates, phytosteryl sinapates showed the highest antioxidant activity which was 20 times that of trolox, followed by phytosteryl ferulates, phytosteryl caffeates and phytosteryl vanillates. Although phytosteryl vanillates had the lowest activity of all four phytosteryl phenolates, their antioxidant activities were ten times higher than that of trolox.

The esterification process had a positive effect on all phytosteryl phenolates as there were significant differences between all starting materials and their corresponding phytosteryl esters. The ORAC values of all four types of phytosteryl phenolates were two times higher than that of their corresponding starting phenolic acids, which indicate their greater hydrogen atom donating ability under the test conditions employed.

6.4 Antioxidant activity in bulk oil model system

Antioxidant activity is also affected by system environment. For example, the same antioxidants showed different activities in bulk oil and oil-water emulsion (Porter 1989). A bulk oil model system was employed to investigate the effect of structure modification on the antioxidant activity of phytosteryl phenolates.

A stripped corn oil devoid of endogenous antioxidants was employed for assessing antioxidant efficiency of phytosteryl phenolates. The bulk oil was heated under accelerated conditions and lipid oxidation was monitored (Perkins, 1992). Volatile organic acids are formed and detected in the Rancimat method as an indicator of

oxidation. Induction time (IT) is used as an important parameter in measuring lipid oxidation in the Rancimat method. It is defined as the time to reach a sudden increase in oxidation rate after a slow initial increase. The changes were monitored by following electrical conductivity caused by the formation of oxidation products. Antioxidant activity of test compounds was expressed as protection factor, which is defined as ratio of IT of the oil with added antioxidants to that of the oil devoid of any additives, as explained in Section 3.2.3.3.

The protection factors of phenolic acids and their derivatives are shown in Figure 6-3. On the basis of polar paradox theory (see below), it was expected that the most potent antioxidant be the most polar one. According to the R_f values in Table 5-1, caffeic acid and its derivatives are the most polar compounds in all groups of the tested compounds, thus caffeic acid and its derivatives had the highest antioxidant activity with a protection factor of 1.5-2 among all four groups of compounds tested. The other three groups of compounds with protection factor of $\approx$ 1 showed a much less effect on oxidative stability of the oil. Vanillic acid and ferulic acid as well as their phytosteryl esters displayed a slight pro-oxidative effect under test conditions employed as their protection factor was less than 1. The esterification process exerted a negative effect on most phytosteryl phenolates, but phytosteryl vanillates appeared to enhance the antioxidant activity of the intermediate products-vinyl phenolates. This may due to the moderate liophobicity of these intermediate compounds.

Except for the number of hydroxyl groups, differences in antioxidant activity of phenolic compounds in bulk oil are usually related to their polarity (Porter et al., 1989). A

higher protection factor of caffeic acid than its esters, i.e, phytosteryl caffeates, may be explained by the interfacial phenomenon of hydrophilic antioxidants in bulk oil. The partially soluble hydrophilic antioxidants tend to be oriented in the air-oil interface where surface oxidation occurs, and therefore protect the system from oxidative changes. The interfacial phenomenon also lends support to the antioxidant "polar paradox" theory that in food systems of low surface-to-volume ratio (e.g. bulk oils) which states that polar antioxidants with high hydrophilic-lipophilic balance (HLB) are more effective than nonpolar lipophilic antioxidants (Frankel et al., 1994; Shahidi and Zhong, 2011).

Although phenolic acids may protect the oil from oxidation better than their corresponding esters, their poor solubility remains a problem since they exist as a suspension in the oil and the appearance of oil products is an important parameter for quality judgment. The phytosteryl phenolates, especially phytosteryl caffeates, with better fat-solubility may still serve as a potential antioxidant in bulk oil systems.

6.5 Antioxidant activity in *β*-carotene-linoleate model system

Antioxidants behave differently when used in different media. Hence, their efficacy in bulk oil may not necessarily reflect that in oil-in-water emulsions. Moreover, it has been recognized that high surface-to-volume ratio emulsions are the natural conditions, while low surface-to-volume bulk lipid is more like an artifact that is less common in foods and biological systems as food matrices are usually multicomponent systems. Therefore, the effectiveness of phytosteryl phenolates in oil-in-water emulsions was also monitored for a more comprehensive assessment of their antioxidant activity.

In this work, the antioxidant activity of phenolic acids and their lipophilic phytosteryl phenolates in oil-in-water emulsion was determined using a β-carotene/linoleic acid emulsion system. Their antioxidant activity was detected by monitoring the bleaching of β-carotene as affected by the test antioxidant compounds. The decolouration of β-carotene is a free radical-mediated phenomenon resulting from oxidation of linoleic acid in the emulsion system, which gives rise to the formation of free radicals and hydroperoxides. The presence of antioxidants can minimize the loss of β-carotene during the coupled oxidation of linoleic acid and β-carotene in the emulsified aqueous system. Under test conditions employed, the antioxidant activity of phenolic acids followed the order of caffeic acid > sinapic acid = ferulic acid > vanillic acid. The better antioxidant performance of caffeic acid compared to ferulic acid in a dispersed system has also been reported (Cuvelier et al., 2000). A similar trend was also observed for vinyl and phytosteryl ester of caffeic acid. The results (Figure 6-4) indicate that phytosteryl phenolates, except phytosteryl ferulates, showed better inhibition of bleaching of β-carotene than their starting phenolic acids counterparts as well as intermediate vinyl esters. Phytosteryl caffeates and sinapates had the highest antioxidant activity with 55-57% inhibition over 100 min incubation, followed by phytosteryl ferulates (20%) and phytosteryl vanillates (2.46%). Thus, the esterification process positively affected the antioxidant activity of the modified phytosteryl phenolates, except for phytosteryl ferulates. The negative value of vanillic acid and vinyl vanillate means that they acted as pro-oxidant under test conditions employed. This is in agreement with the previous findings reported in the ORAC assay for antioxidant activity of vanillic acid.

Esterification of vanillic acid to phytosteryl vanillate successfully changed it from being a pro-oxidant to one having a moderate antioxidant activity. Lower rates of absorbance decay were observed for emulsions containing test compounds than that seen for the control except for vinyl vanillate (Figure 6-5).

Among the various factors affecting the ultimate performance of a phenolic compound in a dispersed system is phase partitioning (McClements and Decker, 2000). In the oil-in-water emulsion system, hydrophilic antioxidants such as phenolic acid tend to move to the water phase and hence provide less protection to the oil. The lipophilic phytosteryl phenolates, in contrast, are more soluble in the oil phase, or oriented in the oil-water interface due to the presence of both hydrophobic aliphatic side chains and hydrophilic hydroxyl groups in their molecules, thus showing higher antioxidant activity than their hydrophilic phenolic acid counterparts.

6.6 Antioxidant activity in cooked ground meat model system

Lipids may also be responsible to for quality deterioration of processed whole tissue foods. These foods generally require a larger amount and different types of antioxidants due to their predisposition to oxidation during precooking and long storage time under high surface-to-volume conditions (Porter, 1993; Shahidi et al., 1986). Cooked ground muscle foods provide an excellent model for assessing the effectiveness of antioxidants in thermally processed whole or modified tissue foods, where heating causes rapid oxidation of lipid and development of "warmed-over flavour" (Shahidi et al., 1987).

Antioxidant activity of phenolic acids and their derivatives has previously been evaluated in a muscle food model system. Phenolic acids inhibited lipid oxidation in cooked fish (mackerel) meat with a higher efficacy than that of commonly used synthetic antioxidant tert-butylhydroquinone (TBHQ) (Ramanathan and Das, 1992). In a cooked pork model system, phenolic acids exhibited a higher antioxidant activity than typical food antioxidants BHT (butylated hydroxytoluene) and α-tocopherol (Shahidi and Alexander, 1998).

In the work reported here, the antioxidant activity of phytosteryl phenolates was determined in a cooked pork model system by monitoring the formation of thiobarbituric acid reactive substances (TBARS) as affected by test compounds and butylated hydroxyanisole (BHA) as a reference antioxidant. During storage at 4°C, all meat samples showed increased content of TBARS with time as a result of lipid oxidation (Figure 6-6). The samples with added phytosteryl phenolates had lower TBARS values than the control over the entire storage period. The antioxidants added to fresh meat exerted inhibitory effect against oxidation during cooking of the meat prior to storage, which explains the higher TBARS value in the control sample on day 0. The antioxidant activity of phytosteryl phenolates followed the trend BHA > phytosteryl caffeates ≈ phytosteryl sinapates > phytosteryl ferulates > phytosteryl vanillates > free phytosterols. The lower TBARS value of free phytosterols indicates their lower antioxidant activity in the model system employed.

6.7 Reducing power test

In addition to hydrogen atom donation, antioxidants may also inhibit oxidation through single electron transfer. Antioxidants can deactivate a free radical or reduce an oxidant by donating an electron and forming an antioxidant radical cation, followed by rapid and reversible deprotonation (Wright et al., 2001). The antioxidant radical formed is then stabilized by electron delocalization (resonance), as in hydrogen atom donation mechanism. Although the net result of electron transfer is the same as the hydrogen atom transfer pathway, the ability of an antioxidant to donate an electron or a hydrogen atom may vary depending on both intrinsic and extrinsic factors. The ability of an antioxidant to act as an electron donor, or its reducing power, is determined by the ionization potential (IP) of the compound and in strongly solvent-dependent (Wright et al., 2001).

The reducing power of phytosteryl phenolates was measured as Fe^{3+}-Fe^{2+} transformation mediated by test compounds and expressed as ascorbic acid equivalents; ascorbic acid is a known reducing agent. Solubility also affects the effectiveness of antioxidants, especially their reducing power since electron transfer mechanism is strongly solvent dependent due to solvent stabilization of charged species (Wright et al., 2001). Phytosteryl phenolates with enhanced lipophilicity have poor solubility in aqueous media and hence compromised activity under the hydrophilic test environment. Similar changes in reducing power caused by acylation have been found for rutin esters (Lue et al., 2010).

Only caffeic acid, sinapic acid and their vinyl ester counterparts exhibited 1.5-2 times greater reducing power than ascorbic acid. All other tested compounds showed either equal or lower reducing power than ascorbic acid. The esterification of phenolic

acids into their vinyl esters and phytosteryl phenolates dramatically decreased their reducing power. The phytosteryl caffeates possessed the highest reducing power among all four groups of phytosteryl phenolates, however, it only had half of the reducing power of caffeic acid. This disagrees with the results from ORAC assay reported above, and may arise from different mechanisms involved as well as the existing differences in the test environment in the two assays. As already mentioned, the electron donating ability of antioxidants is dependent on their IP, which may be altered by structure modification. Esterification of phenolic acids with phytosterols may change not only their BDE, but also their IP. The presence of electron-donating substituents can stabilize the phenoxyl radical cation and hence lowering IP and enhancing electron donating ability. In the contrast, electron-withdrawing substituents such as esters increase IP and decrease the reducing power of the antioxidant (Wright et al., 2001). As a result, phytosteryl phenolates with ester side chains showed lower reducing power than the original molecule. The opposite trend was observed for ORAC, possibly because the effect of the electron-withdrawing side chain on hydrogen atom donation was counteracted by other factors, such as hydrogen bonding and steric changes. Moreover, the BDE of a phenolic antioxidant seems to be less sensitive to substitution than the IP. For example, BDE decreases by 1 kcal/mol, while IP decreases by over 8 kcal/mol when an aminophenol is methylated (Wright et al., 2001).

6.8 LDL cholesterol oxidation test

The LDL-cholesterol oxidation test is often used for the measurement of antioxidant activity. It has been found that atherosclerosis is not caused by LDL-cholesterol but oxidized LDL-cholesterol (Steinbrecher, 1987; Steinberg et al., 1989). Thus, there is great interest in investigating the role of antioxidants in preventing the oxidation of LDL-cholesterol. Subjecting LDL-cholesterol to oxidation in the presence of a known concentration of the test compound and monitoring the progression of oxidation is one of the approaches to study the effect of test antioxidant against LDL-cholesterol oxidation. Conjugated dienes (CD) are often used as an indicator of the level of peroxidation of LDL-cholesterol in antioxidant studies. The inhibition of LDL cholesterol oxidation of the extracts was expressed as percentage inhibition based on the CD value after 100 min of incubation (Madhujith and Shahidi, 2007).

Figure 6-8 shows the results of inhibition of LDL-cholesterol oxidation by different test compounds. Ferulic acid was used as a reference antioxidant and demonstrated high inhibition of LDL-cholesterol oxidation up to 99.08 ± 2.53%. This is in agreement with results of Castelluccio et al. (1995) who reported that hydroxycinnamic acids such as caffeic and ferulic acids increased the resistance of LDL-cholesterol to oxidation (Castelluccio et al., 1995). Among the tested phytosteryl phenolate samples, phytosteryl ferulates showed the lowest inhibition activity while the rest of the tested samples rendered the same extent of inhibition.

6.9 DNA scission assay

DNA damage is often measured as single strand-breaks, double strand-breaks, or chromosomal aberrations (Breimer, 1990). In the present study, the synthesized phytosteryl phenolates together with the starting materials and the intermediates were evaluated for their capacity to inhibit both peroxyl and hydroxyl radical induced DNA supercoiled strand scission.

It is well documented that oxidative stress occurring in biological systems is partially attributed to peroxyl radicals. In the absence of any antioxidant, the peroxyl radical abstracts a hydrogen atom from the nearby DNA to generate new radicals, which in turn evoke a free radical chain reaction leading to the destruction of DNA molecules. The DNA retention capacity of tested compounds against AAPH-derived peroxyl radical damage is shown in Figure 6-9 and Table 6-1. Radicals can cleave supercoiled plasmid DNA to a nicked circular or, at higher concentrations of radicals, to a linear DNA form. The control lanes where the reaction mixture did not contain any antioxidant in Figure 6-9 showed clearly that the presence of peroxyl radical resulted in a dramatic scission of supercoiled DNA to its nicked circular form. However, the radical concentration used in the present study was not sufficient to destroy the nicked circular DNA, which might be more difficult to destroy than supercoiled DNA. All the phenolic acids and vinyl phenolates showed a high level of DNA retention capacity, varying from 96 to 99%, however, the esterification dramatically decreased DNA retention capacity of the phytosteryl phenolates with the exception of phytosteryl caffeates. Only 10-20% DNA retention was observed for phytosteryl vanillates, ferulates and sinapates, while a 100 % retention was rendered by phytosteryl caffeates. Peroxyl radicals are more stable than

other oxygen radicals and have the ability to diffuse relatively far from the site of their generation before they react with a target molecule (Ross and Beilski, 1990; Morrero and Marnett, 1993). Our results indicate that phytosteryl caffeates have great potential to be used as antioxidants for scavenging peroxyl radicals.

Of all the reactive oxygen species (ROS), hydroxyl radical is known to be the most reactive oxidising radical that reacts with biomolecules at diffusion-controlled rates (Cheeseman and Slater, 1993). Thus, it is physiologically harmful and suspected in such pathologies as atherosclerosis and DNA mutation (Malins, 1993). The synthesized phytosteryl phenolates together with their precursors and intermediates were also subjected to the evaluation of their DNA retention capacities against hydroxyl radical damage. The results are presented in Figure 6-10 and Table 6-2. The hydroxyl radical in this assay was generated via Fenton reaction. Compared with the DNA retention capacities of phenolic acids and their derivatives against peroxyl radical damage, much difference existed in their DNA retention ability against hydroxyl radical damage. While up to 90% DNA retention capacities against peroxyl radical was observed for all phenolic acids and their vinyl phenolates, ferulic acids and vanillic acids together with their vinyl phenolates showed moderate 26-40% DNA retention ability against hydroxyl radical damage. As for caffeic acid, sinapic acid and their vinyl phenolates, which showed high levels of antioxidant activity in most previous assays, possessed very low DNA retention ability against hydroxyl radical damage of 0.15-2.35%. The esterification process significantly enhanced the DNA retention ability of phytosteryl caffeates and sinapates against hydroxyl radical damage. However, an opposite effect was reported for

phytosteryl vanillates and no significant differences (α=0.05) existed between ferulic acid and phytosteryl phenolates.

The above results suggest that hydroxycinnamates tested act mainly as peroxyl radical scavengers. This is in agreement with the report of Castelluccio et al. (1996). The esterified phytosteryl phenolates showed either the same/or lower DNA retention ability against peroxyl radical and hydroxyl radical with the exception of phytosteryl caffeates and sinapates, which showed enhanced DNA retention ability against hydroxyl radical.

6.10 Summary

Phytosteryl phenolates exhibited antioxidant activity that was system dependent and followed different antioxidant mechanisms. All derivatives possessed antioxidant activity superior to that of their phenolic acid counterparts in terms of ORAC values and the efficacy in β-carotene-linoleate assay, suggesting their potential use as antioxidants in more lipophilic environments, however, this is not applicable to the bulk oil system as all phytosteryl phenolates showed lower antioxidant activity than their hydrophilic phenolic acid counterparts, in agreement with polar paradox theory. Phytosteryl phenolates with enhanced lipophilicity also exhibited higher antioxidant activities than their parent phenolic acids in scavenging peroxyl radicals, possibly due to combined electronic and steric effects. However, reducing power of the derivatives was lower than that of their phenolic acid counterparts, which may be explained by their poor solubility in the aqueous test medium or micelle formation. The lipophilic derivatives tested possessed a higher antioxidant potential in inhibiting lipid oxidation in emulsion and muscle food

model systems. In the DNA scission test, esterified phytosteryl phenolates showed either the same/or lower DNA retention ability against peroxyl radical and hydroxyl radical with the exception of phytosteryl caffeates and sinapates, which showed enhanced DNA retention ability against hydroxyl radical. Phytosteryl caffeates also possess higher activity on DNA retention ability against peroxyl radical. Moderate inhibition activity of LDL oxidation was observed for phytosteryl phenolates. The antioxidant activity of phytosteryl phenolates was similar or superior to that of their parent phenolic acid counterparts, suggesting their potential use as phenolic acid alternatives in the food industry.

The results reported here suggest that lipophilic derivatives of phenolic acids may be used as potential functional ingredients in foods, cosmetics, drugs and as natural health products for health promotion and disease risk reduction, however, these findings are based on *in vitro* chemical assays, and more research on their bioactivities in real food and biological systems is needed for better understanding and utilization of the derivatives prepared as nutraceuticals or ingredient for functional foods.

Table 6-1. Effect of phenolic acids, vinyl phenolates and phytosteryl phenolates in preventing peroxyl radical-induced DNA scission (DNA retention ability, %).

Samples	Phenolic acids	Vinyl phenolates	Phytosteryl phenolates
CA and derivatives	96.80 ± 1.13^{Aa}	98.65 ± 1.63^{Aa}	100.00 ± 0^{Aa}
FA and derivatives	98.8 ± 0.85^{Aa}	99.85 ± 0.07^{Aa}	17.30 ± 3.11^{Bb}
SA and derivatives	99.25 ± 0.78^{Aa}	99.35 ± 0.64^{Ba}	19.85 ± 3.61^{Bb}
VA and derivatives	97.20 ± 1.84^{Aa}	98.90 ± 0.99^{Ba}	10.60 ± 0.14^{Bb}

[a]Results reported are mean values of two determinations ± SD. Means in each column sharing the same superscript are not significantly ($P > 0.05$) different from one another.

[A]Results reported are mean values of two determinations ± SD. Means in each row sharing the same superscript are not significantly ($P > 0.05$) different from one another.

CA, caffeic acid; FA, ferulic acid; SA, sinapic acid; and VA, vanillic acid.

Table 6-2. Effect of phenolic acids, vinyl phenolates and phytosteryl phenolates in preventing hydroxyl radical-induced DNA scission (DNA retention ability, %).

Samples	Phenolic acids	Vinyl phenolates	Phytosteryl phenolates
CA and derivatives	0.15 ± 0.07^{Bb}	1.30 ± 0.14^{Bb}	11.60 ± 1.56^{Ab}
FA and derivatives	26.65 ± 13.93^{Aa}	34.50 ± 2.83^{Aa}	15.60 ± 7.35^{Ab}
SA and derivatives	2.35 ± 1.91^{Bb}	0.65 ± 0.35^{Bb}	51.05 ± 3.32^{Aa}
VA and derivatives	28.05 ± 4.17^{ABa}	40.35 ± 0.78^{Aa}	15.20 ± 5.52^{Bb}

[a]Results reported are mean values of two determinations ± SD. Means in each column sharing the same superscript are not significantly ($P > 0.05$) different from one another.

[A]Results reported are mean values of two determinations ± SD. Means in each row sharing the same superscript are not significantly ($P > 0.05$) different from one another.

CA, caffeic acid; FA, ferulic acid; SA, sinapic acid; and VA, vanillic acid.

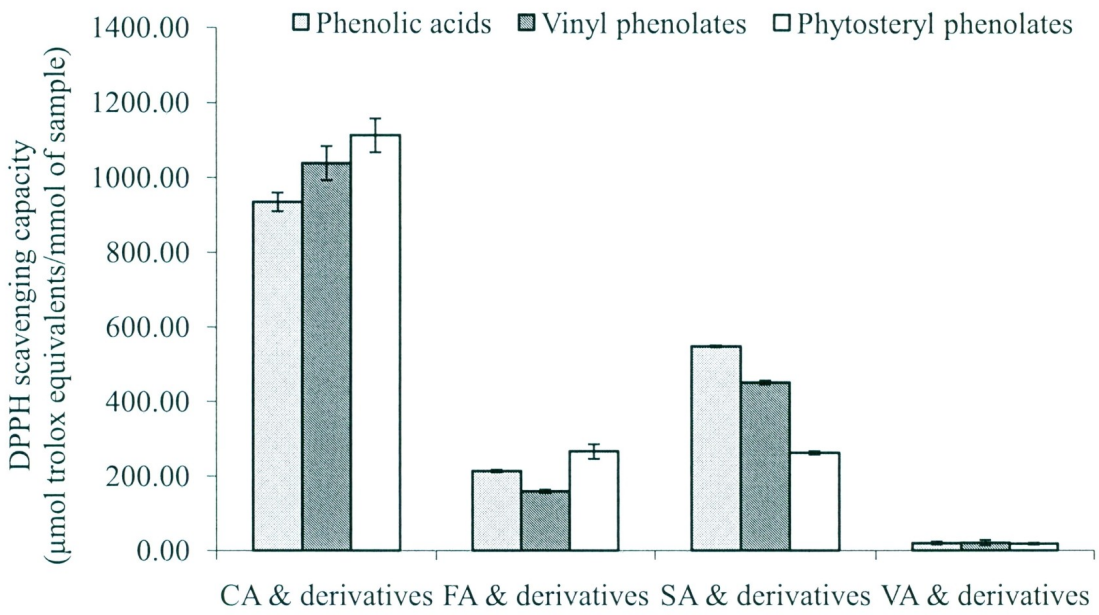


Figure 6-1. DPPH radical scavenging ability of phenolic acids and their derivatives. CA, caffeic acid; FA, ferulic acid; SA, sinapic acid; and VA, vanillic acid.

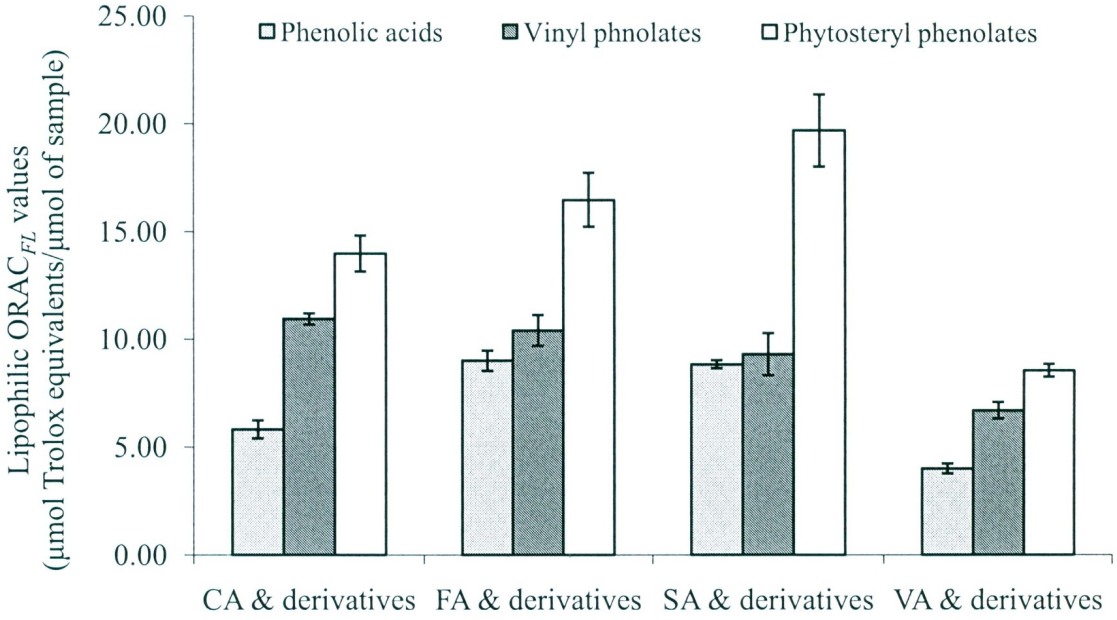


Figure 6-2. Lipophilic-ORAC$_{FL}$ values of phenolic acids and their derivatives as trolox equivalents. CA, caffeic acid; FA, ferulic acid; SA, sinapic acid; and VA, vanillic acid.

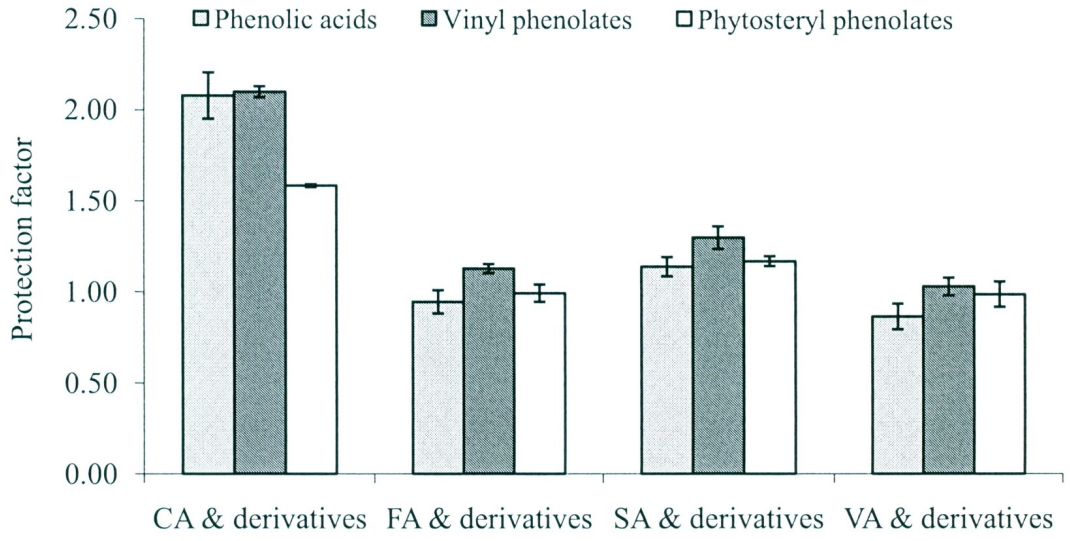


Figure 6-3. Protection factor of phenolic acids and their derivatives in stripped corn oil. CA, caffeic acid; FA, ferulic acid; SA, sinapic acid; and VA, vanillic acid.

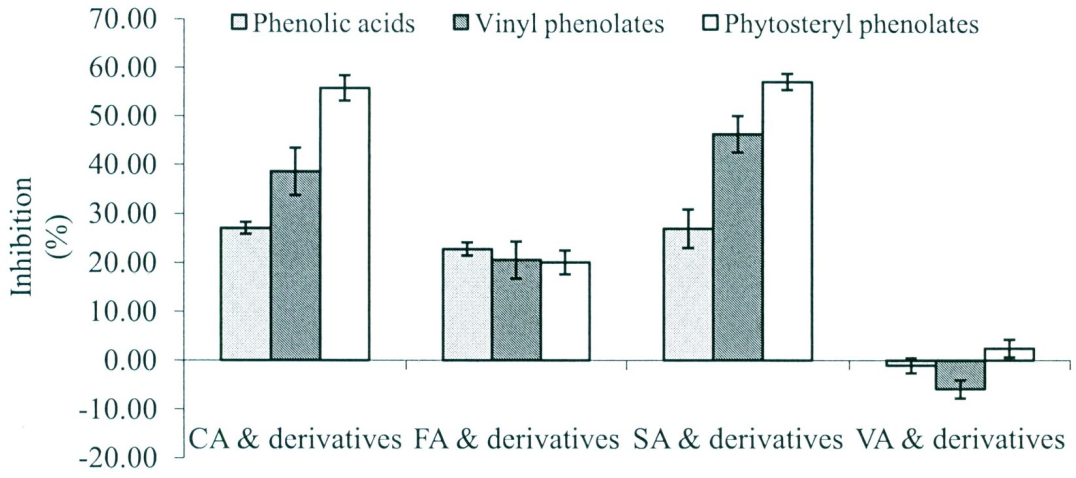


Figure 6-4. Inhibitory effect of phenolic acids and their derivatives against β-carotene bleaching. CA, caffeic acid; FA, ferulic acid; SA, sinapic acid; and VA, vanillic acid.

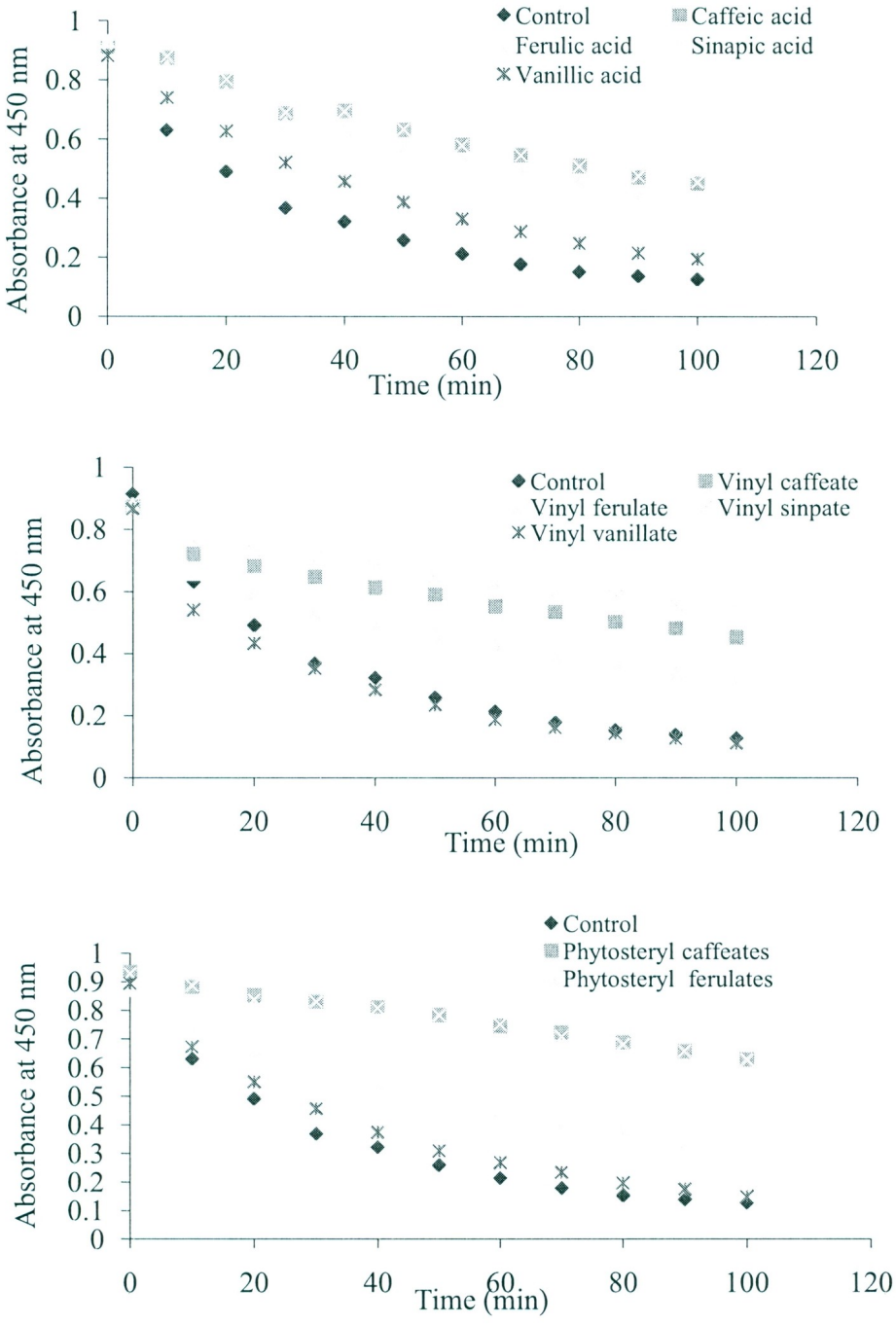


Figure 6-5. β-Carotene bleaching as affected by phenolic acids, vinyl phenolates and phytosteryl phenolates

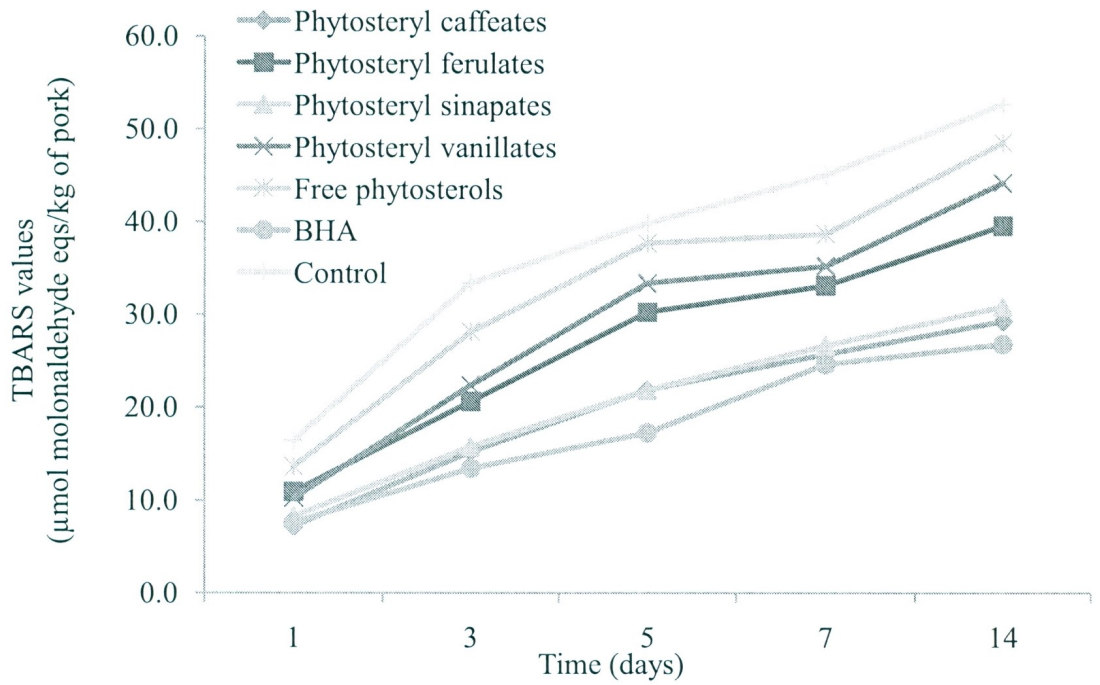


Figure 6-6. Thiobarbituric acid reactive substances (TBARS) values in cooked pork as affected by phytosterols and their derivatives. BHA: butylated hydroxyanisole.

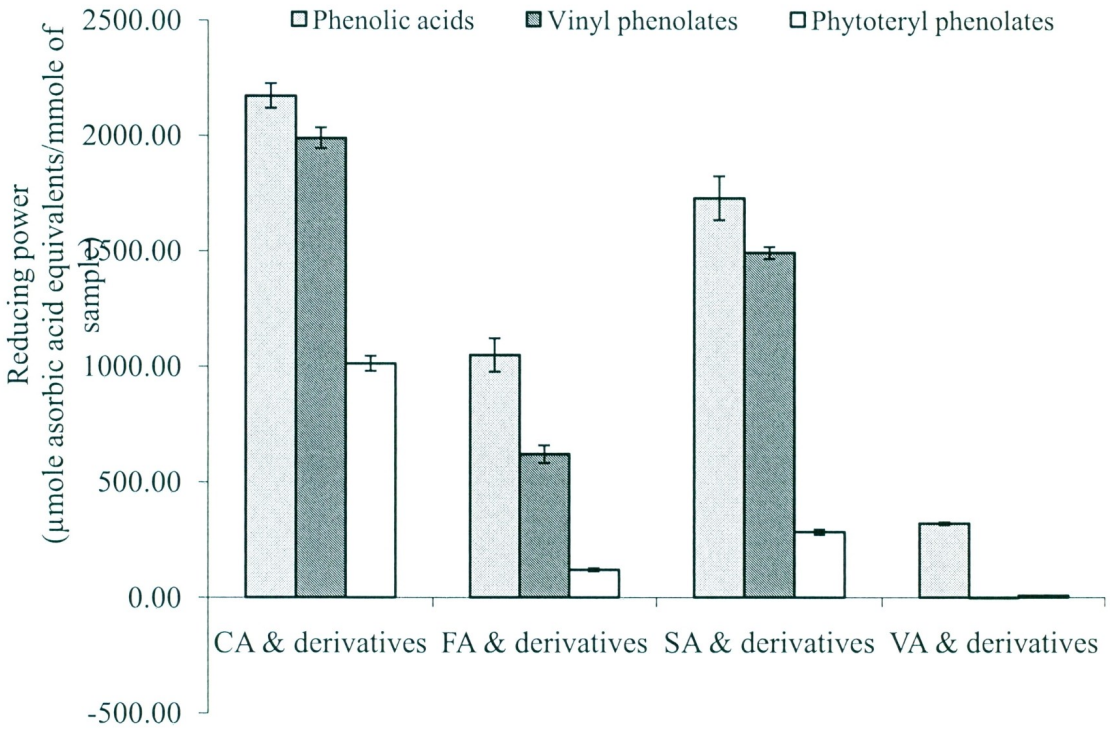


Figure 6-7. Reducing power of phenolic acids and their derivatives. CA, caffeic acid; FA, ferulic acid; SA, sinapic acid; and VA, vanillic acid.

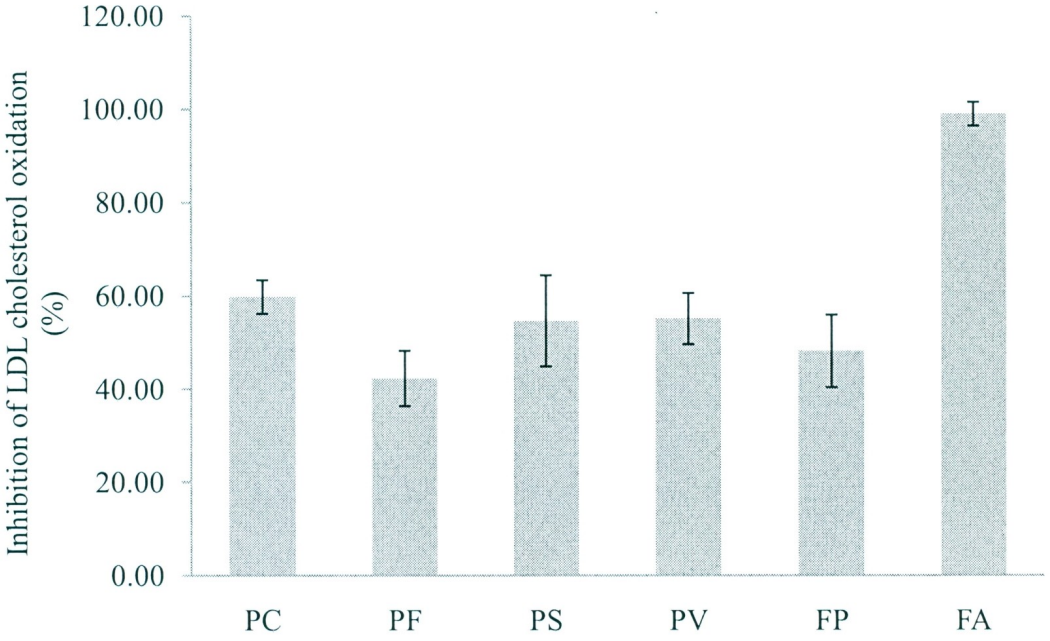
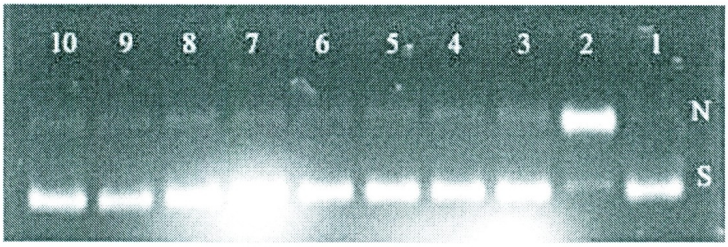
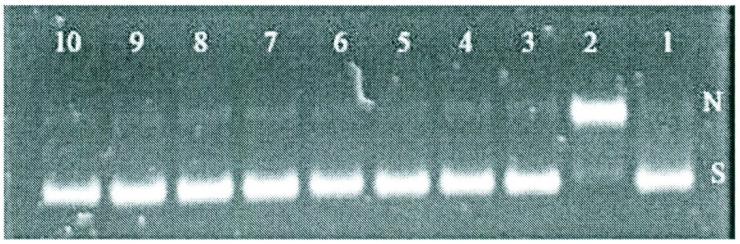


Figure 6-8. Inhibition against LDL-cholesterol oxidation. PC, phytosteryl caffeates; PF, phytosteryl ferulates; PS, phytosteryl sinapates; PV, phytosteryl vanillates; FP, free phytosterols; and FA, ferulic acid.



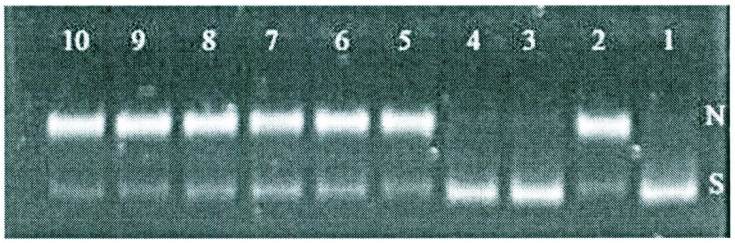
A

Lane 1 = DNA + PBS; lane 2 = DNA + AAPH; lane 3, 4 = DNA + AAPH + caffeic acid; lane 5, 6 = DNA + AAPH + ferulic acid; lane 7, 8 = DNA + AAPH + sinapic acid; lane 9, 10 = DNA + AAPH + vanillic acid.



B

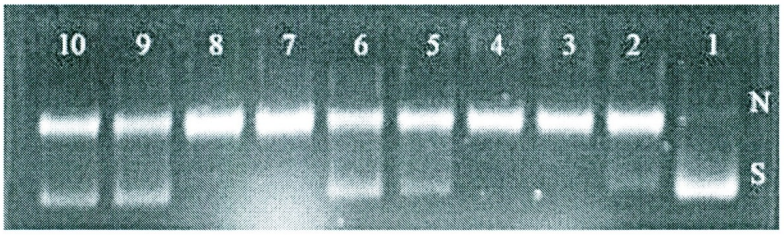
Lane 1 = DNA + PBS; lane 2 = DNA + AAPH; lane 3, 4 = DNA + AAPH + vinyl caffeate; lane 5, 6 = DNA + AAPH + vinyl ferulate; lane 7, 8 = DNA + AAPH + vinyl sinapate; lane 9, 10 = DNA + AAPH + vinyl vanillate.



C

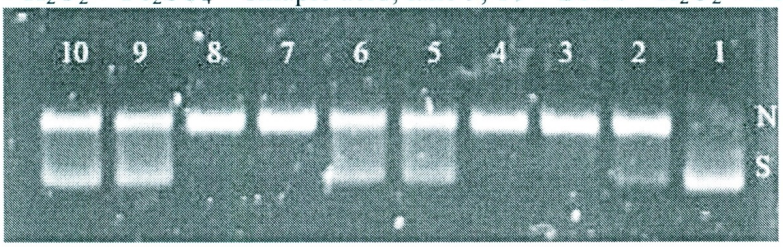
Lane 1 = DNA + PBS; lane 2 = DNA + AAPH; lane 3, 4 = DNA + AAPH + phytosteryl caffeates; lane 5, 6 = DNA + AAPH + phytosteryl ferulates; lane 7, 8 = DNA + AAPH + phytosteryl sinapates; lane 9, 10 = DNA + AAPH + phytosteryl vanillates.

Figure 6-9. Effect of phenolic acids (A), vinyl phenolates (B) and phytosteryl phenolates (C) in preventing peroxyl radical-induced DNA scission. AAPH = 2,2′-azobis(2-methylpropionamidine) dihydrochloride; S, supercoiled DNA; and N, nicked DNA.



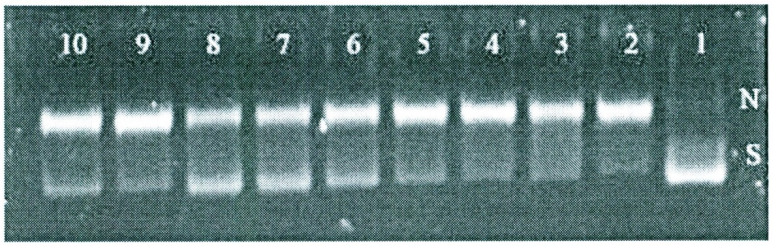
A

Lane 1 = DNA + PBS; lane 2 = DNA + H_2O_2 + Fe_2SO_4; lane 3, 4 = DNA + H_2O_2 + Fe_2SO_4 + caffeic acid; lane 5, 6 = DNA + H_2O_2 + Fe_2SO_4 + ferulic acid; lane 7, 8 = DNA + H_2O_2 + Fe_2SO_4 + sinapic acid; lane 9, 10 = DNA + H_2O_2 + Fe_2SO_4 + vanillic acid.



B

Lane 1 = DNA + PBS; lane 2 = DNA + H_2O_2 + Fe_2SO_4; lane 3, 4 = DNA + H_2O_2 + Fe_2SO_4 + vinyl caffeate; lane 5, 6 = DNA + H_2O_2 + Fe_2SO_4 + vinyl ferulate; lane 7, 8 = DNA + H_2O_2 + Fe_2SO_4 + vinyl sinapate; lane 9, 10 = DNA + H_2O_2 + Fe_2SO_4 + vinyl vanillate.



C

Lane 1 = DNA + PBS; lane 2 = DNA + H_2O_2 + Fe_2SO_4; lane 3, 4 = DNA + H_2O_2 + Fe_2SO_4 + phytosteryl caffeates; lane 5, 6 = DNA + H_2O_2 + Fe_2SO_4 + phytosteryl ferulates; lane 7, 8 = DNA + H_2O_2 + Fe_2SO_4 + phytosteryl sinapates; lane 9, 10 = DNA + H_2O_2 + F Fe_2SO_4 + phytosteryl vanillates.

Figure 6-10. Effect of phenolic acids (A), vinyl phenolates (B) and phytosteryl phenolates (C) in preventing hydroxyl radical-induced DNA scission. S, supercoiled DNA; and N, nicked DNA.

Chapter 7

Synthesis of Phytosteryl Oleates and Docosahexaenoates and Evaluation of their Cholesterol Lowering Effects in ApoE-Deficient Mice

7.1 Introduction

Cardiovascular disease (CVD) continues to be a major cause of mortality and morbidity in the world and ranks first in this respect, especially in the developed countries (Banegas et al., 2003; Jemal et al., 2005). It is well documented that elevated plasma lipid concentration, including increased levels of total and LDL cholesterol, as well as high concentrations of triacylglycerols, are the major risk factors for cardiovascular disease (Gould et al., 1995; Gotto, 1995; Grundy, 1997). Animal models are used for studies of the pathogenesis of atherosclerosis and for evaluation of the beneficial effects of different therapies in its prevention or treatment. ApoE-deficient mice model constitutes a transgenic murine model that is unique and suitable for the study of human atherosclerosis. These mice develop severe hypercholesterolemia and atheroschlerotic lesions that are similar in appearance and distribution to those observed in humans (Nakashima et al., 1994; Plump et al., 1992 and 1994; Zhang et al., 1992; Palinski et al., 1994).

Omega-3 PUFA from fish oil, marine mammal oil and algae sources, have shown hypotriacylglycerolemic effects in animal models and in humans (Harris, 1997a and 1997b). Evidence from epidemiological studies suggests that the Mediterranean diet with a high proportion of monounsaturated lipid may reduce the risk of coronary heart disease (Keys et al., 1986). The addition of phytosterols to the diet lowers plasma cholesterol in animal models and in humans (Becker et al., 1993; Miettinen et al., 1995; Moghadasian et al., 1997; Peterson, 1951). Specifically, phytosterols have been shown to decrease the low-density lipoprotein (LDL) plasma cholesterol levels without significant effects on the level of high-density lipoprotein (HDL) cholesterol in both animal and human models (Moghadasian and Frohlich, 1999; Moghadasian et al., 1999). In addition, they also show the ability to prevent and delay the development of atherosclerotic lesions in animal models (Moghadasian et al., 1997; Moghadasian, 2006; Yeganeh et al., 2005). In a new approach for reducing cardiovascular risk, we enzymatically esterified omega-3 PUFA or oleic acid with phytosterols in the hope of taking advantage of their triacylglycerols and cholesterol lowering effects, respectively.

Various chemical strategies have been explored for the synthesis of phytosteryl esters with fatty acids. For example, phytosterols were first esterified with fatty acyl chlorides (Mattson et al., 1977 and 1982); campesteryl and sitosteryl palmitates were successfully synthesized through transesterification between phytosteryl acetate and methyl palmitate in the presence of sodium ethoxide under reduced atmospheric pressure (Hsieh et al., 1980). Pouilloux et al. (2003) reported a method for the synthesis of phytosteryl esters from natural phytosterols and fatty acid methyl esters in the presence of

alkali oxides as catalysts. Chemical synthesis of phytosteryl esters with n-3 PUFA has also been reported (Edwart et al., 2002; Russell et al., 2002). Although phytosteryl esters with FA can be synthesized by chemical means, the chemical methods suffer from problems associated with the formation of side products (3,5-diene derivatives or oxyphytosterols) (Negishi et al., 2003). In addition, these routes do not always meet the requirements necessary for food applications. Using enzymes as catalysts for synthesis can overcome the disadvantages of chemical methods. Several enzymatic procedures for the preparation of phytosteryl esters of FAs have been developed in recent years (King et al., 2001; Osanai, 1986; Villeneuve et al., 2005; Vu et al., 2004; Weber et al., 2001a; Weber et al., 2001b; Weber et al., 2002). However, most of the studies mentioned above use fatty acid mixtures derived from vegetable oils as substrates.

This chapter reports enzymatic preparation of phytosteryl oleates and phytosteryl docosahexaenoates and evaluation of their effects on plasma lipid levels in the apoE-deficient mice.

7.2 Synthesis of phytosteryl oleates and docosahexaenoates

7.2.1 Preparation of phytosteryl oleates

The method described by Kim and Akoh (2007) was adapted, with modifications, for gram-scale preparation of phytosteryl oleates. Reaction mixtures were separated using flash column chromatography. Fractions were monitored using TLC plate. R_f value corresponding to phytosteryl oleates was 0.93, indicating that the lipophilicity of phytosteryl oleates was considerably higher than that of free phytosterols with R_f value

0.18. The yield of phytosteryl oleates was 95%. The purified phytosteryl oleates were then used in animal studies to evaluate their cholesterol lowering effect.

7.2.2 Preparation of phytosteryl docosahexaenoates

DHA was prepared from DHASCO using the urea complexion method described by Wanasundara and Shahidi (1999). Among the enzymes used for screening for the synthesis of phytosteryl docosahexaenoates, the lipase LPL 311 was the only one that successfully catalyzed the esterification reaction between phytosterols and DHA. Hexanes were used as solvent because hydrophobic solvents generally support a higher lipase activity than the hydrophilic ones (Dordick, 1989; Kvittingen, 1994). A yield of 96% after 24 hours reaction was achieved. Reaction mixtures were separated using flash column chromatography. Fractions were monitored on TLC plates. R_f value corresponding to phytosteryl docosahexaenoates was 0.90. It indicates a higher lipophilicity of phytosteryl oleates compared that of free phytosterols with R_f value of 0.18. The purified phytosteryl docosahexaenoates were then used in animal studies together with phytosteryl oleates to evaluate their cholesterol lowering effect.

7.2.3 HPLC-MS analysis of phytosteryl docosahexaenoates

Free phytosterols and phytosteryl docosahexaenoates, purified by column chromatography, were subjected to HPLC-MS analysis to confirm their structures. GC and GC-MS are the conventional methods used for the analysis of phytosterols and their derivatives. Due to their relatively low volatile nature, GC analysis of phytosteryl esters

requires high temperatures. Therefore, saponification is often applied prior to GC analysis followed by the analysis of phytosterols as their trimethylsilyl derivatives and of fatty acids as their corresponding methyl esters. This approach prevents the identification of the individual phytosteryl esters and thus valuable information may be lost. Direct GC-MS analysis of phytosteryl docosahexaenoates was hindered due to limitations of efficiently separating and eluting these high molecular weight compounds from the GC column as well as low volatility and low oxidative stability of phytosteryl docosahexaenoates at high temperature. Therefore, HPLC-MS methodology was developed for the first time for the separation and identification of the phytosteryl docosahexaenoates. Our preliminary experiments indicated that electrospray ionization (ESI) is not applicable for the determination of phytosterols and their derivatives because no fragmentation occurs for those compounds under conditions employed. Therefore, atmospheric pressure chemical ionization (APCI) was used as the ionization method instead of ESI.

HPLC analysis of the starting free phytosterols was presented in Section 5.3. HPLC chromatogram for phytosteryl docosahexaenoates under reverse-phase condition is shown in Figure 7-1. The retention time for the three phytosteryl docosahexaenoates (compounds 19-21) was 84.1, 94.2 and 108.8 min, respectively. The dramatic increase of the retention time (14.5-17 min for free phytosterols) under the RP-HPLC indicated that the synthesized phytosteryl docosahexaenoates are much more hydrophobic than the starting free phytosterols, in agreement with their increased R_f values. Under the normal phase (NP)-HPLC conditions, all the three components were co-eluted at 6.2 min as

indicated in the HPLC chromatogram in Figure 7-2. This is agreement with the findings of Abidi (2001) who reported that RP-HPLC is more selective for separating free phytosterol homologues and unsaturated analogues than NP-HPLC. This is because in RP-HPLC separation is based on the hydrophobic interactions between the analytes and the stationary phase, which is influenced by the molecular size and the number of double bonds, whereas in NP-HPLC, separation is mainly based on the adsorptive interactions and polarity differences (Abidi, 2001). Both RP-HPLC and NP-HPLC were used to analyze the structure of phytosteryl docosahexaenoates because they showed different ionization patterns with different mobile phases employed in RP-HPLC and NP-HPLC. Mass spectra of phytosteryl docosahexaenoates under APCI positive ion mode following reverse-phase HPLC separation is given in Figure 7-3. The ion peaks of compounds 19, 20 and 21 in phytosteryl docosahexaenoates were at *m/e* 383, 397 and 398, respectively, which correspond to the molecular weight of the moiety of campesterol, sitosterol and sitostanol, respectively, after cleavage of the ester bond. Compound 21 also showed a molecular ion $[M]^+$ at *m/e* 726, which matches the molecular weight of sitostanyl docosahexaenoate. However, no ions corresponding to the DHA moiety in these three mass spectra were observed. Therefore, compounds 19, 20, and 21 can only be tentatively identified as campesteryl, sitosteryl and sitostanyl docosahexaenoates, respectively. To solve the problem of the lack of ions representative of the molecular weight, a normal phase HPLC method was also employed. Although the NP-HPLC resulted in low quality separation of the phytosteryl docosahexaenoates mixtures, the more hydrophobic mobile phase led to different ionization patterns for them, thus providing more relevant structural

information. As indicated in Figure 7-4, the fragment ions so produced indicated the presence of the fatty acid and phytosterol moieties as well as the protonated positive molecular ions for phytosteryl docosahexaenoate mixtures (compound 19, 20 and 21). Ions with *m/e* 727, 725 and 711 are $[M+H]^+$ corresponding to the protonated molecular ions for sitostanyl docosahexaenoate, sitosteryl docosahexaenoate and campesteryl docosahexaenoate, respectively, were also present. The ions with *m/e* 383, 397 and 398, represented the moiety of campesterol, sitosterol and sitostanol, respectively, after cleavage of the ester bond. Finally, the ion at *m/e* 329 represents the protonated DHA moiety after cleavage of the ester bond from the phytosteryl docosahexaenoates. The influence of different solvents (methanol, isopropanol, acetonnitrile, and water) and additives such as ammonium acetate and formic acid on the signal intensity of phytosterols and their derivatives was also reported by Lembcke et al. (2005). These authors concluded that the highest signal intensities could be obtained using pure methanol or isopropanol. The separation of the intact phytosteryl esters have also been achieved by reverse-phase HPLC (Gordon and Griffith, 1992a; 1992b), however, the application of this technique is limited due to the lack of resolution of different phytosteryl esters arising from similar polarities of phytosteryl esters, in accordance with our findings. Thus, the above HPLC-MS information confirms that the synthesized products are indeed phytosteryl docosahexaenoates.

7.2.4 FTIR analysis of phytosteryl docosahexaenoates

The IR spectra of free phytosterols and phytosteryl docosahexaenoates were compared as shown in Figure 7-5. These spectra could provide evidence that the enzymatically synthesized products were phytosteryl esters. The free phytosterols had a strong absorption at 1053 cm^{-1} that was assigned to the C-O vibration via participation of oxygen atom of the hydroxyl group. A broad band around 3300-3600 cm^{-1} corresponded to the O-H vibration of the hydroxyl group of phytosterols. The esters were characterized by a strong sharp band at 1734 cm^{-1} that was assigned to an ester carbonyl group and the absorption at 1163 cm^{-1} corresponded to C-O-C asymmetric stretching vibration and symmetric vibration of the ester linkage. The free phytosterols have a strong absorption at 1053 cm^{-1} that was assigned to the C-O vibration of the hydroxyl group; this band disappeared in the synthesized phytosteryl docosahexaenoates. The broad band around 3300-3600 cm^{-1} corresponding to the O-H vibration of the hydroxyl group of phytosterols has also disappeared.

7.3 Evaluation of the cholesterol lowering effect of phytosteryl oleates and docosahexaenoates in apoE-deficient mice

7.3.1 Body weight

Figure 7-6 summarizes body weight gain of mice in all three experimental groups over the entire course of the experiment. All of the experimental animals gained body weight during the experimental period. The initial mean body weight of the three animal

groups was 17.5 ± 2.6, 18.9 ± 1.3 and 18.8 ±1.0 g, respectively, for the control, phytosteryl oleates and phytosteryl docosahexaenoates-treated groups. Compared with week 0, the body weight of the control, phytosteryl oleates and phytosteryl docosahexaenoates-treated groups at week 4 increased by 24.2 ± 7.2, 23.0 ± 6.4 and 21.3 ± 6.3%, respectively. The corresponding body weight increase in week 7 was 30.0 ± 8.4, 27.8 ± 7.6 and 26.0 ± 7.5%, respectively. Therefore, the control group showed the highest body weight gain, while phytosteryl docosahexaenoates-treated group had the lowest body weight gain. The lower body gain of the phytosteryl docosahexaenoates-treated group may partially be explained by the presence of DHA esters which functioning as hypolipidemic agents. Consumption of long-chain omega-3 polyunsaturated fatty acids (PUFA), which are concentrated in fish and other marine oils, has been reported to favourably affect the plasma triacylglycerols levels (Harris, 1999; Kris-Etherton et al., 2002).

7.3.2 Total plasma cholesterols

The levels of plasma total cholesterol at baseline and during the experimental period have been illustrated in Figure 7-7. The three groups of animals showed similar plasma total cholesterol levels at week 0 (≈ 6 mmol/l). The level of plasma total cholesterol was all increased after consumption of the 0.1% (w/w) cholesterol-enriched diet among the three experimental groups, however, at week 4, phytosteryl oleates treated animals showed a significantly lower plasma total cholesterol level than the control group (9.41 ±1.65 versus 13.23 ± 1.58 mmol/l, $P < 0.01$), followed by phytosteryl

docosahexaenoates-treated animals, which had a total plasma cholesterol level of 10.73 ± 2.02 mmol/l at week 4 ($P < 0.05$ compared with control). A significant difference ($P < 0.01$) remained among the phytosteryl oleates-treated animals and the control group until the end of the study. However, at week 7 there was no significant difference in total plasma cholesterol levels between the animals of phytosteryl docosahexaenoates-treated and the control group.

In the present study, plasma total cholesterol concentrations were significantly decreased in animals fed phytosteryl oleates and phytosteryl docosahexaenoates compared with the control. This confirms the recent findings that dietary phytosterol-fish oil conjugates decrease the plasma cholesterol concentration in guinea pigs (Edwart et al., 2002), hamster (Demonty et al., 2005) and obese, insulin-resistant rats (Russell et al., 2002). To the best of our knowledge, this is the first report about the cholesterol lowering effect of pure phytosteryl oleates. The results from animal studies indicated its better ability in lowering total plasma cholesterol than the counterpart phytosteryl docosahexaenoates used here. The 27-29% lower total plasma cholesterol in apo-E-deficient mice fed phytosteryl oleates (compared to control) is considerably greater than 9-14% reductions reported for humans ingesting 2 g of free phytosterols daily (Law, 2000). Further studies are needed to confirm this finding in both animal and human subjects.

7.3.3 Total triacylglycerols

In addition to the elevated levels of low-density lipoprotein (LDL) cholesterol, high plasma triacylglycerol levels have also been reported to be one of the main factors for the pathogenesis of CVD (Hokanson and Austin, 1996). Thus, to prevent or treat CVD, it is necessary to reduce the plasma triacylglycerol levels by dietary and/or pharmaceutical agents. Figure 7-8 shows the level of total plasma triacylglycerols at baseline and during the experimental period for all three groups of animal. All groups of mice had similar level of total plasma triacylglycerols at baseline. At week 4 and week 7, both groups treated with phytosteryl oleates and phytosteryl docosahexaenoates showed dramatically increased level of total triacylglycerols as compared to the controls. We hypothesized that the synthesized phytosteryl docosahexaenoates may have additive or synergistically triacylglycerol lowering effect since the triacylglycerol-lowering effects of omega-3 fish oils have been well-established in both humans and animal models (Harris, 1997 a; 1997b; Ikeda et al., 2001). However, experimental results contradict this hypothesis. This paradoxical plasma triacylglycerol increase of phytosteryl oleates and phytosteryl docosahexaenoates treatment may be partially explained the apo-E deficient mice model employed. Studies reported that the animal model (apo E-deficient mice) do not respond to triacylglycerol-lowering effects of fish oils (Asset et al., 2001; Xu et al., 2007). Therefore, other animal models, such as rabbit or hamster, may be preferred instead of rodent models to study the triacylglycerol lowering effect of these compounds. The results may also suggest that phytosteryl docosahexaenoates are possibly broken down in the intestine and the docosahexaenoic acid may be the reason for the increase of

triacylglycerols. Asset et al. (2001) and Xu et al. (2007) observed a similar pattern of triacylglycerols increase after feeding fish oils to apo-E deficient mice.

7.3.4 Atherosclerotic lesion development

Figure 7-9 summarizes the morphometric analysis of the atherosclerotic lesions in the aortic roots of apo-E deficient mice. The lesion size in the phytosteryl oleates and phytosteryl docosahexaenoates-treated groups was smaller than those in the control group at $P < 0.01$ and $P < 0.05$ levels, respectively. The lesion size in the phytosteryl oleates-treated group was around four times smaller than that of the control group, while that in the phytosteryl docosahexaenoates-treated group was around three times smaller than that of the control group. This demonstrates the strong antiatherogenic properties of phytosteryl oleates and phytosteryl docosahexaenoates.

It is reported that omega-3 PUFA, after digestion, can be incorporated into phospholipids of red blood cell membranes. Thus it may improve erythrocyte lipid fluidity and deformability, and consequently reduce their fragility (Cartwright et al., 1985; Poschl et al., 1996; Terano et al., 1983). It may also be worthwhile to investigate the effect of phytosteryl oleates and docosahexaenoates on red blood cell fragility using animal studies.

7.4 Summary

Enzymatic synthesis of phytosteryl oleates and docosahexaenoates was successfully achieved. The structures of phytosteryl docosahexaenoates were confirmed

by both normal phase and reverse phase HPLC-MS and FTIR. This study shows that dietary phytosteryl oleates and docosahexaenoates reduce plasma total cholesterol in apoE-deficient mice as compared to the control. The changes are associated with significant reductions in atherogensis. Moreover, results indicate that apoE-deficient mice model is a robust model with accelerated atherosclerosis and severe hypercholesterolemia, it also significantly responds to cholesterol lowering and antiatherogenic effects of phytosteryl derivatives. However, phytosteryl oleates and docosahexaenoates exhibited no triacylglycerol lowering effect in this animal model. Controversially they increased the total plasma triacylglycerol level in apoE-deficient mice. Therefore, other animal model may be employed to determine the triacylglycerol lowering effect of these compounds.

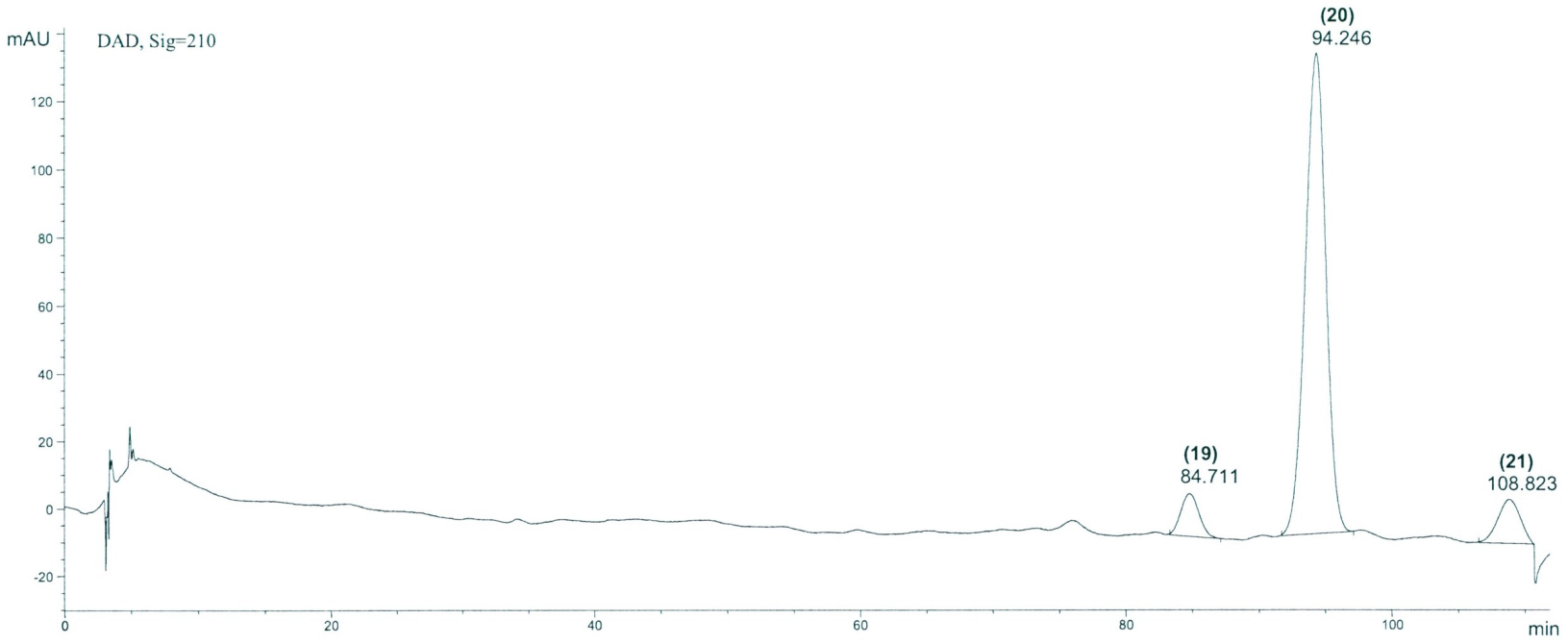


Figure 7-1. Reverse-phase HPLC chromatogram for phytosteryl docosahexaenoates. (compounds 19, 20, 21 are campesteryl docosahexaenoate, sitosteryl docosahexaenoate, and sitostanyl docosahexaenoate, respectively).

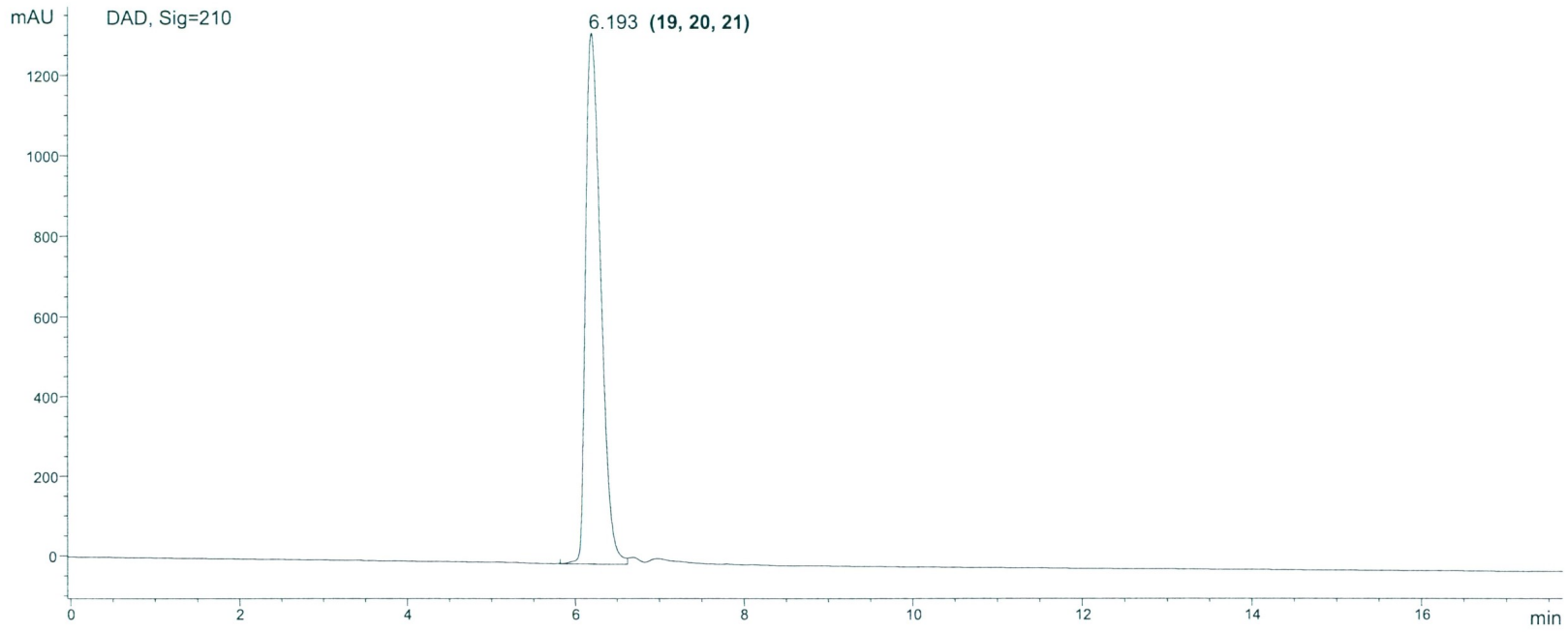
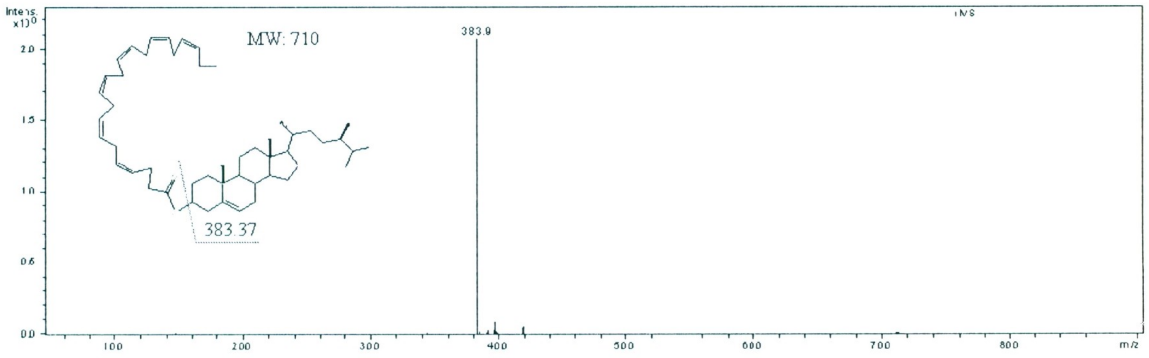
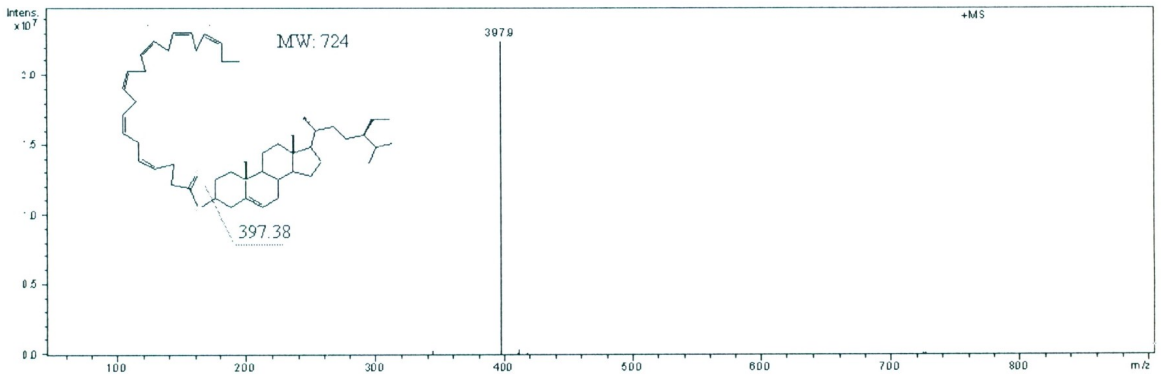


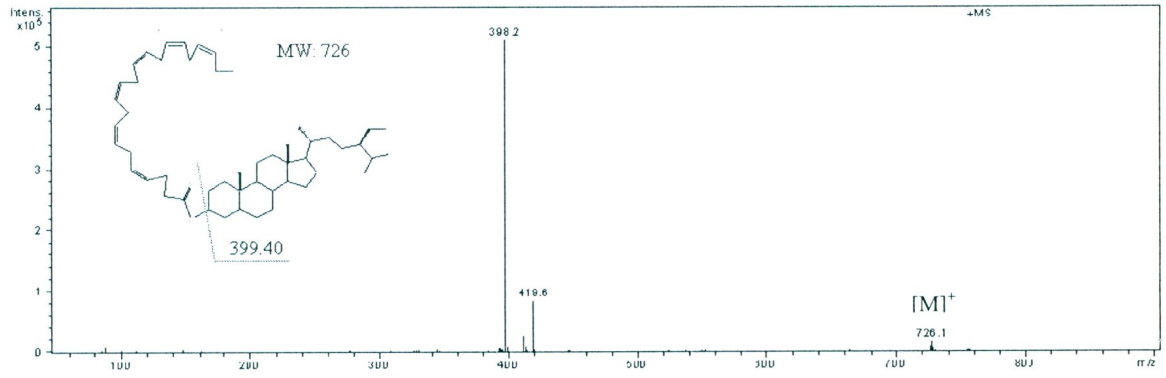
Figure 7-2. Normal-phase HPLC chromatogram for phytosteryl docosahexaenoates. (compounds 19, 20, 21 are campesteryl docosahexaenoate, sitosteryl docosahexaenoate, and sitostanyl docosahexaenoate, respectively).



(a) Campesteryl docosahexaenoate (19)



(b) Sitosteryl docosahexaenoate (20)



(c) Sitostanyl docosahexaenoate (21)

Figure 7-3. Mass spectrum of phytosteryl docosahexaenoates under APCI positive ion mode after reverse-phase HPLC separation.

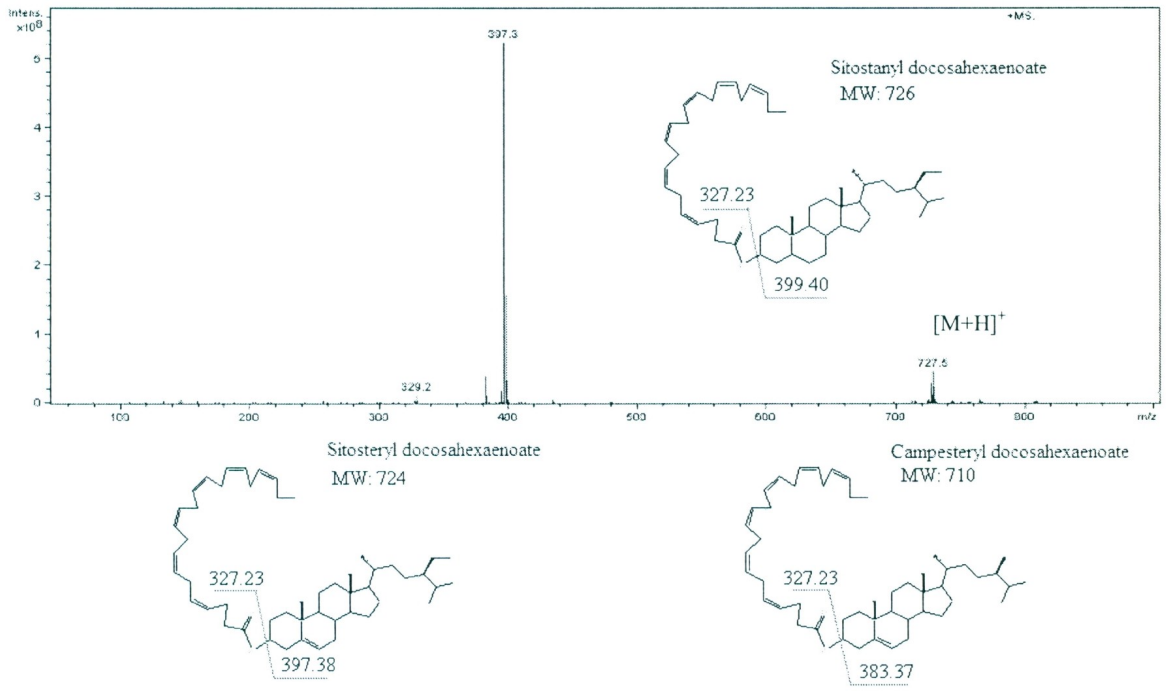


Figure 7-4. Mass spectrum of phytosteryl docosahexaenoates under APCI positive ion mode after normal phase HPLC separation.

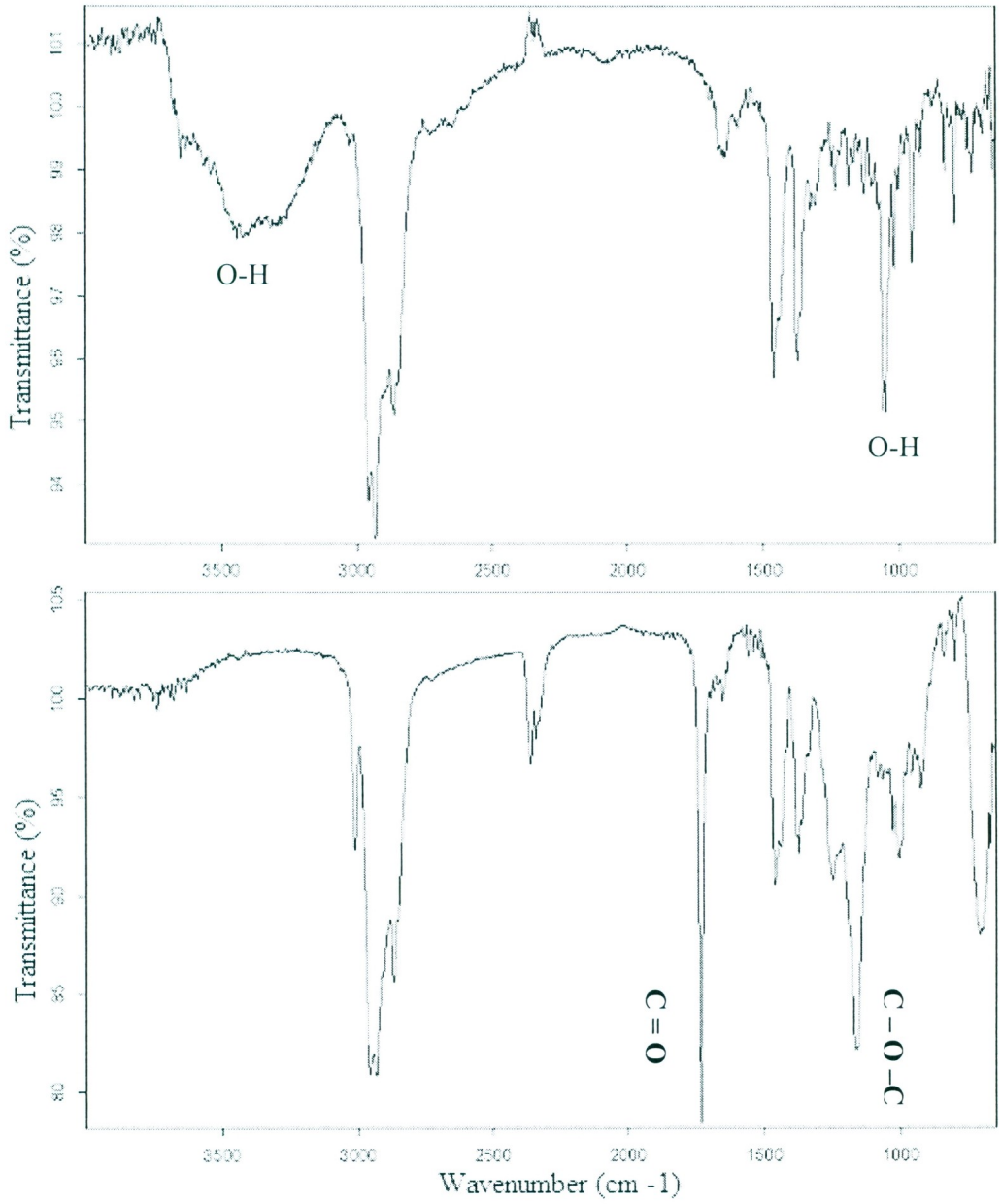


Figure 7-5. IR spectrum for free phytosterols (top) and phytosteryl docosahexaenoates (bottom).

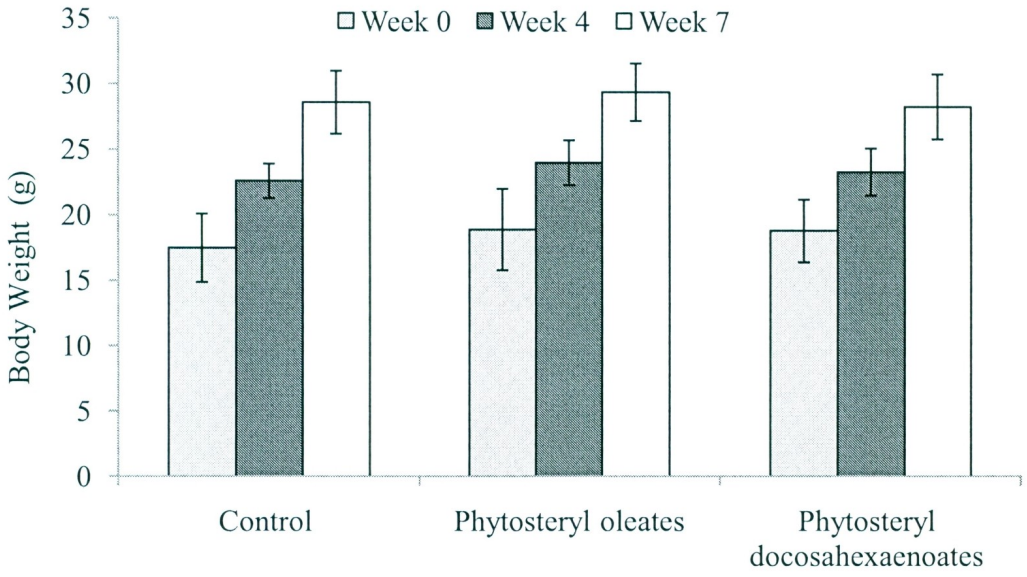


Figure 7-6. Body weight measurements (mean ± SD, g) at baseline and during the experimental course in the control, phytosteryl oleates treated and phytosteryl docosahexaenoates treated apo E-KO mice.

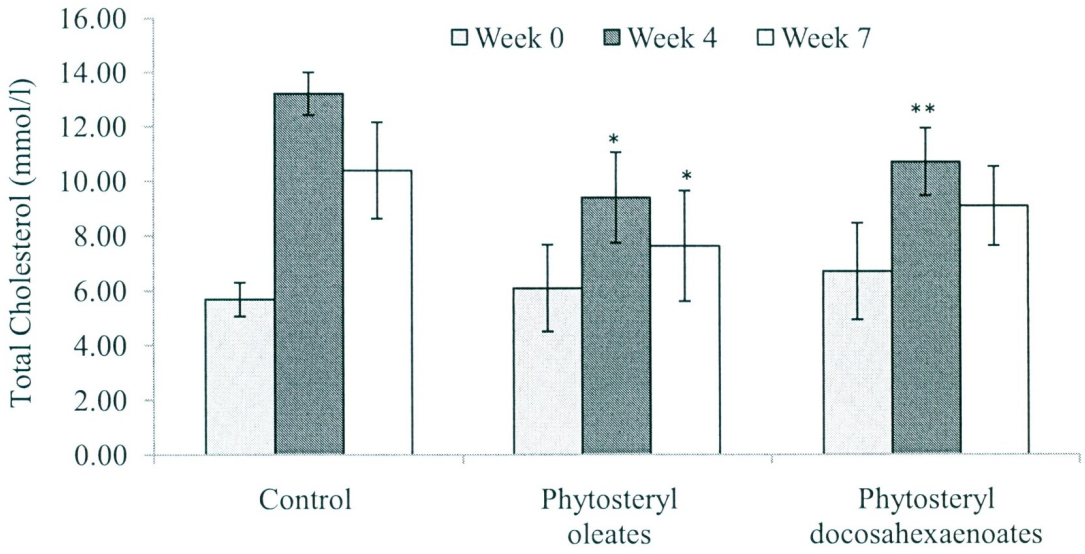


Figure 7-7. Plasma total cholesterols (TC) concentration (mean ± SD, mmol/l) of control, phytosteryl oleates treated and phytosteryl docosahexaenoates treated apo E-KO mice. * $P < 0.01$ as compared to the control; ** $P < 0.05$ as compared to the control.

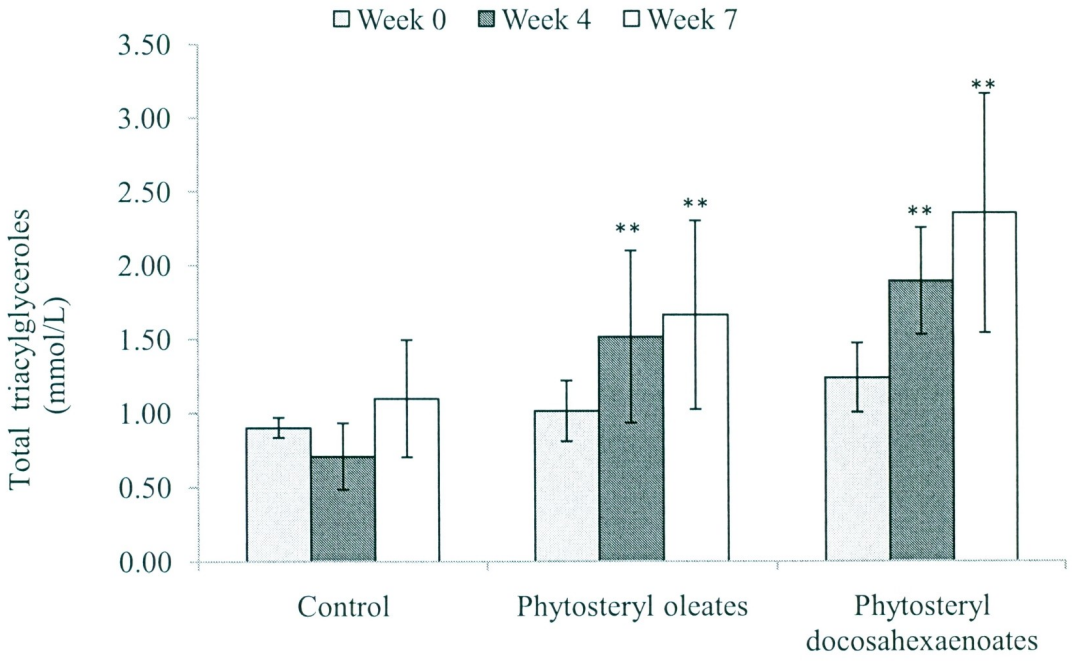


Figure 7-8. Total plasma triacylglycerols concentration (mean ± SD, mmol/l) of control, phytosteryl oleates treated and phytosteryl docosahexaenoates treated apo E-KO mice.

** $P < 0.05$ as compared to the control.

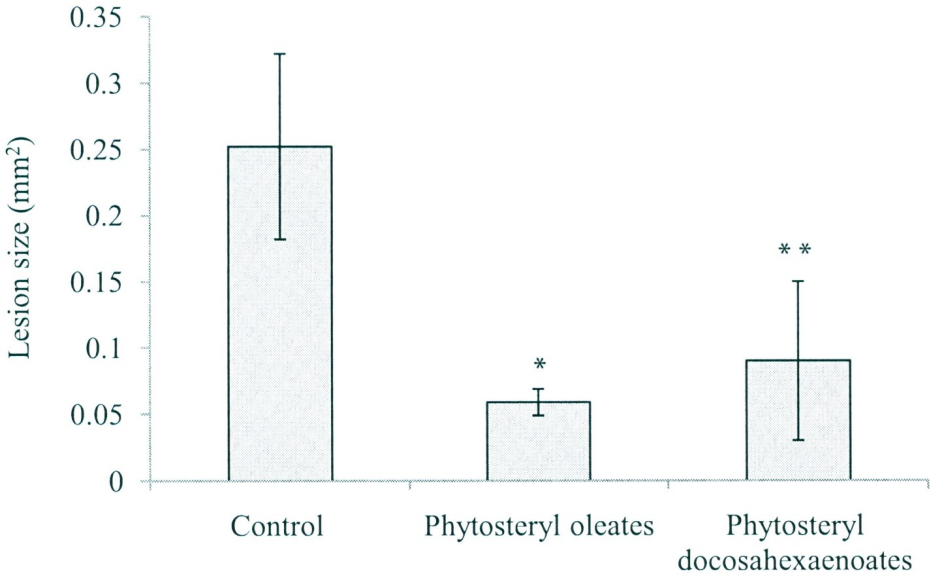


Figure 7-9. Morphometrical features of the atherosclerotic lesions in the aortic roots of control, phytosteryl oleates treated and phytosteryl docosahexaenoates treated apo E-KO mice. * $P < 0.01$ as compared to the control; ** $P < 0.05$ as compared to the control.

Chapter 8

Conclusions and Recommendations

Response surface methodology (RSM) was successfully employed to evaluate the effect of reaction time, enzyme load, and substrate mole ratio on the degree of esterification of phytosteryl caprylates. A face-centred CCD determined the optimized conditions as reaction time (9.25 hours), enzyme load (7.93%), and substrate mole ratio (caprylic acid to total phytosterols, 2.15), which led to a high conversion yield of 98% for phytosteryl caprylates. Spectroscopic methods and mass spectral studies were successfully employed to confirm the structures of phytosteryl caprylates.

The feasibility of a simple two-step chemoenzymatic synthesis of phytosteryl phenolates was successfully established in this work. An intermediate was first chemically produced and subsequently esterified with phytosterols via alcoholysis with lipase as a catalyst. The structures of phytosteryl phenolates were confirmed by Fourier-transform infrared (FTIR) and high performance liquid chromatography-mass spectroscopy/mass spectroscopy (HPLC-MS/MS) using atmospheric-pressure chemical ionization (APCI) under both positive and negative modes. The antioxidant activity of synthesized phytosteryl phenolates was evaluated by different assays, including DPPH radical scavenging capacity test, $ORAC_{FL}$ assay, bulk oil model system using the Rancimat test, β-carotene-linoleate model system, cooked pork model system, reducing power test, LDL oxidation and DNA scission assays. The results indicated that their

antioxidant activity was system-dependent and followed different antioxidant mechanisms. The large molecular size and relatively non-polar structures of phytosteryl phenolates may affect their partitioning in matrices and thus their accessibility and potential as antioxidants. The antioxidant activity of phytosteryl phenolates can be similarly explained by the formation of resonance-stabilized structures. Most phytosteryl phenolates possessed antioxidant activity which was superior to that of their phenolic acid counterparts in terms of ORAC value and efficacy in β-carotene-linoleate assay, suggesting their potential use as antioxidants in more lipophilic environments. However, this was not applicable to the bulk oil system as all phytosteryl phenolates showed lower antioxidant activity than their hydrophilic phenolic acid counterparts, in agreement with polar paradox theory. Phytosteryl phenolates with enhanced lipophilicity also exhibited higher antioxidant activities than their parent phenolic acids in scavenging peroxyl radicals, possibly due to combined electronic and steric effects. However, the reducing power of the derivatives was lower than that of their phenolic acid counterparts, which may be explained by their poor solubility in the aqueous test medium or micelle formation. The lipophilic derivatives tested possessed a higher antioxidant potential in inhibiting lipid oxidation in emulsion and muscle food model systems. In the DNA scission test, esterified phytosteryl phenolates showed either the same/or lower DNA retention ability against both the peroxyl and hydroxyl radicals with the exception of phytosteryl caffeates and sinapates, which showed enhanced DNA retention ability against hydroxyl radical. Phytosteryl caffeates also possess higher activity on DNA retention ability against peroxyl radical. Moderate inhibition activity of LDL oxidation

was observed for phytosteryl phenolates. The antioxidant activity of phytosteryl phenolates was similar or superior to that of their parent phenolic acid counterparts, suggesting their potential use as phenolic acid alternatives in the food industry. The approach presented here for preparation of phytosteryl phenolates may facilitate their industrial application as functional food ingredients and nutraceuticals. This would also facilitate further investigation of the biological properties of these products. The phytosteryl phenolates could also be potentially used as an ingredient for waterproof sunscreen products due to their strong UV absorption and relatively water-insoluble nature.

Enzymatic synthesis of phytosteryl oleates and docosahexaenoates was also successfully achieved. The structures of phytosteryl docosahexaenoates were confirmed by normal and reverse phase HPLC-MS and FTIR. Phytosteryl oleates and docosahexaenoates reduced plasma total cholesterol in apoE-deficient mice. The changes are associated with significant reductions in atherogensis. Thus they could be used as nutraceuticals or functional foods ingredients for cholesterol-lowering purposes, as confirmed by experiments. Moreover, results indicate that apoE-deficient mice model is a robust model with accelerated atherosclerosis and severe hypercholesterolemia, it significantly responds to cholesterol-lowering and antiatherogenic effects of phytosterol derivatives. However, phytosteryl oleates and docosahexaenoates exhibited no triacylglycerol-lowering effect in this animal model. Controversially they increased the total plasma triacylglycerol level in apoE-deficient mice. Therefore, other animal model may be employed to determine the triacylglycerol-lowering effect of these compounds.

Future studies may be focused on the following areas:

1. Examine the cholesterol-lowering effect of phytosterol caprylates and their potential antimicrobial properties.
2. Optimize the chemical synthesis of vinyl phenolates or alternatively searching for enzymatic methods to prepare them.
3. Optimize the synthesis of phytosteryl phenolates and to further study their biological activities, such as cholesterol-lowering, antimicrobial, antipolymerization, and antiinflammatory effects, among others.
4. Incorporate phytosteryl esters other than phytosteryl oleates and phytosteryl docosahexaenoates in the apo-E deficient mice studies to compare their cholesterol-lowering effects.
5. Identify the possibility of the natural existence of certain novel phytosteryl esters synthesized in this thesis work, such as phytosteryl sinapates in canola oil or seeds, phytosteryl docosahexaenoates in mussels or other marine plant sources.
6. Evaluate the effect of esterification on the stability of phytosterols and to determine the oxidation products of phytosterols and their corresponding esters.

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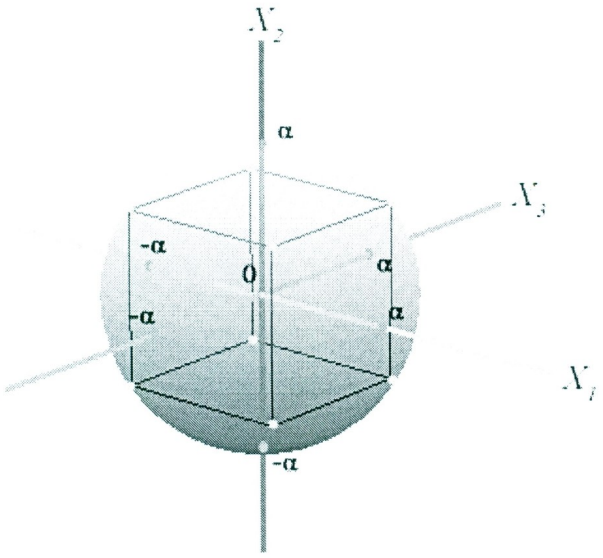
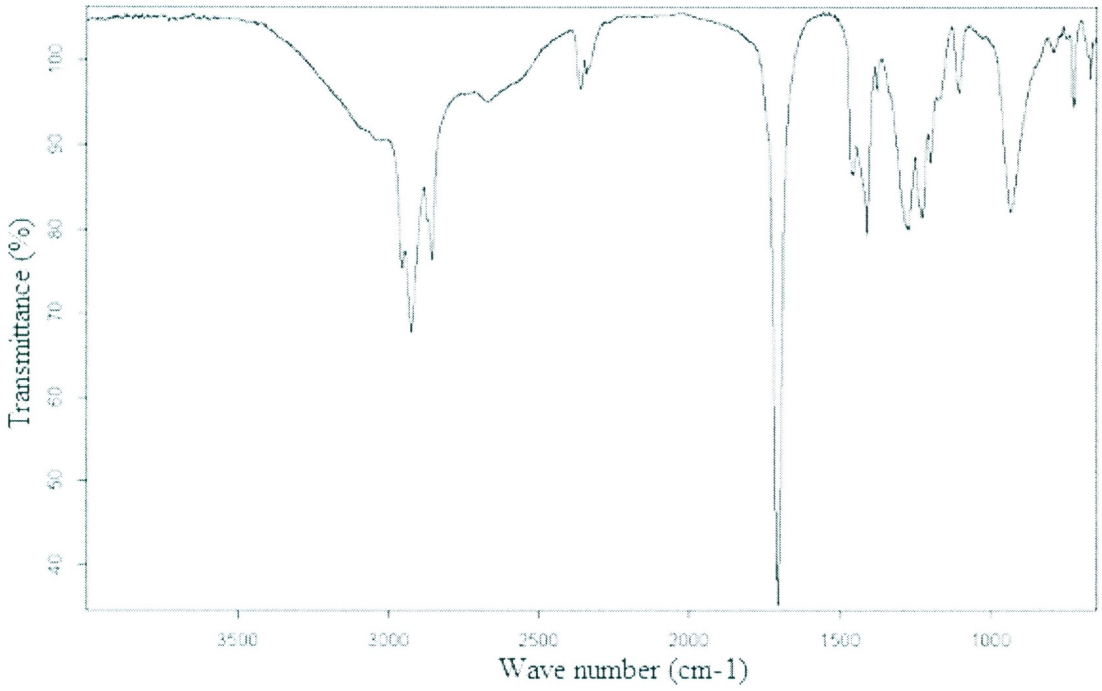
Appendix

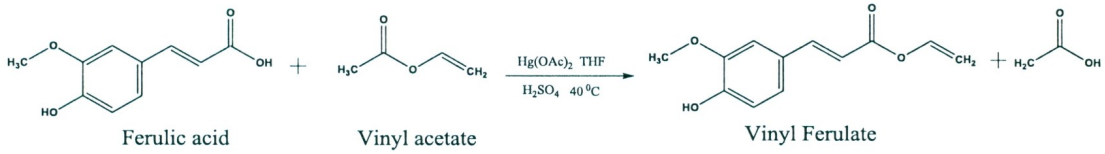


Appendix 3-1. Experimental points for a face-centred three variables and three level central composite design (CCD) (α=1).

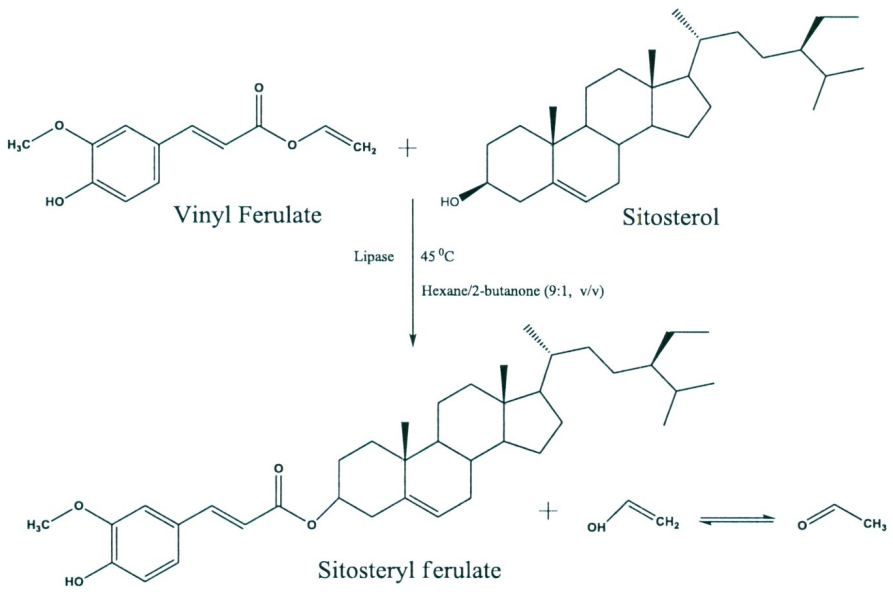


Appendix 4-1. IR spectrum of caprylic acid.

Step one

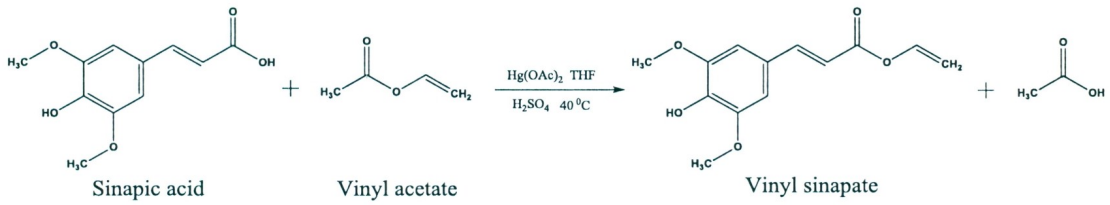


Step two

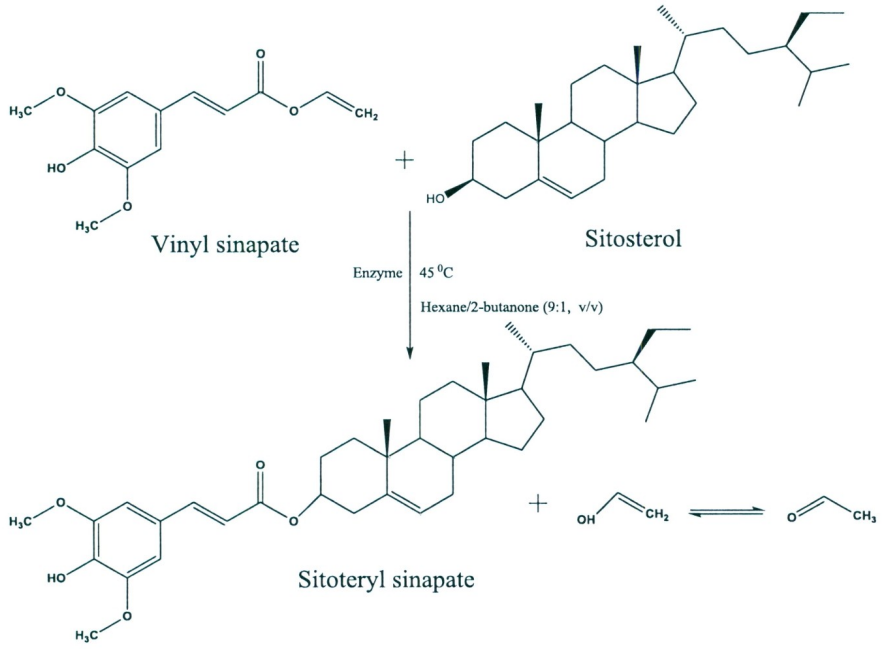


Appendix 5-1. Two step chemoenzymatic synthesis of phytosteryl ferulates.

Step one

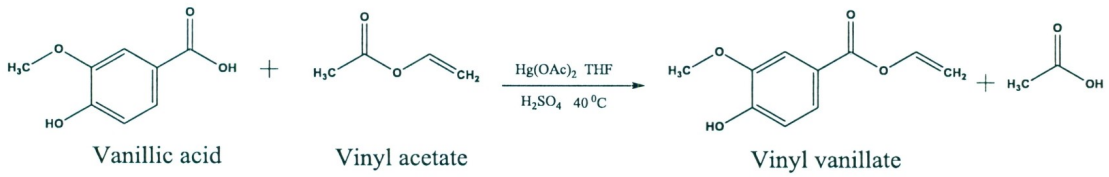


Step two



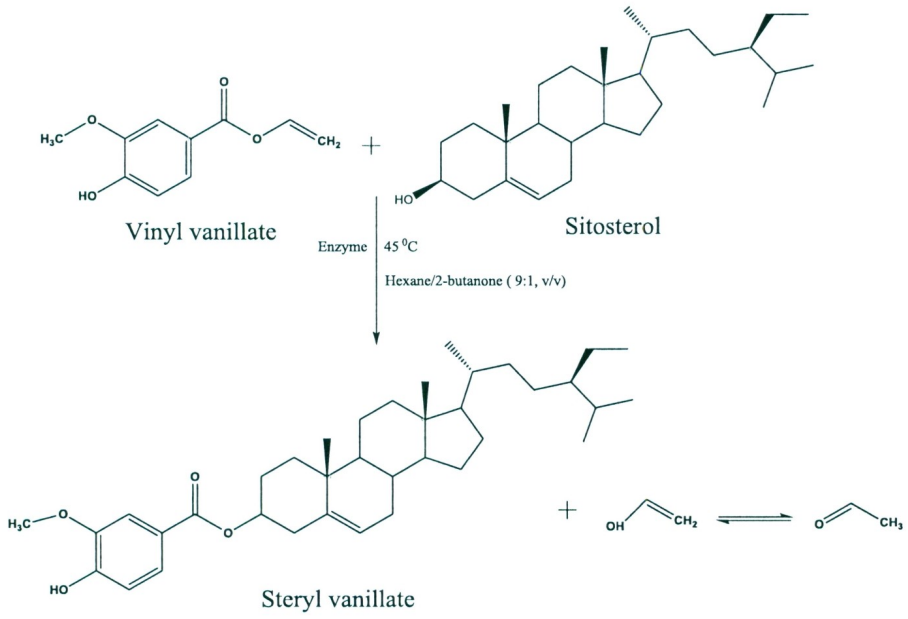
Appendix 5-2. Two step chemoenzymatic synthesis of phytosteryl sinapates.

Step one



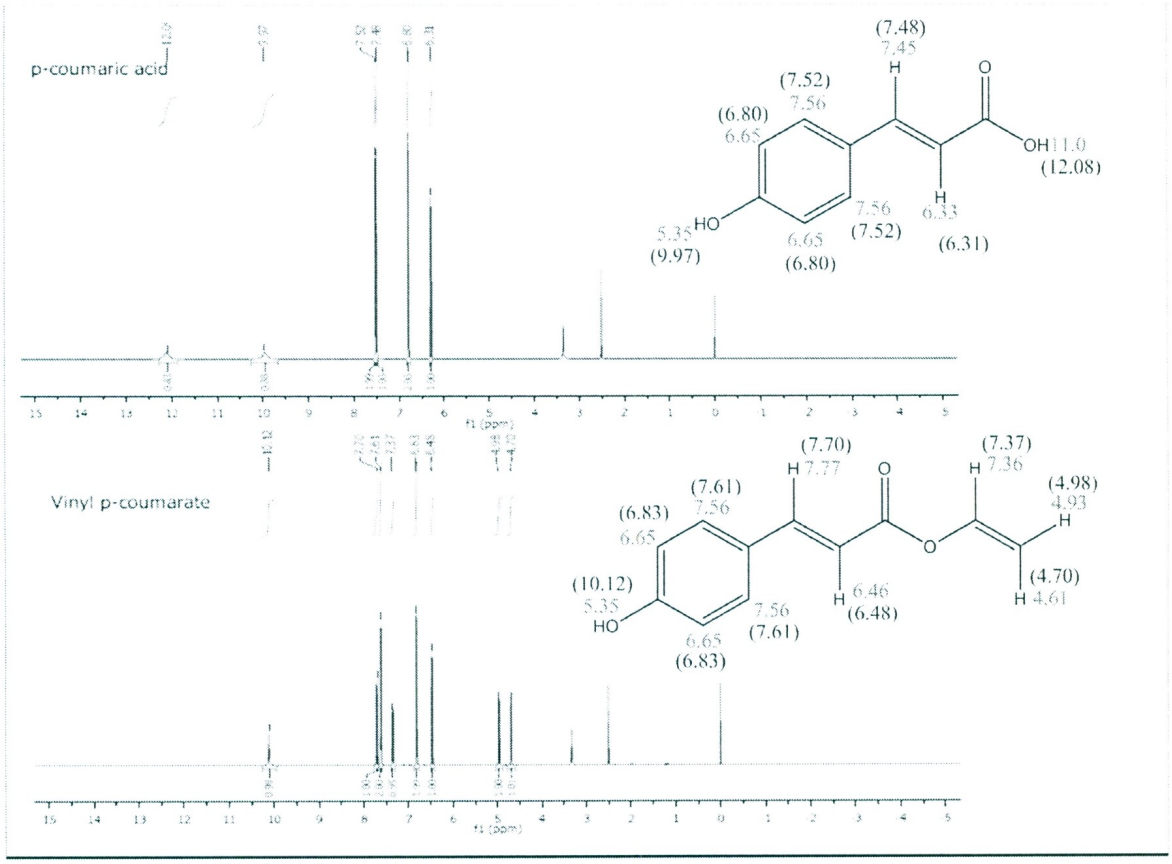
Vanillic acid Vinyl acetate Vinyl vanillate

Step two



Vinyl vanillate Sitosterol Steryl vanillate

Appendix 5-3. Two step chemoenzymatic synthesis of phytosteryl vanillates.



Appendix 5-4. The ^{1}H-NMR spectra of *p*-coumaric acid and vinyl *p*-coumarate. Structures provide the assignment for the predicted and experimental (in bracket) chemical shifts.

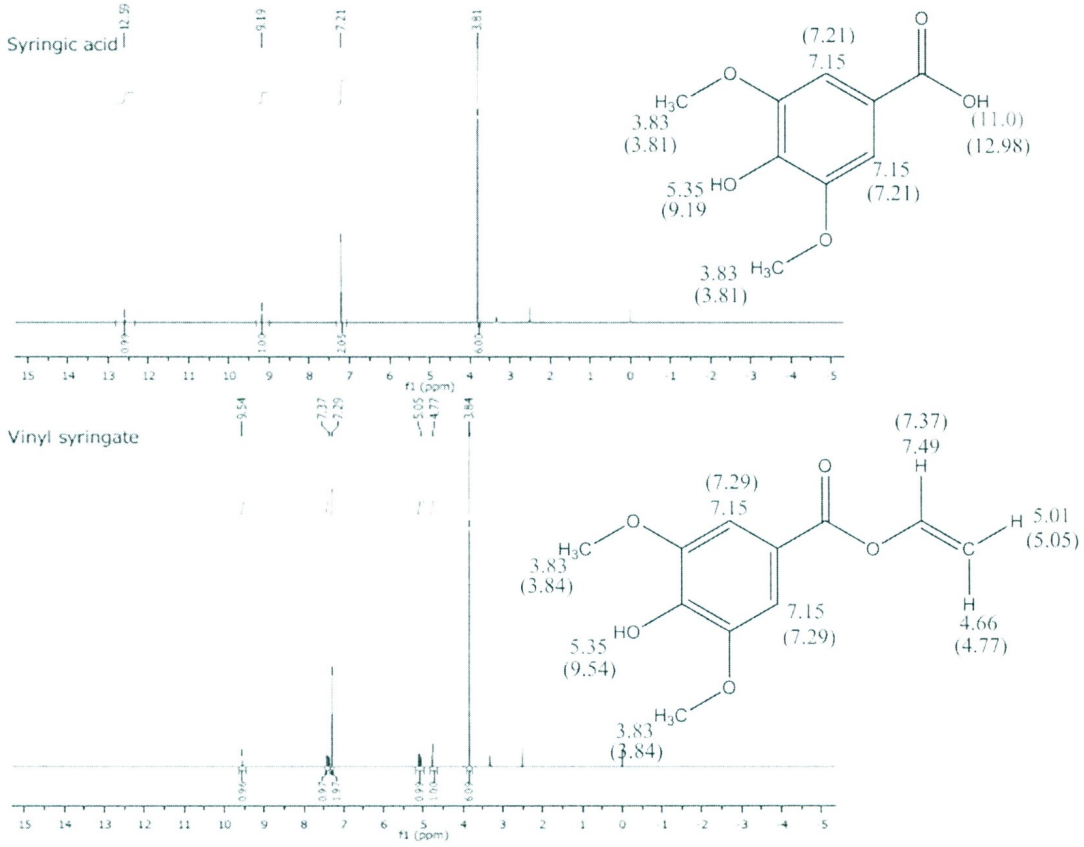


Figure 5-5. The ^{1}H-NMR spectra of syringic acid and vinyl syringate. Structures provide the assignment for the predicted and experimental (in bracket) chemical shifts.

Appendix 6-1. DPPH radical scavenging ability of phenolic acids and its derivatives (μmol trolox equivalents/mmol of sample).

Samples	Phenolic acids	Vinyl phenolates	Phytosteryl phenolates
CA and its derivatives	934.96 ± 24.61 ^{Ba}	1038.63 ± 45.20 ^{Aa}	1112.57 ± 45.09 ^{Aa}
FA and its derivatives	213.47 ± 3.61 ^{Bc}	159.28 ± 4.57 ^{Cc}	265.78 ± 19.71 ^{Ab}
SA and its derivatives	547.55 ± 2.46 ^{Ab}	451.24 ± 5.17 ^{Bb}	261.7 ± 4.85 ^{Cb}
VA and its derivatives	20.78 ± 3.85 ^{Ad}	21.45 ± 6.67 ^{Ad}	18.16 ± 2.53 ^{Ac}

[a]Results reported are mean values of three determinations ± SD. Means in each column sharing the same superscript are not significantly ($P > 0.05$) different from one another.

[A]Results reported are mean values of three determinations ± SD. Means in each row sharing the same superscript are not significantly ($P > 0.05$) different from one another.

CA, caffeic acid; FA, ferulic acid; SA, sinapic acid; and VA, vanillic acid.

Appendix 6-2. Lipophilic-ORAC$_{FL}$ values for phenolic acids and its derivatives (μmol trolox equivalents/μmol of sample).

Samples	Phenolic acids	Vinyl phenolates	Phytosteryl phenolates
CA and derivatives	5.81 ± 0.42^{Cb}	10.95 ± 0.26^{Ba}	13.99 ± 0.83^{Ac}
FA and derivatives	9.01 ± 0.47^{Ba}	10.42 ± 0.73^{Bab}	16.49 ± 1.25^{Ab}
SA and derivatives	8.83 ± 0.19^{Ba}	9.31 ± 0.98^{Bb}	19.71 ± 1.67^{Aa}
VA and derivatives	4.00 ± 0.22^{Cc}	6.70 ± 0.37^{Bc}	8.54 ± 0.30^{Ad}

[a]Results reported are mean values of four determinations ± SD. Means in each column sharing the same superscript are not significantly ($P > 0.05$) different from one another.

[A]Results reported are mean values of four determinations ± SD. Means in each row sharing the same superscript are not significantly ($P > 0.05$) different from one another.

CA, caffeic acid; FA, ferulic acid; SA, sinapic acid; and VA, vanillic acid.

Appendix 6-3. Protection factor of phenolic acids and its derivatives in stripped corn oil.

Samples	Phenolic acids	Vinyl phenolates	Phytosteryl phenolates
CA and derivatives	2.08 ± 0.13^{Aa}	2.10 ± 0.03^{Aa}	1.58 ± 0.01^{Ba}
FA and derivatives	0.94 ± 0.06^{Bbc}	1.13 ± 0.02^{Ac}	0.99 ± 0.05^{Bc}
SA and derivatives	1.14 ± 0.05^{Bb}	1.30 ± 0.06^{Ab}	1.17 ± 0.03^{Bb}
VA and derivatives	0.87 ± 0.07^{Bc}	1.03 ± 0.05^{Ac}	0.99 ± 0.07^{ABc}

[a]Results reported are mean values of three determinations ± SD. Means in each column sharing the same superscript are not significantly ($P > 0.05$) different from one another.

[A]Results reported are mean values of three determinations ± SD. Means in each row sharing the same superscript are not significantly ($P > 0.05$) different from one another.

CA, caffeic acid; FA, ferulic acid; SA, sinapic acid; and VA, vanillic acid.

Appendix 6-4. Inhibition effect of phenolic acids and its derivatives against β-carotene bleaching (%).

Samples	Phenolic acids	Vinyl phenolates	Phytosteryl phenolates
CA and derivatives	27.05 ± 1.21^{Ca}	38.5 ± 4.85^{Ba}	55.77 ± 2.63^{Aa}
FA and derivatives	22.79 ± 1.32^{Aab}	20.55 ± 3.77^{Ab}	20.07 ± 2.43^{Ab}
SA and derivatives	26.94 ± 3.92^{Cb}	46.32 ± 3.71^{Ba}	57.10 ± 1.65^{Aa}
VA and derivatives	-1.07 ± 1.53^{Bc}	-5.92 ± 1.84^{Cc}	2.46 ± 1.79^{Ac}

[a]Results reported are mean values of five determinations ± SD. Means in each column sharing the same superscript are not significantly ($P > 0.05$) different from one another.

[A]Results reported are mean values of five determinations ± SD. Means in each row sharing the same superscript are not significantly ($P > 0.05$) different from one another.

CA, caffeic acid; FA, ferulic acid; SA, sinapic acid; and VA, vanillic acid.

Appendix 6-5. Reducing power of phenolic acids and its derivatives (μmol ascorbic acid equivalents/mmol of sample).

Samples	Phenolic acids	Vinyl phenolates	Phytosteryl phenolates
CA and derivatives	2173.28 ± 53.70 ^{Aa}	1989.75 ± 44.75 ^{Aa}	1013.86 ± 33.12 ^{Ba}
FA and derivatives	1050.57 ± 72.50 ^{Ac}	621.48 ± 38.49 ^{Bc}	119.93 ± 6.27 ^{Cc}
SA and derivatives	1729.00 ± 94.87 ^{Ab}	1492.31± 25.06 ^{Bb}	282.89 ± 11.19 ^{Cb}
VA and derivatives	319.92 ± 6.27 ^{Ad}	-2.22 ± 0.00 ^{Cd}	8.86 ± 2.24 ^{Bd}

[a]Results reported are mean values of three determinations ± SD. Means in each column sharing the same superscript are not significantly ($P > 0.05$) different from one another.

[A]Results reported are mean values of three determinations ± SD. Means in each row sharing the same superscript are not significantly ($P > 0.05$) different from one another.

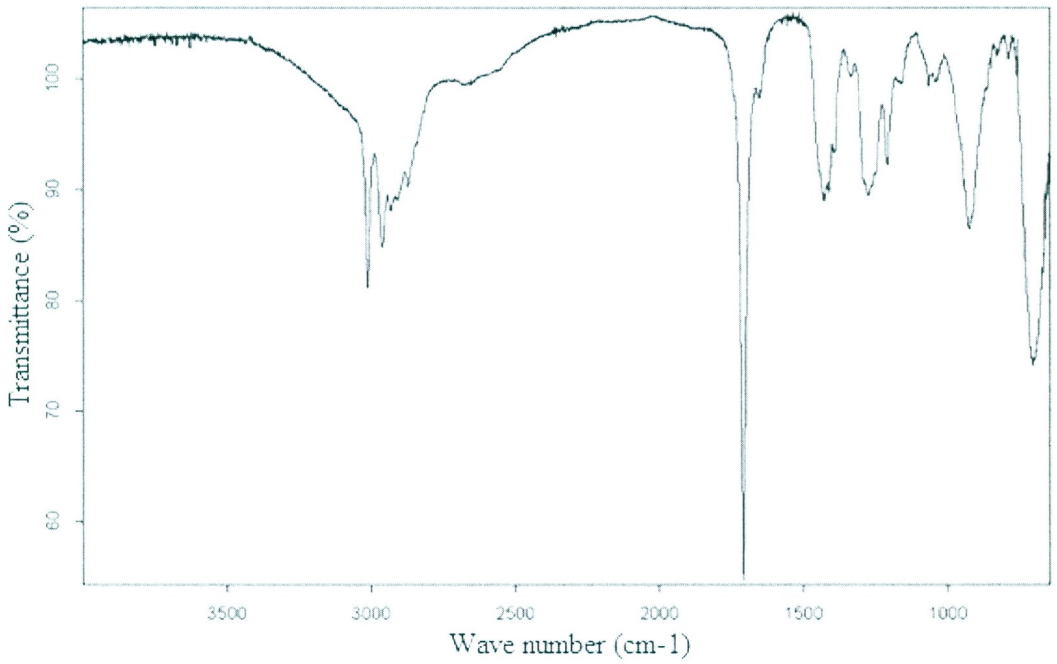
CA, caffeic acid; FA, ferulic acid; SA, sinapic acid; and VA, vanillic acid.

Appendix 6-6. Inhibition of LDL cholesterol oxidation (%) by free phytosterols and phytosteryl phenolates.

Samples	Inhibition of LDL cholesterol oxidation (%)
Phytosteryl caffeates	59.80 ± 3.62 [b]
Phytosteryl ferulates	42.27 ± 5.93 [c]
Phytosteryl sinapates	54.67 ± 9.79 [bc]
Phytosteryl vanillates	55.10 ± 5.51 [bc]
Free phytosterol	48.11 ± 7.78 [bc]
Ferulic acid	99.08 ± 2.53 [a]

[a]Results reported are mean values of three determinations ± SD.

Means in each column sharing the same superscript are not significantly ($P > 0.05$) different from one another.



Appendix 7-1. IR spectrum of docosahexaenoic acid.