Mass Spectrometric Methods for Analysis of Sterols and Steryl Esters in Biological Samples

by

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Abstract

The composition of free sterols in cultivated blue mussels obtained commercially and from laboratory feeding experiments has been investigated. Total lipids were extracted from mussels using a modified Folch procedure followed by isolation of free sterols using column chromatography. Sterols were identified using GC/MS and quantified using (GC/MS-SIM) and GC/FID. The most abundant free sterols in commercially cultivated mussels were cholesterol, desmosterol, and brassicasterol. Mussels were fed on algae or fish waste for six months. The concentrations of sterols in fish waste-fed mussels were compared with those in algae-fed mussels. However, fish waste-fed mussels had significantly higher cholesterol concentrations compared to algae-fed mussels. Mussels fed algae had significantly higher campesterol compared to fish waste-fed mussels.

Free sterols are neutral molecules that are difficult to ionize by MALDI or ESI. Therefore, in order to increase their ionization efficiency, sterols were converted into their corresponding picolinyl esters, *N*-methylpyridyl ethers and sulphated esters. Using MALDI-MS, sterol picolinyl esters were identified as $[M+Na]^+$, the signal being significantly enhanced after the addition of sodium acetate. Sterol *N*-methylpyridyl ethers were detected as $[M]^+$ while sulphated sterols were detected as $[M-H]^-$. The ester bonds of picolinyl and sulphated esters cleaved during MALDI-CID MS/MS resulting in diagnostic fragments at m/z 146 and 97, respectively.

Picolinyl esters of sterols were also analyzed using ESI/APCI-QIT MS^n. The Picolinyl esters were detected as $[M+H]^+$ in ESI and APCI sources. The ester bonds of picolinyl esters cleaved during CID MS^2, resulting in diagnostic fragments corresponding to steryl cation moieties $[M+H-C_6H_5NO_2]^+$. The CID MS^3 of $[M+H]^+ \rightarrow [M+H-C_6H_5NO_2]^+$ of picolinyl esters was found to be useful for structural elucidation and for distinguishing among steryl isomers.

Steryl esters were identified in margarine and corn using ESI-QIT MS^n. Sterols and other lipids extracted from samples using hexane were subjected to solid phase extraction. The steryl ester fraction was eluted with hexane: diethyl ether (98:2, v/v). Fatty acid steryl esters were detected as $[M+NH_4]^+$ using ammonium acetate as dopant. Steryl esters, including molecular isomers, were identified using ESI-QIT MS^2. ESI-QIT MS^3 was carried out on the intact steryl fragmentation cation. The resulting CID spectra of the steryl cation were found to be unique and similar to those from APCI-QIT MS^2 CID of free sterol standards.

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Dedication

To the soul of my father
To my mother, brothers and sisters

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List of Abbreviations

APCI: atmospheric pressure chemical ionization

BSTFA: *N,O*-bis(trimethylsilyl) trifluoroacetamide

CHCA: α-cyano-4-hydroxycinnamic acid

CID: collision-induced dissociation

DESI: desorption electrospray ionization

DHB: 2,5-Dihydroxybenzoic acid

EI: electron impact

ELSD: evaporative light scattering detector

ESI: electrospray ionization

FFA: free fatty acids

GC-MS: gas chromatography mass spectrometry

HPLC-MS: high performance liquid chromatography- mass spectrometry

HPTLC: high performance thin layer chromatography

IR: infrared

LDL: low-density lipoprotein

LOD: limit of detection

LOQ: limit of quantification

m/z: mass-to-charge ratio

MALDI-TOF: matrix-assisted laser desorption/ionization time-of-flight

MS/MS: Tandem mass spectrometry

MS^2: the second order of tandem mass spectrometry

MS^n: The n order of tandem mass spectrometry

PAHs: polycyclic aromatic hydrocarbons

PL: phospholipids

QIT: quadrupole ion trap

QqQ: triple quadrupole

RSD: relative standard deviation

S/N: signal-to-noise ratio

SA: sinapinic acid

SIM: selected-ion monitoring

SPE: solid phase extraction

SRM: selected reaction monitoring

TAG: triacylglycerols

THAP: 2,4,6-trihydroxyacetophenone monohydrate

TLC-FID: thin layer chromatography-flame ionization detection

TMCS: trimethylchlorosilane

TMS: trimethylsilyl ether

UV: ultraviolet

Chapter One: Introduction and Overview

1.1 Lipids and their chemical structures

Lipids comprise a wide range of biomolecules ranging from polar to nonpolar compounds. However, lipids are insoluble in water but soluble in organic solvents [1]. Lipids include several classes such as aliphatic hydrocarbons, polycyclic aromatic hydrocarbons (PAHs), wax esters, steryl esters, methyl esters, acylated glycerol ethers, triacylglycerols, free fatty acids, aliphatic alcohols, sterols, diacyglycerols, monoacyglycerols, glycoglycerolipids, pigments and phospholipids [2]. Figure 1.1 shows the chemical structures of some of these classes.

Lipids are considered the most abundant biomolecules present in the brain constituting 50% of its dry weight. Moreover, lipids are considered one of the most common components of cellular membranes and they have a significant role in signal transduction. Lipids serve as a reservoir of excess energy in biological systems [3, 4]. Physiological energy can be obtained from the oxidation of fatty acids and triacyglycerols inside biological systems [5]. Triacylglycerols which are present in vegetable oils are considered one of the renewable resources of energy [5]. Also, lipids are important in nutrition where studies have shown that sterols have a potential for reducing the level of cholesterol in blood [6].

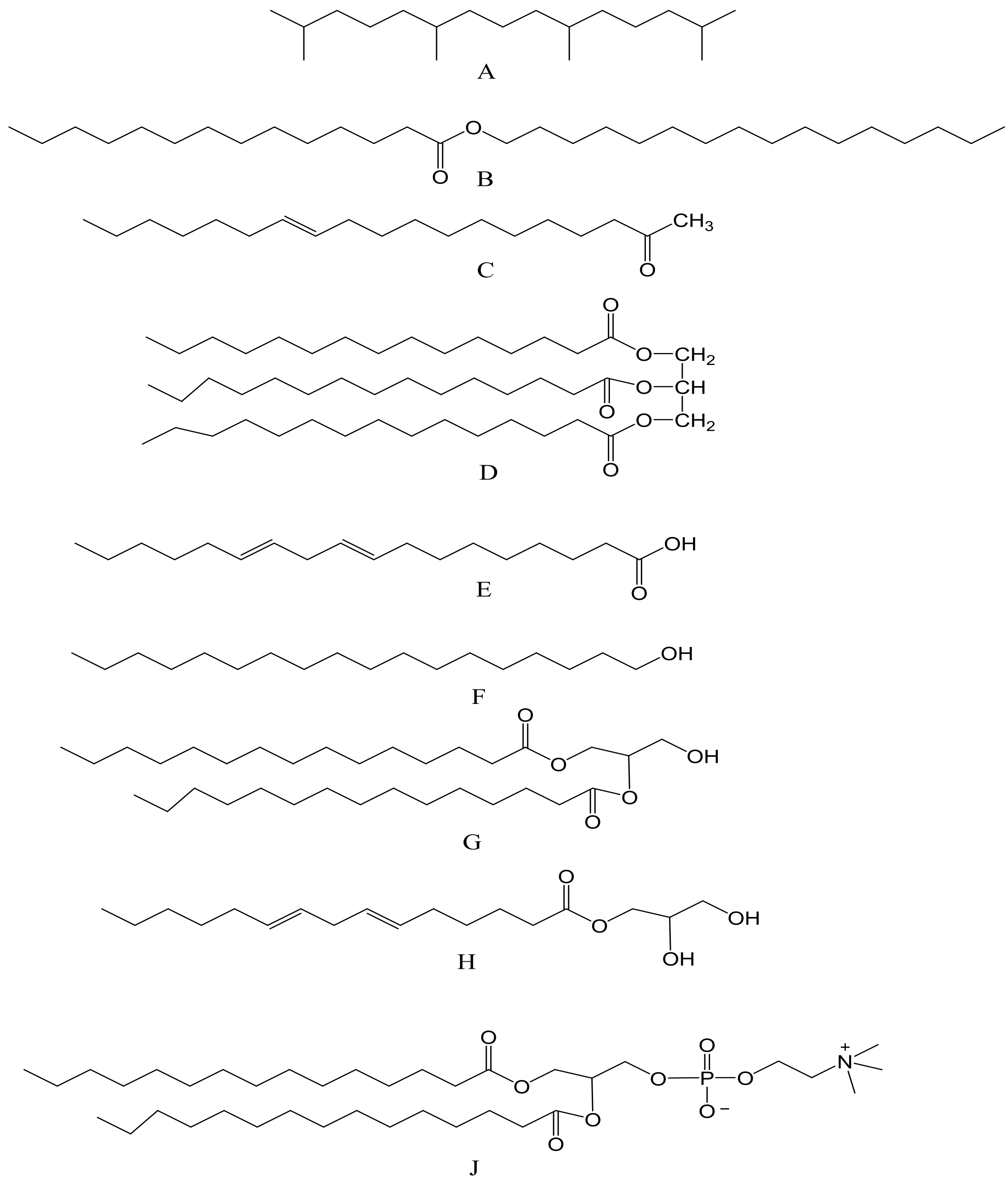


Figure 1.1. Classes of lipids and their structures, (A) aliphatic hydrocarbon, (B) wax ester, (C) fatty acid methyl ester, (D) triacylglycerol, (E) free fatty acid, (F) aliphatic alcohol, (G) 1,2-diacylglycerol, (H) monoacylglycerol, (J) phospholipid.

1.2 Chemistry of sterols

Sterols are natural substances present in plants, animals and fungi. β-sitosterol, stigmasterol and 24-methylenecholesterol occur abundantly in plants [7], while in animals it is mainly cholesterol, and in fungi, ergosterol [8]. Plant-derived sterols are often called phytosterols [9, 10], which belong to a type of natural product called "triterpenes". The triterpene family contains more than 100 different phytosterols and there are more than 4000 other types of triterpenes [11]. Sterols can be naturally synthesized via cyclization of squalene epoxide [12] giving a tetracyclic structure that can be subjected to many transformations to give a wide variety of chemical compounds which have a common steroid nucleus (perhydrocyclopentanophenanthrene), and which differ in the structure of the alkyl side chain [13].

Two numbering systems have been introduced by IUPAC. Figure 1.2 shows nomenclatures (numbering system) of sterols according to IUPAC-IUB 1976 and IUPAC-IUB 1989. The form according to IUPAC-IUB 1976 is still used in the literature more than the later one [11] and therefore will be used here.

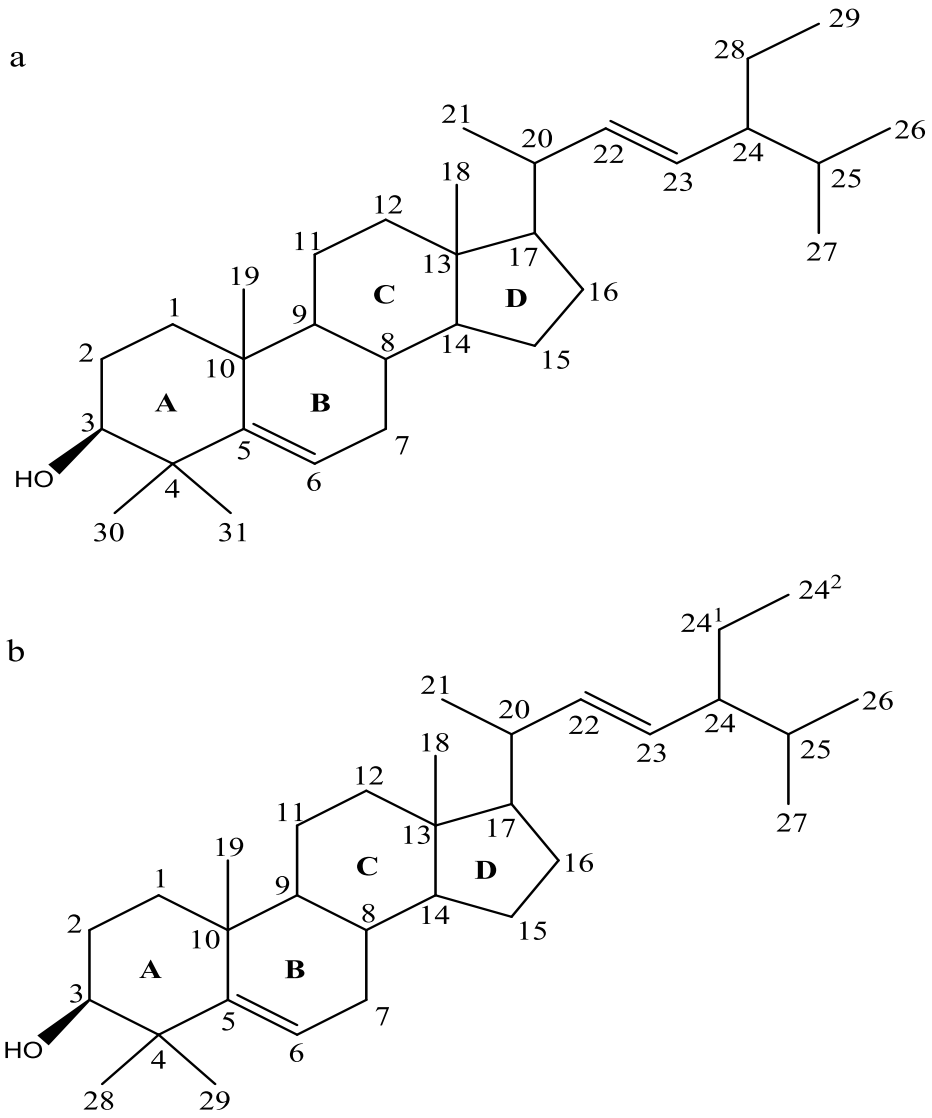


Figure 1.2. Sterol structure and its numbering system according to (a) IUPAC-IUB 1976 and (b) IUPAC-IUB 1989

Cholesterol contains 27 carbon atoms while other sterols including phytosterols contain an additional methyl or ethyl group at carbon number 24 in the alkyl side chain and/or at carbon number 4 in the sterol nucleus (Figure 1.3). Sterols are classified into three groups according to the number of methyl groups on carbon number 4. These groups include 4-dimethyl sterols which contain two methyl groups on carbon number 4, 4-monomethyl sterols with one methyl group, and 4-desmethyl sterols that do not contain any methyl groups attached to carbon number 4. Most common sterols belong to the 4-desmethyl group, such as cholesterol, β-sitosterol and stigmasterol [9]. Sterols are also classified depending on the location of the double bond in the sterol nucleus into Δ^5-sterols, Δ^7-sterols and $\Delta^{5,7}$-sterols. In Δ^5-sterols, the double bond is located between carbons 5 and 6 whereas in Δ^7 the double bond exists between carbons 7 and 8, and in $\Delta^{5,7}$-sterols, the two double bonds are located in both of these positions. However, sterols can contain another double bond in the alkyl side chain either between carbons 22 and 23 such as in brassicasterol, or between 24 and 25 such as in desmosterol or between carbons 24 and 28 such as in fucosterol [10].

The substituent methyl or ethyl group at carbon 24 converts this position to a chiral center; therefore, two epimers can be found. The chirality is designated by the symbols α and β where α is equivalent to R and β is equivalent to S. 24-methyl sterol epimers could be R or S [10]. Campesterol and epibrassicasterol are examples of 24α epimers while, 22-dihydrobrassicasterol and brassicasterol are examples of 24β epimers. However, the ethyl substituent at carbon 24 could be unsaturated and, in this case, the ethylidene group could be *cis* which is represented by 24Z (isofucosterol) or *trans* which is designated by 24E

(fucosterol) [9]. Also, α and β symbols can be used to show whether the substituent is located below or above the plane of the sterol nucleus since α reveals that the substituent is located below the plane and β above the plane. i.e., 5α in 5α-cholestanol reveals that the H substituent at carbon 5 is located below the plane of the saturated sterol nuclei [14].

Sterols can be designated by their trivial (common) name or by their systematic name. As an example, the systematic name of cholesterol (trivial name) is expressed as cholest-5-en-3β-ol which indicates that the double bond is located between carbons 5 and 6, and the hydroxyl group is located at carbon number 3 above the plane of sterol nucleus.

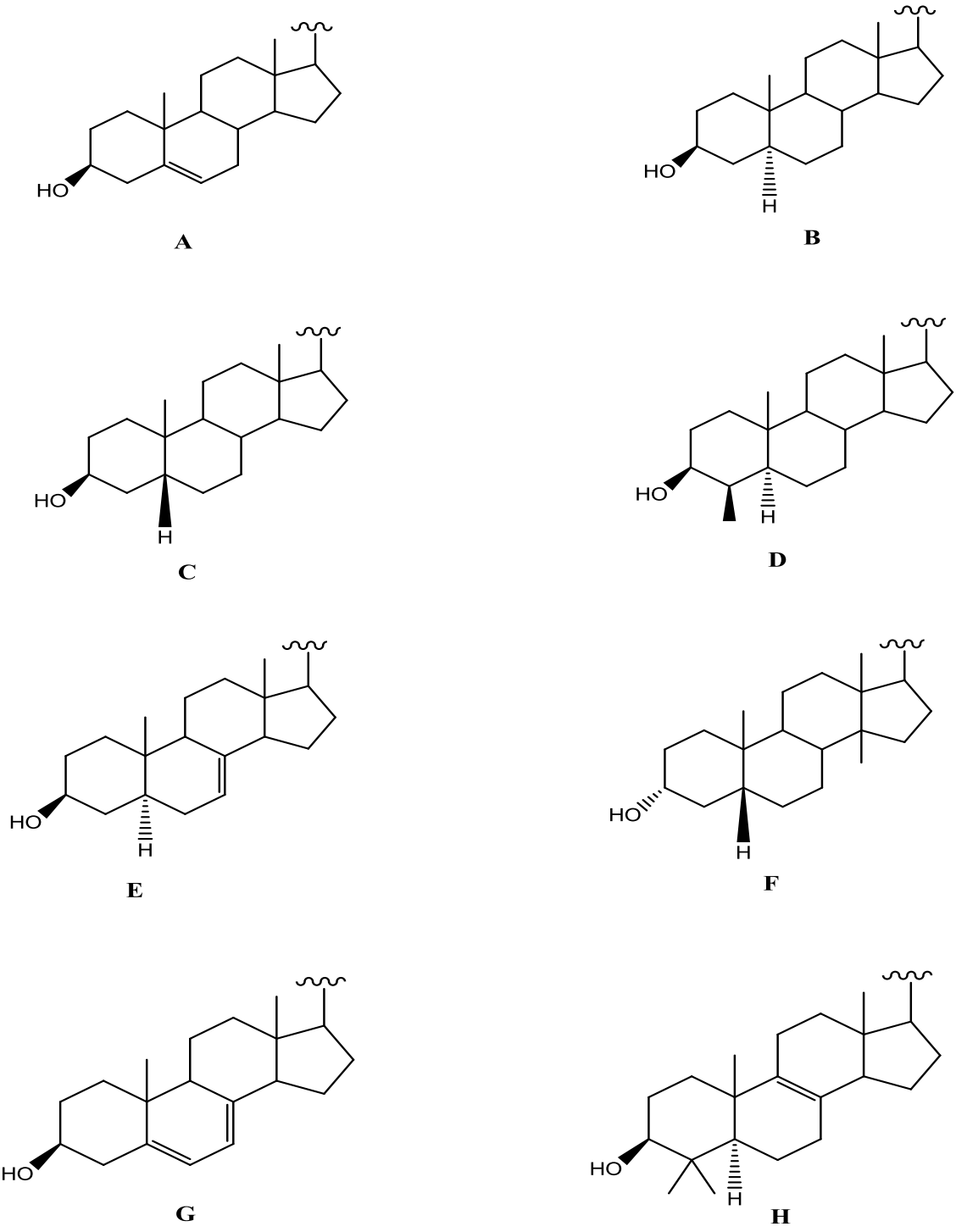


Figure 1.3. The most common sterol chemical structures. Letters from A to H show the structures of sterol nucleus

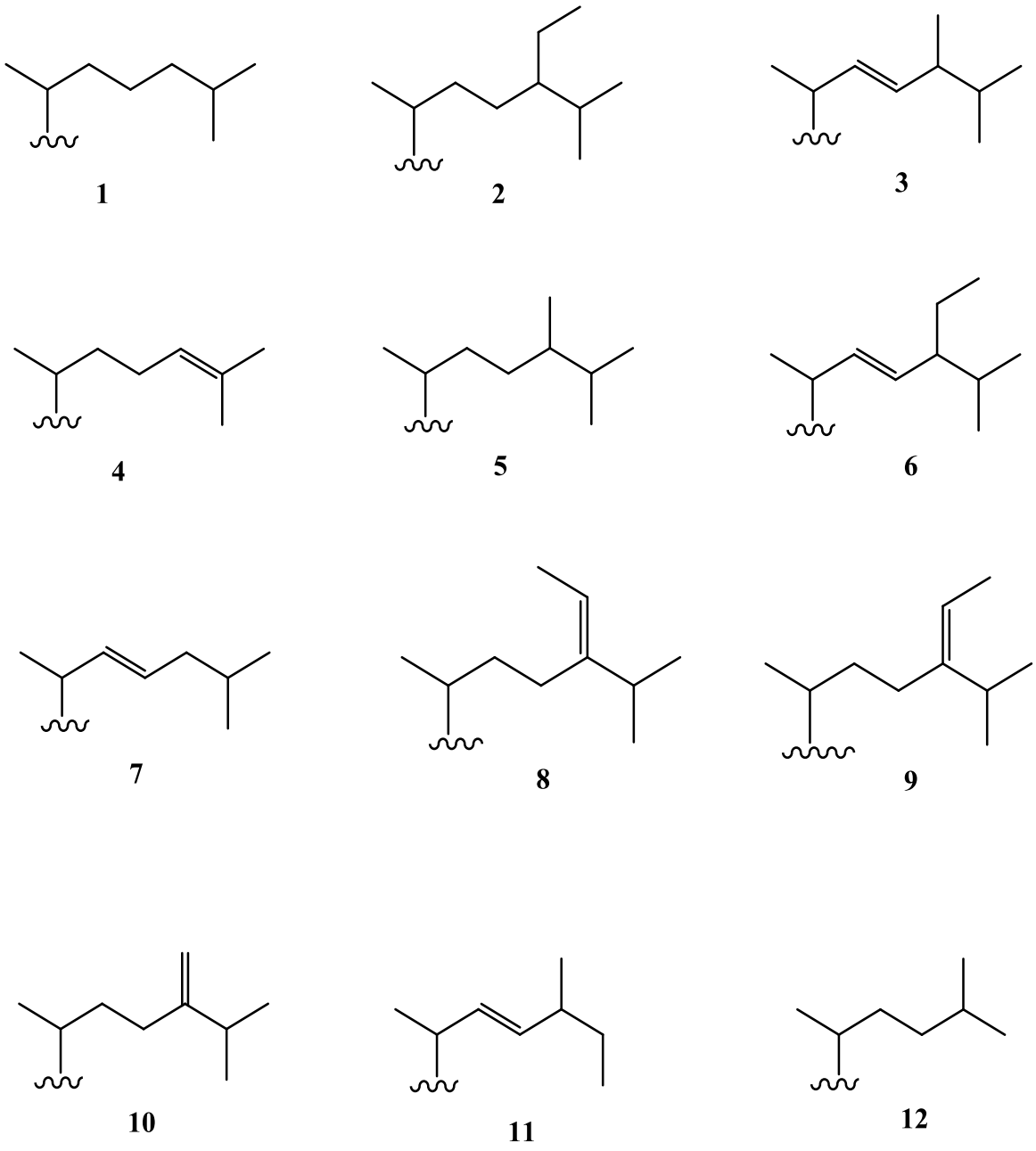


Figure 1.3 (Continued). The most common sterol structures. The numbers from 1-12 show the structures of sterol alkyl side chains

1.3 Abbreviation system in sterols

Sometimes abbreviations can be used to simplify the names of sterols. The form 24-Me-28$\Delta^{5,22E}$ can be used instead of 24-methyl cholesta-5,22(E)-dien-3β-ol. This abbreviation indicates that this sterol contains 28 carbon atoms with a methyl group substituent at carbon number 24. The symbol $\Delta^{5,22E}$ reveals that this sterol possesses 2 double bonds, one of them between carbons 5 and 6 and the other between carbons 22 and 23 with E configuration. Cholesterol can be abbreviated as 27Δ^{5} which indicates that cholesterol contains 27 carbon atoms and a double bond between carbons 5 and 6. The prefix "24-nor" in 24 nor-26Δ^{22E} reveals that carbon number 24 is deleted from the sterol containing 27 carbon atoms [14]. Figure 1.3 shows the chemical structures of most common sterols and Table 1.1 shows the abbreviations of these sterols along with their trivial and systematic names [14].

Table 1.1. Trivial and systematic names of most common sterols along with their abbreviations

	Trivial name	Systematic name	Abbreviation
A1	cholesterol	Cholest-5-en-3β-ol	$27\Delta^{5}$
B1	cholestanol	5α-cholestan-3β-ol	$27\ \Delta^{0}$
A4	desmosterol	Cholesta-5,24-dien-3β-ol	$27\ \Delta^{5,24}$
G3	ergosterol	24-methylcholesta-5,7,22E-trien-3β-ol	$24Me\text{-}28\Delta^{5,7,22E}$
A11	occelasterol	24-methyl-27-norcholesta-5,22(E)-dien-3β-ol	$24Me\text{-}27nor\text{-}27\Delta^{5,22E}$
A8	fucosterol	24-ethylcholesta-5,24(28)(E)-dien-3β-ol	$24Et\text{-}29\ \Delta^{5,24(28)E}$
A9	isofucosterol	24-ethylcholesta-5,24(28)(Z)-dien-3β-ol	$24Et\text{-}29\ \Delta^{5,24(28)Z}$
B8	fucostanol	24-ethylcholest-24(28)(E)-en-3β-ol	$24Et\text{-}29\ \Delta^{24(28)E}$
B9	isofucostanol	24-ethylcholest-24(28)(Z)-en-3β-ol	$24Et\text{-}29\Delta^{24(28)Z}$
A5	campesterol	24-methylcholest-5-en-3β-ol	$24Me\text{-}28\Delta^{5}$
A12	24-nordehydrocholesterol	24-norcholesta-5,22(E)-dien-3β-ol	$24\text{-}nor\text{-}26\Delta^{5,22E}$
B12	24-nordehydrocholestanol	24-nor-5α-cholesta-22(E)-en-3β-ol	$24nor\text{-}26\Delta^{22E}$
A7	*trans*-22dehydrocholesterol	Cholesta-5,22(E)-dien-3β-ol	$27\Delta^{5,22E}$
B7	*trans*-22dehydrocholestanol	5α-cholesta-22(E)-dien-3β-ol	$27\Delta^{22E}$
A6	stigmasterol	24-ethylcholesta-5,22(E)-dien-3β-ol	$24Et\text{-}29\Delta^{5,22E}$
A2	β-sitosterol	24-ethylcholest-5-en-3β-ol	$24Et\text{-}29\Delta^{5}$
A10	24-methylenecholesterol	24-methylcholesta-5,24(28)-dien-3β-ol	$24Me\text{-}28\Delta^{5,24(28)}$
B10	24-methylenecholestanol	24-methylcholest-24(28)-en-3β-ol	$24Me\text{-}28\Delta^{24(28)}$
A3	brassicasterol	24-methylcholesta-5,22(E)-dien-3β-ol	$24Me\text{-}28\Delta^{5,22E}$

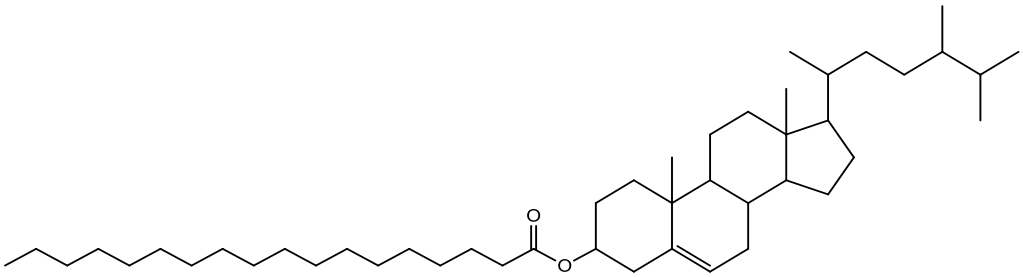
Table 1.1. (Continued)

B3	brassicastanol	24-methyl-5α-cholest-22(E)-en-3β-ol	24Me-28Δ^{22E}
C1	coprostanol	5β-cholestan-3β-ol	5β-27Δ^{0}
F1	*epico*prostanol	5β-cholestan-3α-ol	3α,5β-27Δ^{0}
A6	ethylcholesta-5,22E-dienol	24-ethylcholesta-5,22(E)-dien-3β-ol	24Et-29$\Delta^{5,22E}$
B6	ethylcholest-22E-enol	24-ethyl-5α-cholest-22(E)-en-3β-ol	24Et-29Δ^{22E}
B2	ethylcholestanol	24-ethyl-5α-cholestan-3β-ol	24Et-29Δ^{0}
C2	ethylcoprostanol	24-ethyl-5β-cholestan-3β-ol	24Et-5β-29Δ^{0}
B5	24-methylcholestanol	24-methyl-5α-cholestan-3β-ol	24Me-28Δ^{0}
D1	4-methylcholestanol	4α-methyl-5α-cholestan-3β-ol	4αMe-28Δ^{0}
E1	lathosterol	5α-cholest-7-en-3β-ol	27Δ^{7}
H4	----------	4,4-dimethyl-5α-cholesta-8,24-dien-3β-ol	4,4diMe-29$\Delta^{8,24}$

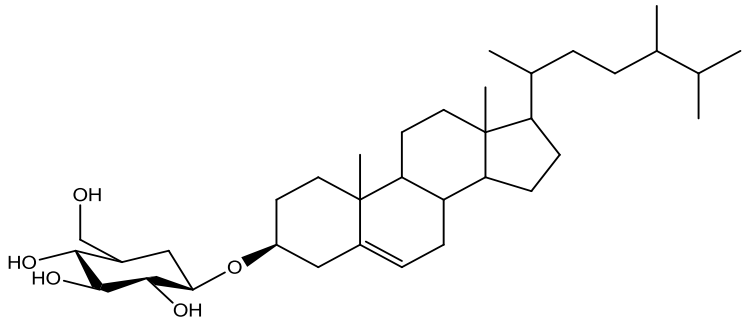
1.4 Conjugated sterols

In addition to the free form, sterols can be found in nature conjugated to other natural chemical compounds. Conjugated sterols include steryl esters, steryl glycosides and acylated steryl glycosides. In steryl esters, the alcohol group of the sterol is esterified to a fatty acid [14]. Cholesteryl stearate in Figure 1.4 is an example of a fatty acid steryl ester. However, the systematic name of this steryl ester is cholest-5-en-yl octadecanoate. In steryl glycosides (SG), the alcohol group is glycosylated with a sugar monomer, usually the pyranose form of D-glucose, while in acylated steryl glycosides, the sugar moiety of SG is acylated at C_6 with fatty acids [15]. The diversity of chemical structures in these conjugated sterols comes from differences in the type of sterol, fatty acid or sugar and the number of sugar groups [15]. Figure 1.4 shows the chemical structures for each type of conjugated sterol.

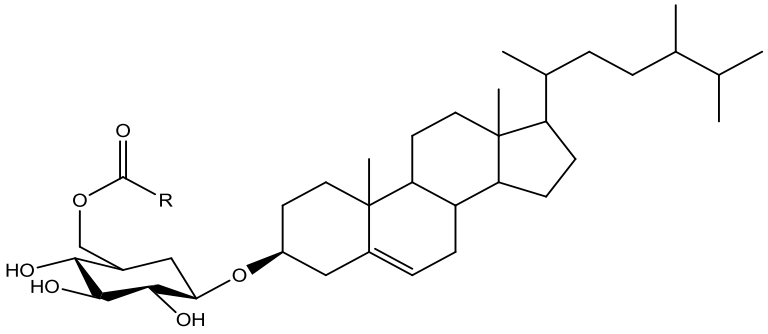
Several studies have shown that steryl esters can inhibit the absorption of cholesterol inside the small intestine and thus decrease the level of cholesterol in blood [16, 17]. Therefore, there is growing interest in analysis of phytosterols and steryl esters as healthy ingredients in various foods within both the scientific community and commercial organizations [10]. Steryl glycosides and acylated steryl glycosides occur in the plasma membrane of plants and some yeasts species [18]. It has been found that up to 80% of total phytosterols occur as glycosylated sterols in foods. However, steryl glycosides can be added to the diet to improve lipid metabolism and immune function [19].



Steryl ester (SE)



Steryl glycoside (SG)



Acylated steryl glycoside (ASG)

Figure 1.4. Chemical structures of some conjugated sterols

1.5 The importance of sterols

Plant-derived sterols are often called phytosterols, and are important constituents of plant membranes where they assist in maintaining the phospholipid bilayers in a stable form. This function is similar to that of cholesterol in animal cell membranes [20].

However, phytosterols have many positive impacts on human health. It has been found that their consumption can reduce levels of cholesterol in blood [6, 21] via inhibition of the absorption of cholesterol in the small intestine [22], and can protect against cardiovascular and heart disease [23]. The average consumption of phytosterols in northern European countries from common food sources is about 200 mg/day, but this amount of phytosterol is not sufficient to reduce the levels of cholesterol in blood [24]. Therefore, phytosterols have recently gained an important role in the nutraceutical and pharmaceutical industries where they are extracted from plants and added to prepared food as a health ingredient [23].

1.6 Sterols in mariculture

1.6.1 The origin of sterols in molluscs and bivalves

Bivalves are a subgroup of molluscs which belong to marine invertebrates. Mussels belong to bivalves which can live in either salt or fresh water. Sterol profiles of marine invertebrates have been identified by many analytical techniques including gas-liquid chromatography, argentation chromatography, mass spectrometry and nuclear magnetic resonance spectrometry. Dietary sources of sterols are necessary for some molluscs to grow and survive due to their limited ability to synthesize sterols *de novo*. On the other hand, mammals have the ability to synthesize cholesterol from precursors having low

molecular weight such as acetate or mevalonate [25, 26]. However, in some marine organisms, cholesterol is a precursor of steroid hormones, vitamin D, bile salts and molting hormones. It has been found in spiny lobsters that exogenous cholesterol is biologically converted to sex hormones such as progesterone, 17α-hydroxy progesterone, androstendione and testosterone, and to molting hormones such as 20-hydroxyecdysone [27]. Teshima and Kanazawa reported that the mollusc is capable of synthesizing cholesterol but this capability was governed by the life cycle, sexual maturation and sex of mussels [28].

Molluscs have different abilities to metabolize sterols [29]. For example, *chitons* which belong to the *Amphineura* class have the ability to synthesize cholest-7-enol from precursors having low molecular weight. In addition, they can biologically convert cholesterol to cholest-7-enol. Some other molluscs such as gastropods can synthesize cholesterol *de novo* and have the ability for dealkylating some phytosterols to cholesterol. Therefore most gastropods do not need an external source of cholesterol to grow. However, it has been found that mussels and pelecypods have a limited ability for *de novo* sterol synthesis [28, 30]. Teshima and Patterson reported that the oyster has the ability to synthesize 24-methylenecholesterol and 24-ethyldiencholesterol from acetate [31]. However, in spite of sea scallops having the ability to synthesize sterols, dietary phytosterols are considered the main sources of the sterol composition of sea scallops [32]. It has been shown that scallops have the ability to take up cholesterol from cultured microalgae [32].

It has been found by Knauer et al. [33] that Pacific oyster spat have the ability to assimilate dietary phytosterols after feeding on microalgae for a period of six weeks.

Also, they observed that the sterol profile of spat tissues reflected their diets and the overall content of sterols was less in un-fed spat. Berenberg and Patterson [34] reported that the composition of sterols in oysters reflected their diets. Moreover they observed that the oyster has the ability to synthesize cholesterol *de novo* and it was able to dealkylate phytosterols to cholesterol. However, the contribution of the synthesis of sterols to the sterol composition of oysters is low which indicates that most sterols found in oysters are from their diet.

1.6.2 Sterol composition of molluscs

Different classes of molluscs have different sterol compositions. For example, chitons which belong to the *Amphineura* class contain mainly Δ^7 sterols [35], while Δ^5 sterols are predominant in other marine invertebrates such as mussels. However, more than 45 sterols were identified in the oyster. This reflects the complexity of dietary sterols in the food webs [35].

1.6.3 Sterols and their biosynthesis in mussels

The *de novo* synthesis of sterols in marine bivalves is a controversial issue; since some authors revealed that marine bivalves can synthesize sterols *de novo* while others demonstrated that they cannot [36]. However, Voogt [37] showed that sterols can undergo *de novo* alkylation in mussels. It has been shown that cholesterol in marine bivalves can be biologically converted to 24-methylenecholesterol via alkylation. Popov et al. [38] reported that mussels can slowly synthesize sterols *de novo* and they have the ability to metabolize cholesterol via esterification, oxidation and Δ^{22}-dehydrogenation. Idler et al.

[39] reported that bivalves neither have the ability to synthesize sterols *de novo* nor the ability to convert them. However, it has been found that mussels are capable of converting desmosterol to cholesterol, 22-dehydrocholesterol and 24-methylenecholesterol. Also, it was observed that fucosterol is converted to cholesterol via desmosterol [34]. However, it was reported that only cholesterol can be synthesized by mussels [40].

1.6.4 Sterols in microalgae

Microalgae are considered one of the most important sources in the diet of molluscs. Both marine and fresh water mussels feed on plankton and other microscopic sea creatures. Water, that contains the diet, is drawn by the mussels into their body using their incurrent siphon. The water is then pumped into the branchial chamber using their cilia. The wastewater then exits via the excurrent siphon. Ultimately, the food reachs their mouth where digestion occurs [41].

Sterols are present in plankton in the free form, esterified to fatty acids (steryl esters), linked to sugars (steryl glycosides) or linked to acylated sugars (acylated steryl glycosides) [42].

The sterol composition of microalgae is affected by the season in which they grow [43]. It was reported that the sterol composition of red algae varies from season to season. For example, in fonds of red algae, the major sterol in spring was cholesterol, while sitosterol was the major sterol in September. This may be attributable to cholesterol being synthesized by fonds in spring [43]. However, each type of algae has a particular sterol profile which varies also based on seawater quality [44]. Sterols may be used as

biomarkers since bivalves take up the sterols of microalgae without changes or with limited change. Therefore, bivalves living in different marine environments which contain different types of microalgae that synthesize different suites of sterols will exhibit a particular sterol composition based on the sterol composition of microalgae [45]. Gatenby et al. [46] showed that Δ^5, C_{29} sterols are dominant in *B. grandis* (type of algae), whereas $\Delta^{5,7}$, $\Delta^{5,7,22}$ and Δ^7, C_{28} sterols are predominant in *N. oleoabundans*. The biochemical composition of algae differs according to their species, light, temperature and growth stage [46]. Cholesterol and fucosterol are predominant in brown algae. Fucosterol is the major sterol in *Padina crassa Yamada* (type of brown algae), while in *Padina pavonia* the predominant sterol is cholesterol. In most brown algae, fucosterol constitutes over 70% of total sterols [47]. The presence of a particular sterol profile in marine samples can be evidence of the presence of a particular type of microalgae. For example, the high level of 4-methyl sterols such as dinosterol in marine samples is evidence of the presence of dinoflagellates [48].

1.7 A brief introduction to mass spectrometry

Mass spectrometry presently is a common chemical analytical techniques used by chemists and biochemists. It can be used in many areas such as chemistry, physics, geology, material science, the petroleum industry, forensic science, and environmental science. Mass spectrometry possesses many advantages which include ultrahigh detection sensitivity and high molecular specificity. It is used for many purposes such as the determination of molecular mass and structural elucidation of compounds [49]. In

addition, it can be used for quantification at trace levels and identification of compounds present in a complex mixture [50]. A mass spectrometer consists of three physically separated components. These components are an ionization chamber, a mass analyzer, and the detector. A mass spectrometer is not able to detect neutral molecules; therefore, firstly, the analyte must be converted to gaseous ions inside the ionization chamber. These ions are accelerated into the mass analyzer using a suitable voltage, in which the ions are separated based on their mass to charge ratio. The separated beam of ions are converted to an electrical signal using a suitable detector and displayed in the form of a mass spectrum [50]. All of these steps are carried out under high vacuum ($10^{-4} - 10^{-8}$ torr) in order to prevent the analyte ions from interacting with other ions [49].

1.7.1 Types of ionization techniques

Ionization techniques are categorized into soft and hard where soft ionization techniques exhibit little or no fragmentation while hard ionization techniques result in significant fragmentation. Electron impact (EI) is classified as a hard ionization technique. In EI, the analyte in the gaseous state interact with an accelerated beam of electrons emitted from a heated metallic filament (e.g, tungsten), resulting in ejection of an electron from the analyte and the formation of a positively charged radical cation [51]. Electron impact is widely coupled to gas chromatography (GC) which is especially suitable for volatile and thermally stable compounds [51]. Chemical ionization (CI) also is utilized coupled to a GC. In CI, ionization is carried out via the interaction between energetic electrons and a neutral gas such as methane resulting in charged ions which then interact with the target analyte resulting in protonated or deprotonated ions as well as

adduct ions. However, CI is complementary to EI since the molecular ion is much more likely to be observed. The main drawback of both techniques is that they are not suitable for thermally unstable or non-volatile compounds including polar and large biological samples [51].

Matrix-assisted laser desorption/ionization (MALDI), electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are the most common soft ionization techniques used for analysis of biological compounds such as proteins, DNA, and lipids. These ionization methods will be highlighted since they were used in this work.

1.7.2 Electrospray ionization mass spectrometry

Over the last three decades, electrospray ionization mass spectrometry has become widely used in clinical laboratories because it is a sensitive, reliable and robust technique as well as suitable for thermally labile bio-molecules. The coupling of ESI with HPLC enables the analysis of a wide range of molecules including small and large molecules having wide ranges of polarities [50].

Electrospray ionization-mass spectrometry (ESI-MS) was developed by Dole and coworkers in the late of 1960s [52]. In ESI-MS, the sample, usually in polar solvents, is infused into the ionization chamber using a thin needle at atmospheric pressure. Charged droplets of the analyte are formed at the end of the needle as a result of applying an electrical potential with a value of 3-4 kV [49].

There are two proposed models explaining the mechanisms of the formation of the gas-phase ions from the charged droplets. These models involve the charge residue model (CRM) and the ion desorption model (IDM). In CRM model, the evaporation of a solvent causes the droplet to shrink in size resulting in the increase of the charge density on the surface of the droplet until it reaches the Rayleigh instability limit. At this limit, the repulsive columbic forces become slightly higher than the droplet surface tension resulting in the rapid break down of the large droplet into smaller droplets with higher charge density. The evaporation of the solvent continues to take place until the droplet contains only one analyte ion which is followed by the transfer of this analyte into the gas phase [49, 53]. Figure 1.5 shows the schematic of the CRM model. In the IDM model, due to the evaporation of solvent, the size of the droplet becomes too small which leads to increased the charge density on the surface of the droplet thus increasing the electric field of the droplet. At this point, the electric field on the surface exceeds the droplet's cohesive forces causing desorption of the analyte from the droplet surface [49, 53].

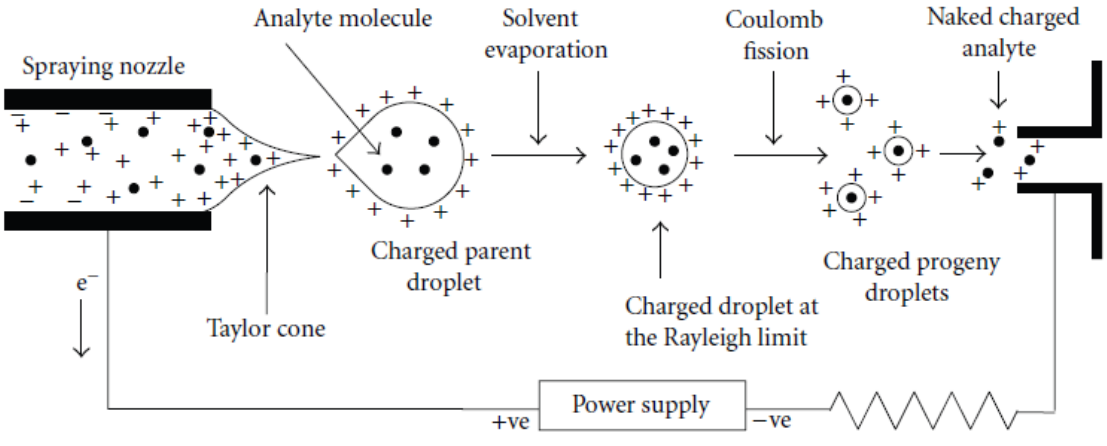


Figure 1.5. Schematic of an electrospray ionization source (reproduced with permission from ref. 56)

1.7.3 Atmospheric pressure chemical ionization

Atmospheric pressure chemical ionization-mass spectrometry (APCI-MS) was developed in the mid 1960s by Shahin [54] who used a discharge chamber with a platinum wire as an anode for the investigation of ion-molecule reactions. In the 1970s, APCI was interfaced to liquid chromatography and equipped with a corona discharge needle [49]. As shown in Figure 1.6, an APCI source consists of a sample inlet capillary, nebulizer capillary, and corona discharge needle. An auxiliary gas is pumped into the surrounding region of the needle to prevent interaction between the analyte and the tube wall [55].

In APCI-MS, the sample emerging from the fused-silica capillary tube is nebulized into very small droplets using N_2 as a nebulizer gas. An auxiliary gas is pumped to minimize the interaction between the analyte and the wall of the source. The electrons which are emitted from the corona discharge needle ionize gases existing in the the source and forms primary ions (N_2^+, $N_4^+$and H_2O^+). The reactant ions such as ($H_3O^+(H_2O)_n$) are formed as a result of the interaction of these primary ions with H_2O that is present in the APCI source as an aerosol. The ionization of the analyte occurs via transfer of a proton from the reactant ion (($H_3O^+(H_2O)_n$) to the analyte to form a protonated molecular ions $[M+H]^+$. However, in negative ion mode, the ionization takes place as a result of the interaction of O_2^-(reactant ion) with the analyte via electron or proton transfer. Cluster ions can be formed and by ramping up the temperature of the source, these clusters can be eliminated [49].

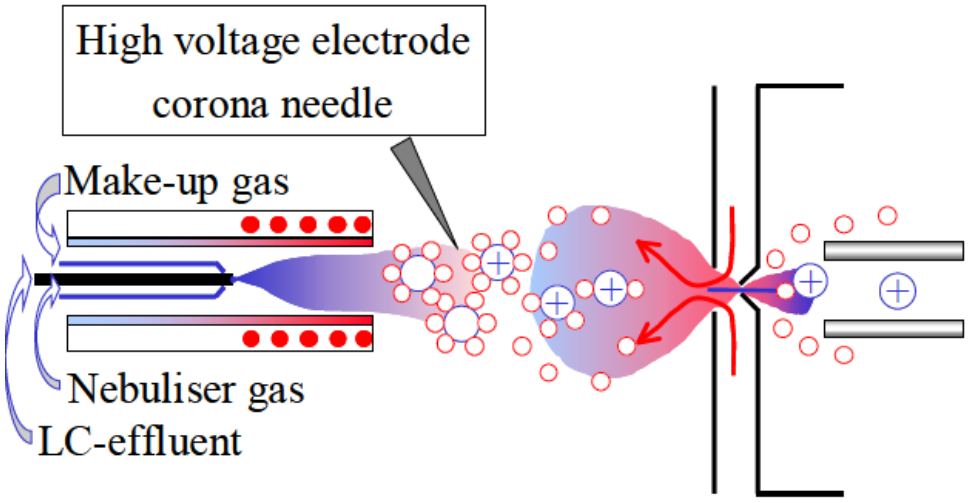


Figure 1.6. Schematic of an atmospheric pressure chemical ionization source (reproduced with permission from ref. 55)

1.7.4 Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF)

1.7.4.1 Basic concepts

Matrix-assisted laser desorption/ionization (MALDI) is a soft ionization technique that can be used for analysis of biomolecules having high molar mass with little or no decomposition and thus, the molecular ion of the target analyte can be observed [58]. MALDI-TOF has become a common technique for the analysis of a wide range of biomolecules including proteins, lipids, oligosaccharides and other chemical and biochemical molecules [59].

In MALDI, the matrix absorbs the energy of the laser resulting in desorption of the matrix and the sample and transmission of both the matrix and charged analyte into the gas phase. The most common type of laser used in MALDI is a UV laser (e.g. nitrogen laser that emit radiation at a wavelength of 337 nm). The Nd: YAG lasers which

emit radiation wavelength of 355 nm can also be used [60]. The laser pulse-width ranges used are from 1 to 100 ns and this is short enough not to sufficiently decompose the analyte [61].

The ionization in MALDI results from the complex interaction between the matrix and the analyte. However, the presence of acidic or basic groups in the analyte plays a minor role in the ionization process [62]. The mechanism of the formation of ions in MALDI is not fully understood. However, several proposed models have been introduced to explaning ion formation. These models include: gas-phase photoionization, excited state proton transfer, gas-phase ion-molecule reactions and desorption of performed ions. The proton transfer in the solid phase before desorption and gas-phase proton transfer in the plume from phtoionized matrix are the most accepted models. The gas-phase ions are then accelerated into the mass analyzer via applying an electrostatic field [63]. In many cases when the analyte cannot form protonated or deprotonated ions, adduct ions such as sodiated $[M+Na]^+$ and potasiated $[M+K]^+$ can be formed by adding salts of these alkali metals. However, in biological samples containing lipids, Na^+ and K^+ ions are normally present and $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ can be formed [64]. In cases where analytes have low ionization potential or are not able to undergo proton-transfer reactions they can be ionized via direct photoionization or by charge transfer from the matrix to the analyte [65]. Another strategy is to derivatize the analyte with a permanently charged or an easily ionizable group. This study takes this approach to the analysis of free sterols.

1.7.4.2 The role of matrix

Besides the nature of sample preparation, the type of matrix affects the results for certain molecules that can be analyzed by MALDI-TOF. Matrix selection is mainly based on the nature of the target analyte as well as the experimental conditions [66]. The matrix absorbs the laser energy instead of the analyte resulting in a decreased probability of degradation of the sample and it minimizes the probability of froming cluster ions. This can be achieved by mixing a very small quantity of analyte with a large quantity of matrix (usually 100 to 1000 fold molar excess of the matrix) [67].

There are specific properties that are required for a chemical to be used as a matrix. They are as follows: it must have the ability to absorb the energy at the laser wavelength, it should be soluble in solvents compatible with the analyte, it must have the capability to isolate the analyte molecules after co-crystallization, it should be vacuum stable, it must cause co-desorption of the analyte upon laser irradiation and finally it must stimulate analyte ionization [66]. The most common matrices used in positive mode are carboxylic acids which have the ability to protonate the analyte and form positively charged ions [68]. However, some analytes cannot be protonated due the lack of basic groups. They are used with basic matricies which result in proton abstraction to form negatively charged ions. 2,5-Dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (CHCA) and sinapinic acid (SA) are considered the most common acidic matrices whereas dithranol and *p*-nitroaniline are basic matrices.

1.7.4.3 The addition of the salts as dopants in MALDI-TOFMS

When the analyte cannot form protonated or deprotonated ions, adduct ions such as sodiated $[M+Na]^+$ and potasiated $[M+K]^+$ can be formed by adding salts of these alkali metals. However, in biological samples containing lipids, Na^+ and K^+ ions are normally present and $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$ can be readily observed [64]. Also, addition of excess alkali salt can be used to reduce the complexity of the mass spectrum of a complex mixture and possibily facilitate quantification. In one study by Stubiger and Belgacem [64] three different salt additives were used in MALDI analysis of lipds. They found that the addition of ammonium citrate to a solution of THAP in methanol formed both sodiated and/or protonated lipid molecules. However, by the addition of sodium salt the potasiated adducts were suppressed with mainly sodaited adducts present. Finally, if lithium salts were added, both sodium and potassium adducts were suppressed and only lithium adducts were observed.

1.7.4.4 Time-of-flight (TOF) mass analyzer

MALDI is interfaced to a time-of-flight (TOF) mass analyzer due to its unlimited mass range and ion pulse capabilities. The ions are accelerated to a field-free drift tube by applying an electric field with a voltage of 20 kV [8]. The ions then are transmitted to the drift tube with the same kinetic energy. The kinetic energy for any particle is proportional to its mass and the square of its velocity. The time that takes each ion to reach the detector is measured according to the following equation [69]:

$$t = [mL/(2zeV_{acc})]^{1/2}$$

Where t is the flight time, m is the mass of the ion in kg, L is the length of the drift tube in m, e is the elementary charge (1.6×10^{-19} C), and V is the accelerating voltage in V.

The mass resolution of a linear TOF mass analyzer is relatively poor compared to magnetic/electric sector or FTICR instruments. The main reason is that ions with the same m/z can have different kinetic energies due to desorption/ionization processes. In order to decrease the difference in kinetic energy that leads to improve resolution, a delayed extraction method was introduced [70]. This method can decrease the distribution in kinetic energies of the ions and thus improve the resolution [71].

Reflectron-TOF was also introduced to improve MALDI-TOF resolution. It is based on increasing the flight path of the drift tube by applying a constant electrostatic field (i.e., electrostatic mirror) at the end of the drift tube (Figure 1.7). Since, these mirrors help to decrease the difference in flight times of the same m/z ions, this leads to minimizing the distribution of kinetic energies and thus focuses ions of the same m/z in space and time [72].

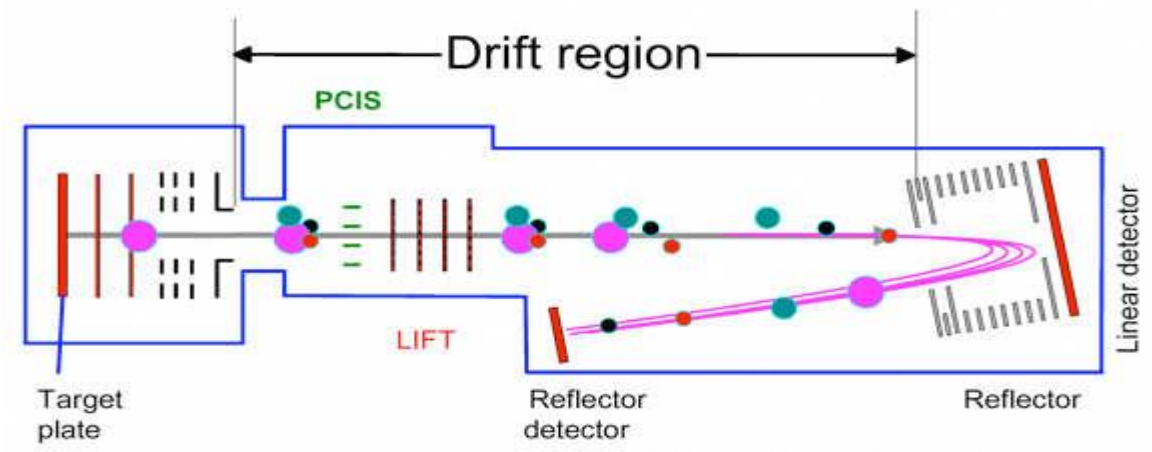


Figure 1.7. Schematic of a reflectron-TOF mass analyzer (reproduced with permission from ref. 73)

1.8 Quadrupole ion-trap mass spectrometry (QITMS)

QITMS consists of three electrodes: a ring electrode and two end cap electrodes. Bascially ions having a wide range of m/z are trapped simultaneously by applying a *rf* voltage to the ring electrode. After ramping up the *rf* voltage, ions with a particular m/z value move in a stable trajectory. The ions sequentially become destabilized and are ejected through perforations that exist in the end cap electrode and are ultimately detected by the electron multiplier [49, 63].

QIT can be operated in several MS modes that perform different ion analysis. These modes include the mass-selective instability mode, the mass-selective stability mode and resonance ion ejection mode [49, 63].

In the mass-selective instability mode, *dc* (*U*) and *rf* (*V*) voltages are applied to the ring electrode and as a result, ions with a wide range of m/z are trapped within the boundaries of the electrodes. The 3D quadrupole field within the trap is created as a result

of applying direct and alternative potentials $\varphi = (U\text{-}V \cos \omega t)$ to the ring electrode and keeping the end cap electrode at ground potential. This causes moving of ions in three-dimensional trajectories (Figure 1.8). Introducing helium gas into the trap with a pressure of 10^{-3} torr helps minimize the high energy which can result from the collisions of the trapped ions, and focuses the ions at the center of the trap [49, 63].

The mass spectrum can be acquired via sweeping the *dc* and *rf* voltages and the frequency of the *rf* signal (ω). As a result, the ions with higher m/z values become sequentially destabilized in the axial direction and ultimately exit the trap to be detected by the detector [49, 63].

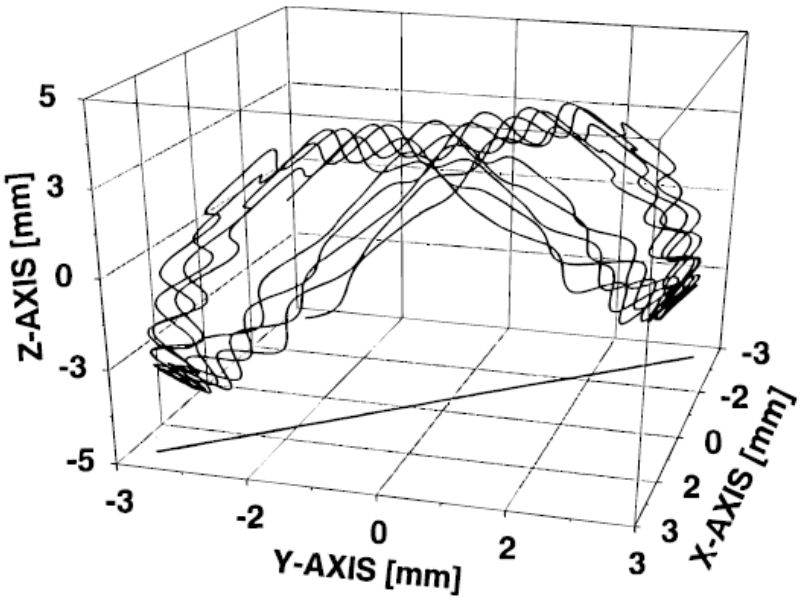


Figure 1.8. Three-dimentional trajectory of the ions in QIT (reproduced with permission from ref. 57)

Figure 1.9 shows the stability diagram which demonstrates the mechanism of ion detection. At particular values of the *dc* voltage, the amplitude and angular frequency of the *rf* signal, and the electrode dimensions, ions with certain range of m/z are confined within the boundaries of the stability diagram. However, in a particular point, only *rf* voltage is applied to the ring electrode, while the end-cap electrodes are kept at ground potential (i.e., U and a_u are both equal to 0). In this point, all trapped ions are located on the q_z axis and by scanning the *rf* voltage, the ions move from one point to another along the q_z axis [49, 63]

The mass analysis is based on the following equation

$$m/z = 4V/(q_{max}\,\omega^2 r_0^2)$$

Where q_{max} is the maximum value of q_z at which ions exit the stability diagram and it is equal to 0.908, ω: angular frequency of the rf signal and r_0: the dimensions of the trap. At a particular value of V, all ions that possess q_z values between 0 and 0.908 have stable trajectories. By increasing V, ions are sequentially ejected from the trap according to their m/z and ultimately detected.

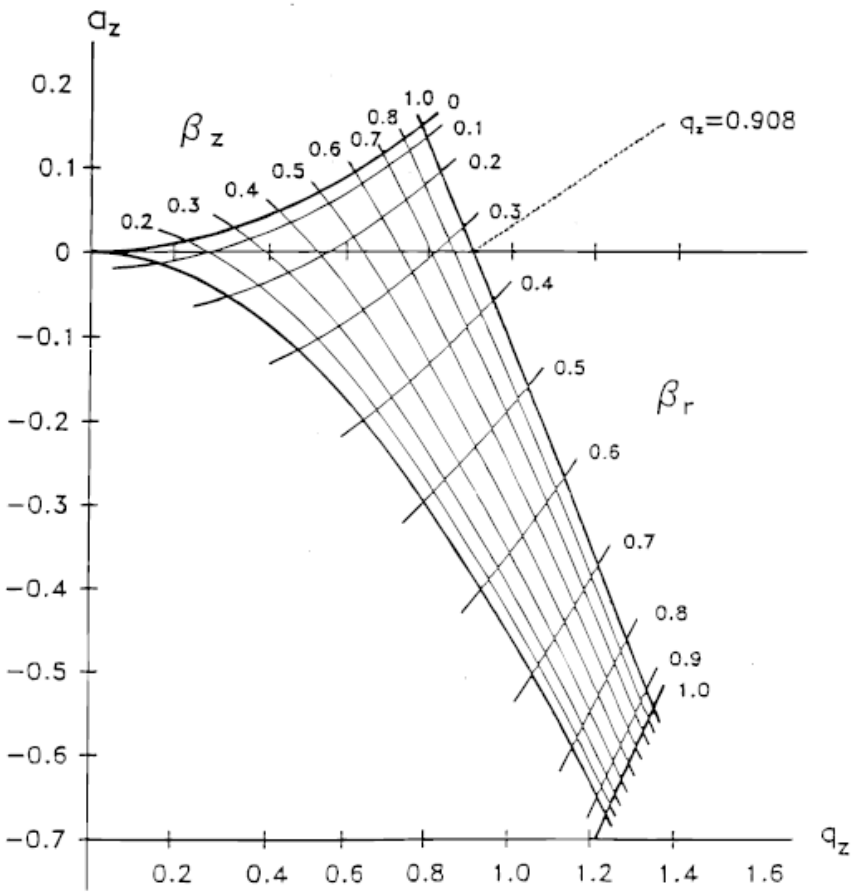


Figure 1.9. The stability diagram in a_z and q_z space in QIT (reproduced with permission from ref. 57)

The second operational mode of a QITMS is the resonance ejection mode. In this mode, an additional *rf* voltage (50 V) is applied to the end-cap electrodes resulting in the excitation of the ions. All the ions inside the trap oscillate with a characteristic frequency (secular frequency) in the radial and axial directions. The ion absorbs the energy only if its secular frequency matches with the frequency of the *rf* voltage resulting in the increase of the velocity of the ion inside the trap until it is ejected from the trap along z direction [49, 63].

The third operational mode is the mass–selective stability mode. In this mode, a potential φ is applied to the ring electrode and – φ to the end-cap electrodes. Only a particular m/z value is stored at a time. The mass spectra are obtained by scanning the *dc* and *rf* voltages, while keeping their ratio constant [49, 63].

QITMS has many of advantages, which include the high sensitivity (attomole range), the high scan speed and the smaller size than other mass analyzers. It can be used to investigate the gas-phase ion-molecule reactions. However, the drawbacks include its low dynamic range due to the space-charge effect, and its poor resolution which is approximately equal to unit mass. However, the mass resolution can be improved by decreasing the scanning speed, and by manipulating the frequency and the amplitude of the resonance ejection signal [49, 63].

1.9 Tandem mass spectrometry

Tandem mass spectrometry (i.e., MS/MS) was introduced for the first time in the late 1960s. So called "tandem in space" involves connecting two or more mass spectrometers in order to obtain more detailed structural information and imporved chemical elucidation of the target analyte. This technique can perform many tasks which include identification of the chemical compounds in complex mixtures, structural elucidation/confirmation, elucidation of fragmentation pathways and quantification [49].

Tandem mass spectrometers involve using the first mass analyzer to isolate a particular ion (precursor ion). In the second stage, the preselected ion is transmitted to the

collision cell in which the precursor ion is subjected to fragmentation as a result of collision with a neutral gas. The third stage involves mass analyzing all product ions by scanning the second mass analyzer [49]. The most common tandem MS is the triple quadrupole (QqQ). Other tandem configurations include quadrupole-TOF and TOF-TOF mass analyzers. Tandem mass spectrometry can also be performed in an ion storage cell ("tandem in time"). The quadruple ion trap and FTICR are common instruments for this purpose [63].

1.9.1 Types of tandem scans

Tandem mass spectrometry (in space) can involve four types of scan modes used for different tasks. These modes include product ion scan, precursor ion scan, neutral loss scan and selected reaction monitoring (SRM). In product ion scan, the mass spectrum of product ions is obtained as a result of the collision of the preselected ion (precursor ion) with inert gas inside the CID cell and scanning of the second mass analyzer. In a precursor ion scan, the mass spectrum of all precursor ions that cleave to generate a specific preselected product ion mass is obtained. The second mass spectrometer is fixed at a particular product ion and the first mass spectrometer is scanned and records all of precursor ions that generate the specific product ion. This scan mode is utilized for identification of compounds that are similar in their chemical structures and exist in a complex mixture. In a neutral loss scan, a particular neutral fragment is preselected by the second mass analyzer and the instrument records all precursor ions that lose that particular neutral fragment. Both mass spectrometers are scanned at the same time and differ by a particular mass (neutral loss) offset. In selected reaction monitoring (SRM),

both mass analyzers are fixed to record a precursor ion that generates a specific product ion. In addition to chemical identification, this mode is frequently used for quantification of target analyte present in complex mixtures [49, 63].

1.9.2 Collision-induced dissociation

Collision-induced dissociation (CID) occurs as a result of the collision of an analyte ion with a neutral gas. The most popularly used collision gases are helium, nitrogen and argon and in some modern instruments, air can be used (i.e., MALDI). CID involves fragmentation of the activated ions as a result of transformation of the kinetic energy of the ions into internal energy [63]. Two collision energy domains are employed in tandem mass spectrometry. These domains include high-energy (3-10 keV) CID which is used in magnetic sector and TOF/TOF instruments, and low-energy (~ 100 eV) CID which is employed in FTICR, ion trap and quadrupole instruments [49].

1.9.3 Tandem mass spectrometry using a quadrupole ion trap MS

In contrast to the triple quadrupole (QqQ), all steps of MS/MS (or MS^n) in a QITMS which include precursor ion selection, excitation and product ion scan, take place in the same space. All of the ions, except the precursor ion, are ejected from the trap by applying the required *rf* signal to the end-cap electrodes (resonance ejection mode). The precursor ion is then excited by applying an excitation pulse resulting in the increased probability of an ion collision with the inert gas. As the result of collision, the precursor ion cleaves into smaller fragments, which are detected via ejection out of the trap. For doing higher order MS/MS (i.e., MS^3), a particular fragment ion is chosen following

ejection of all other fragments. The excitation of the fragment ion is then carried out as before by applying another excitation pulse resulting in cleavage of this fragment into smaller fragment ions. In contrast to the triple quadrupole configuration, only product ion sacns and selected reaction monitoring can be carried out in QITMS [49, 63].

The coupling of a soft ionization technique such as (ESI) in a quadrupole ion trap mass spectrometry ((ESI-QIT MS) can be a very efficient way for rapid analysis of complex mixtures even those containing similar chemical structures and isomers.

1.10 Analysis of free sterols and steryl esters

In order to analyze sterols, lipids must first be extracted from target samples using, most commonly, either the Folch procedure [74] or the Bligh and Dyer procedure [75]. In the Folch procedure, a mixture of chloroform: methanol: water with a ratio of 8:4:3 is used. However, in the Bligh and Dyer method, a mixture of chloroform: methanol: water with a ratio of 2:2:1.8 is used.

During sample preparation, some precautions must be taken. These precautions include the storage of lipid extract samples under nitrogen to prevent the oxidation of sterols. However, an antioxidant may be added, such as pyrogallol, to prevent the oxidation. The fact that the stability of sterols is influenced by increasing the temperature requires that sterols must be stored at -20 ºC to ensure preservation [76].

1.10.1 Preparative chromatography

After lipid extraction, the lipid extract is usually subjected to fractionation into its classes either by column chromatography, thin-layer chromatography or solid-phase extraction. Preparative chromatography has many benefits which facilitate the analysis of lipids, removes matrix interferences and concentrates the analyte. The fractionation can be either simple, in which lipids are separated into polar or nonpolar fractions, or complex in which lipid classes, such as sterols, steryl esters, triacylglycerols, and phospholipids, can be separated [77].

1.10.1.1 Column chromatography (CC)

In column chromatography (CC), Pasteur pipette is filled with a stationary phase. The most common stationary phases used in CC are silica gel, silicic acid, and alumina. The separation in CC occurs as a result of absorption and/or partitioning of the lipid classes between the solid and mobile phases. Elution of a particular lipid class can be achieved by varying the polarity of the mobile phase [77]. For example, nonpolar lipids such as sterols can be eluted, from a column packed with silica gel, with a mixture of diethyl ether/hexane, while polar lipids can be eluted with diethyl ether [77]

1. 10.1.2 Thin-layer chromatography (TLC)

Silica gel TLC is frequently used for separation of lipid classes. For separation of neutral lipids, a mixture of solvent of hexane, diethyl ether and acetic acid with different proportions can be used. Free sterols, steryl esters, and free fatty acid can be separated

with this system of solvent. For separation of polar lipids such as phospholipids, a mixture containing chloroform: methanol: water (65:25:4, v/v/v) can be used [77].

1.10.1.3 Solid phase extraction (SPE)

Solid-phase extraction is a separation method in which the target compounds can be separated from other compounds existing in a mixture based on their interaction with a stationary phase. Also, it can be used to concentrate and clean up the analyte. The separation of the target analyte is affected by the affinity of the analyte to the stationary phase. Both the target analyte and the impurities are differentially retained on the stationary phase and the target analyte is eluted using an appropriate solvent. In SPE, a vacuum system can be used to speed up the elution flow [78].

The stationary phase is composed of silica particles that have a particular functional group bonded to it such as hydrocarbon chains with different lengths or amino groups (for anion exchange), and sulphonic acid or carboxyl groups (for cation exchange).

SPE usage is classified according to the types of stationary phase:

1. Normal phase SPE. The cartridge is conditioned with a non-polar or slightly polar solvent and the cartridge is then finally washed with water. The aqueous sample is then loaded onto the cartridge. The polar analytes will spend more time on stationary phase than non-polar impurities. These non-polar impurities are eluted with a non-polar solvent and discarded. The analyte is then eluted with polar solvent [78].

2. Reversed phase SPE. The stationary phase is usually silica bonded to hydrocarbon chains. The phase used to separate or isolate compounds of mid to low polarity. The analytes usually are eluted with a non-polar solvent [78].

SPE is commonly used for fractionation of lipid mixtures into its individual components. It is superior over other preparative techniques in that it is simple to use, inexpensive and rapid. Moreover, it doesn't require high volumes of eluting solvents. The most common stationary phases used for fractionation of lipid components are silica and neutral alumina [76]. For separating steryl esters, a mixture of diethyl ether/hexane (1:1, v/v) can be used with an alumina cartridge, while for separating sterols, a mixture of ethanol/hexane/diethyl ether (50:25:25) is used [79].In this study a Strata silica cartridge was used for separation of lipids into nonpolar and polar components. Sterol and steryl esters can be eluted with chloroform, while SG and ASG can be eluted with acetone/ 2-propanol (1:1) [18].

1.10.2 Techniques for analysis of free sterols and steryl esters

A variety of different techniques have been used for the analysis of free sterols including gas chromatography/mass spectrometry (GC/MS) [80], GC-flame ionization detection (GC/FID) [81], liquid chromatography/ultraviolet detection (LC/UV) [8], HPLC/atmospheric pressure chemical ionization (HPLC/(APCI)-MS) [6], HPLC/atmospheric pressure photoionization (HPLC/(APPI)-MS) [82] and HPLC/ electrospray ionization (HPLC/ESI)-MS) [83].

The analysis of sterols by GC requires derivatization to enhance their volatility which results in increasing resolution and sensitivity as well as improved peak shape [84]. Sterols can be easily converted into trimethylsilyl (TMS) ethers, using the derivatizing agent *N*,*O*-bis(trimethylsilyl)trifluoracetamide (BSTFA). When sterols are analyzed without derivatization, they suffer from dehydration and decomposition reactions and poor chromatography exhibiting tailing and poor resolution [9]. Although the GC is the most common analytical technique for free sterols, reversed phase HPLC can be successfully used. However, the chromatographic separation and detector sensitivity are poorer than with GC/FID [9]. During the last decade, HPLC-MS/MS has gained a special interest in steroid analysis and has become a very common analytical technique in clinical diagnostic laboratories [85]. Electrospray ionization (ESI), atmospheric pressure chemical ionization and atmospheric pressure photoionization (APPI) are the most common ionization techniques utilized in HPLC. For analytes such as free sterols that require derivatization, permanently charged or easily ionizable groups are attached to the analyte using ESI, while for APCI a group with high proton or electron affinity is attached [86].

1.10.2.1 Analysis of sterols using ESI-MS and APCI-MS

Atmospheric pressure chemical ionization is suitable for the analysis of neutral and low molecular weight compounds such as sterols. However, electrospray ionization can be used for sterol analysis but the sensitivity is poor compared with that of APCI. Sterols are detected in either ESI or APCI as dehydrated ions ($[M+H-H_2O]^+$) which is diagnostic of sterols. The intensity of this fragment is very low in ESI-MS [76]. However, in order to increase the ionization efficiency and ultimately to enhance the sensitivity in ESI, sterols

are converted into more polar structures. Several studies were carried out for the analysis of sterols in biological samples via derivatization into more polar structures. A method for analysis of sterols in serum using liquid chromatography tandem mass spectrometry (LC-ESI-triple quadruple-MS/MS) after their derivatization to picolinyl esters was developed by Honda et al [87]. It was found that picolinyl esters formed adduct ions with sodium and acetonitrile $[M+Na+CH_3CN]^+$. Sandhoff et al. [88] have developed a method for determination of sterol sulphates using nanoESI-MS/MS equipped with a triple quadrupole mass analyzer. They found that the precursor ion scanning of hydrogen sulphate ion $[HSO_4]^-$ at m/z 97 gave a deprotonated sterol sulphate profile $[M-H]^-$. Cholesterol was analyzed by ESI-MS as its *N*-methylpyridyl ether and, since this derivative is positively charged, it was detected as $[M]^+$ [89]. The analysis of all of these derivatives by ESI-MS resulted in enhancement of the ionization efficiency and thus resulted in increased sensitivity.

Negative APCI-MS can be used for analysis of sterols via introducing a moiety with a strong electron affinity. Determination of cholesterol in tissue by negative APCI-MS via introducing a group having strong electron affinity was reported by Kuo et al. [90] where cholesterol was converted into cholesteryl pentaflurophenyl carbamate. In positive APCI mode, the introduction of a moiety with good proton affinity can increase the ionization efficiency, while derivatization to introduce a group with high polarity leads to a decrease in the sensitivity [91].

1.10.2.2 Analysis of sterols by derivatization MALDI-TOF

Lipids and most matrices used in MALDI easily dissolve in common solvents resulting in a homogeneous mixture with a high degree of crystallization that leads to excellent reproducibility and good signal-to-noise (S/N) ratio [92]. Because of the presence of alkali salts in biological samples and the affinity of lipids for alkali metals, lipids tend to form adduct ions (sodiated or potasiated ions) [64].

Free sterols are neutral molecules that are difficult to ionize by MALDI and their molecular ions have been found to easily fragment. Schiller et al [93] demonstrated that cholesterol does not exhibit its protonated molecular ion $[M+H]^+$, but its $[M+H-H_2O]^+$ at m/z 369.3 with low signal intensity. In order to increase their ionization efficiency and selectivity, sterols can be converted into more polar derivatives [87]. However, analysis of derivatized sterols using MALDI-TOFMS should not require clean up since the method is more tolerant to sample complexity and contaminants. For example, Wang et al [94] described a MALDI-TOF/TOF method for direct analysis of oxosteroids after their derivatization using Girard P (GP) hydrazine to give GP hydrazones without the need of sample cleanup.

1.10.2.3 Analysis of steryl esters

Several techniques have been used for determining steryl esters in biological samples. These techniques included gas chromatography (GC). However, the analysis of steryl esters using GC requires alkaline hydrolysis to obtain the free sterol. The free sterol

fraction is then isolated by solvent extraction, solid phase extraction or preparative thin layer chromatography [95-97]. These techniques are time consuming and information on the fatty acid of the steryl esters is missing. Reversed phase HPLC with different detectors has been used for analysis of intact steryl esters. HPLC with UV detection was used for determining cholesteryl esters [98]. Also, HPLC coupled to an evaporative light scattering detector (ELSD) has been used for analysis of intact steryl esters [99]. In both methods, the triglyceride fraction, which can interfere with the analysis of steryl esters, is removed using preparative chromatography. Mezine et al [100] used HPLC with APCI MS for identification of intact steryl and stanyl esters in cholesterol-lowering spreads and beverages. HPLC coupled to ESI in single ion extraction mode and HPLC-ELSD have been used for identification of fatty acid steryl esters in wheat samples [101].

In samples with a larger number of steryl esters, analysis by HPLC is challenging due to peak overlap and co-elution. This problem can be partially resolved using a MS detector since co-elution or non-resolved peaks can be readily identified. Selected ion moniotoring can be used to differentiate between the co-eluting peaks. However, tandem mass spectrometry possesses the ability to distinguish among steryl ester isomers even without need for chromatographic separation.

Since steryl esters are nonpolar and resist electrospray ionization, dopants may be added to facilitate their ionization. Ammonium acetate is the most common dopant used for analysis of steryl esters using ESI-MS where they form ammonium adducts $[M+NH_4]^+$ which are easily detected in positive mode. However, steryl esters cannot be

identified in atmospheric pressure chemical ionization (APCI) as intact molecules since they easily fragment and form $[M+H-HOOCR]^+$ ions [100].

1.11 Thesis Objectives

The overall objective of this thesis was to develop mass spectrometric techniques for analysis of sterols and steryl esters in real-life biological samples. The specific aims of this thesis were as follow:

1. Examine the influence of different diets on the levels of phytosterols in blue mussels and determine the origin of sterols.
2. Develop a rapid method for analysis of sterols using derivatization MALDI-TOFMS and MALDI-TOF/TOFMS, and ESI-Ion Trap MS^n by converting them into their corresponding picolinyl esters, *N*-methylpyridyl ethers and sulphated esters to enhance their ionization efficiency and to facilitate their analysis.
3. Test derivatization MALDI-TOF and MALDI-TOF/TOF for analysis of sterols in blue mussels.
4. Differentiate among sterol isomers using ESI/APCI-QIT MS^3 via converting them into picolinyl esters.
5. Develop an analytical method based on ESI-QIT tandem mass spectrometry (CID-MS^2 and CID-MS^3) for identification of steryl esters in real samples without need for chromatographic separations.

1.12 Co-authorship statement

The first part of chapter three and chapter four were published in peer-reviewed journals with Iyad Hailat as the principal author and Dr. Robert Helleur as the author of correspondence. It is appropriate to clarify both authors' contributions to these papers.

All experimental work was performed by the principal author. This includes extraction of lipids from mussels, margarine and corn, and preparation of samples for analysis using TLC-FID, GC-MS, GC-FID, MALDI-TOF, ESI/APCI-QIT-MS. The principal author addressed the data analysis with advice from Drs. Helleur and Parrish. This included the interpretation of mass spectra and chromatograms.

The principal author also drafted both manuscripts and replied to comments of peer reviewers with some editing by Dr. Helleur.

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Chapter Two: Lipid and sterol compositions of blue mussels fed algae and culture finfish effluent diets

2.1 Introduction

Dietary sterols, known as phytosterols, are important alicyclic molecules present in plants, algae and fungi. In humans, their consumption reduces levels of cholesterol in blood [1, 2]. Phytosterols exist in plant membranes, where they assist in maintaining the phospholipid bilayers in a stable form. This function is similar to the function of cholesterol in animal cell membranes [3].

The average consumption of phytosterols in northern European countries from common food sources is about 200 mg/day, but this amount of phytosterol is not sufficient to reduce the levels of cholesterol in blood [2]. Recently, oils derived from plants (*e.g.* canola oil) have been used in aquafeeds instead of fish oil and they contain considerable levels of dietary phytosterols [4]. As well, it was found that some marine organisms like bivalves and fish are not as enriched in cholesterol (about 50 mg 100 g^{-1} wet weight) as are in beef (about 100 mg 100 g^{-1} wet weight). Although the proportion of cholesterol in bivalves is 25% of total sterols, they are considered excellent sources of beneficial dietary sterols [5]. Bivalves have a wide variety of phytosterol profiles exceeding 20 different structures [5]. Typically, the chemical structure of phytosterols is similar to cholesterol but the difference between them is in the structure of the side chain; the side chain in cholesterol consists of 8 carbon atoms while in phytosterols, it consists of 9 or 10 carbon atoms with an alkyl substitution at C_{24} [6].

Blue mussels (*Mytilus edulis*), are an excellent source of dietary phytosterols. The diversity in content and composition of phytosterols in blue mussels depends mainly on their diets. In addition, the season and the life cycle of mussels affect the levels and composition of phytosterols [7, 8]. Although the proportion of total lipids in mussels is

low (about 2% of the wet weight), they contain a considerable proportion of beneficial phytosterols [7].

There is increasing interest in growing mussels adjacent to finfish aquaculture cages, such as those that are part of open-water Integrated Multi-Trophic Aquaculture (IMTA) systems where mussel are able to partially consume uneaten fish feed and fish fecal particulates [9]. The extractive organism (the mussel) essentially feeds on the wastes generated by the fed organism (the finfish). A recent study examined the absorption efficiency of blue mussels (*Mytilus edulis* and *M. trossulus*) feeding on Atlantic salmon (*Salmo salar*) feed and fecal particulates and they suggested that organic material in particulate salmon culture waste can effectively be utilized by blue mussels [10]. This study goes further and examines the absorption efficiency and preferences by blue mussels for the phytosterols that are present in aquaculture fish waste.

Microalgae are used in aquaculture as diets to grow larval fish as well as marine invertebrates such as oysters. They contain different concentrations and compositions of dietary sterols depending on the conditions in which the phytoplankton grows such as temperature and the type of light [11]. The main phytosterol in brown algae is fucosterol and, in many red algae, cholesterol and desmosterol dominate [12]. 4α-Methyl phytosterols such as dinosterol are most abundant in dinoflagellates, while 24-methylenecholesterol and brassicasterol (24-methylcholesta-5,22E-dien-3β-ol) occur in diatoms [12]. Other common phytosterols found in plants are β-sitosterol (24-ethylcholesterol), stigmasterol (24-ethylcholesta-5,22-dien-3β-ol) and campesterol (24-methylcholesterol). In contrast, cholesterol is the most abundant sterol found in animals and ergosterol (24-methylcholesta-5, 7, 22E-trien-3β-ol) is the most common phytosterol

found in fungi [12]. Besides algae, bacteria and detritus are the other food sources for blue mussels [13].

Studies have been carried out to determine the content and composition of phytosterols in mussels. Murphy et al. [7] investigated the sterol composition of green and blue mussels. It was observed that the sterol composition of these mussels depended on their diet. The levels of phytosterols in blue mussels were higher compared to green mussels due to dinoflagellates being the major food source of blue mussels, while green mussels depended mainly on a diatom diet. Cholesterol, brassicasterol, 22-methylcholesterol, *trans*-22-dehydrocholesterol and desmosterol (cholesta-5, 24-dien-3β-ol) were the main phytosterols in both mussels.

The composition of phytosterols in blue mussels was also studied by Copeman et al. [5]. Cholesterol, *trans*-22-dehydrocholesterol, brassicasterol, 24-methlenecholesterol and campesterol were found to be dominant. In addition, the study revealed that there was a high level of cholesterol in blue mussels compared with cockles and scallops.

This is the first study that examines the influence of controlled and different diets on the levels of phytosterols in blue mussels. The composition and levels of phytosterols in locally cultivated mussels, and those fed algae or fish waste or those unfed were investigated to determine the origin of phytosterols in blue mussels and to determine which of these treatments contain higher levels of phytosterols compared to the level of cholesterol.

2.2 Materials and methods

2.2.1 Feeding experiments

The feeding experiment was designed by Both et al. [14] in the Ocean Sciences Centre of Memorial University of Newfoundland. Two A-frames each containing three containers (each with a volume of 49 L) were used for this experiment. They were supplied with sand bed filtered water from Logy Bay NL at a rate of 1.9 L/min. Each container contained blue mussels (1.8-2.2 cm shell length) fed algae or fish waste for six months and unfed mussels (4.5-6.5 cm shell length) for 2 months. The mussels were fed 1.5% of their dry weight daily. Effluent (feces and uneaten feed) was collected from a single tank containing 1 year old Atlantic cod fed a commercial diet (Skretting Europa). The effluent was passed through a 500 μm screen followed by a 70 μm screen before being fed to the mussels. The algal diet was a commercial shellfish diet (Shellfish Diet 1800, Instant Algae® Reed Mariculture) that contained a mix of *Isochrysis* (30%), *Pavlova* (20%), *Tetraselmis* (20%) and *Thalassiosira weissflogii* (30%). Food was added manually to the containers daily and the water supply turned off (to prevent flushing of food) for two hours or until any coloration of the water was removed. Unfed mussels received only sand bed filtered sea water.

2.2.2 Chemical reagents

Cholesterol, campesterol, ergosterol, β-sitosterol, desmosterol and brassicasterol were purchased from Steraloids Inc (Newport, USA). 5α-Androstanol, used as an internal standard, was obtained from Sigma Aldrich (St. Louis, Mo, USA). *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane

(TMCS), used for preparation of trimethylsilyl ether derivatives of sterols, was obtained from Supelco (Bellefonte, PA, USA). All organic solvents used in extraction and preparation of samples were of analytical grade.

2.2.3 Lipid extraction

Total lipids were extracted from the meat of the whole mussel using a modified Folch procedure [15]. In this procedure, the meat of the mussel was placed in a test tube and a mixture of chloroform/methanol 2:1(v/v) was added. The content of the tube was homogenized using a polytron PCU-2-110 homogenizer (Brinkmann Instruments, Ontario, Canada). Chloroform extracted water was added to get a final ratio of chloroform/methanol/water (8:4:3, by vol) followed by sonication for 10 min. Samples were then centrifuged at a speed of 5000 rpm for 5 min. The lower organic layer, which contained the lipids, was then drawn into a vial. The extraction process was repeated three times to ensure that most lipids were extracted. The organic solvent was then evaporated using a flash evaporator (Bucheler Instruments, Fort Lee, NJ), followed by addition of 1.5 mL chloroform. Samples were kept at -20°C until analysis.

2.2.4 Lipid classes

Lipid classes were determined using a modified procedure described by Parrish [16]. In this procedure, an Iatroscan Mark V (TLC-FID) was used for separation of lipid classes. One μL of lipid extract was spotted on Chromarods coated with a thin layer of silica gel. Lipid classes were separated using three different solvent mixtures; each mixture was used to separate a group of lipid classes that have similar polarity. The

separation of hydrocarbons, steryl esters and ketones was carried out using two consecutive developments of a mixture of hexane/diethyl ether/formic acid (99:1:0.05, by vol) for 25 and 20 min. Triacylglycerols, free fatty acids and sterols were separated by a mixture of hexane/diethyl ether/formic acid (80:20:1, by vol) for 40 min. The last separation was performed using 100% acetone twice for 15 min each, and then using a mixture of chloroform/methanol/water (5:4:1, by vol) twice for 10 min each, for separation of acetone mobile polar lipid and phospholipids. The Chromarods were dried and scanned after each separation.

2.2.5 Free sterols

Free sterols were isolated from total lipid extracts using column chromatography. The bottom of a Pasteur pipette was plugged with glass wool. It was then placed in an oven at 250°C overnight to remove contaminants, cooled and filled with activated silica gel which had been heated at 150°C for 1 h. The column was washed with 3 mL of diethyl ether then with 6 mL of hexane. Lipid extracts were applied to the top of the column. The free sterol fraction was eluted with 15 mL of a mixture of diethyl ether/hexane (1:1, by vol). Samples were evaporated until dryness using a stream of nitrogen. The trimethylsilyl ether derivatives (TMS) of sterols were obtained by adding 100 μL of *N,O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% of trimethylchlorosilane (TMCS) and heating at 85°C for 20 min. Samples were then dried under nitrogen and 500 μL of hexane/dichloromethane (1:1, by vol) was added followed by addition of 100 μL of 100 mg/L of 5α-androstanol as internal standard and then analysis by GC-MS and GC-FID. The same volume with the same concentration of

internal standard was added to 500 μL of a mixture of sterol standards prepared at different concentrations which was then analyzed by GC-MS and GC-FID to produce a calibration curve.

2.2.6 Gas chromatographic separation and mass spectrometric detection conditions for sterols

Sterols were identified using an Agilent 6890 N GC with an Agilent 5973 mass selective detector. The column used for separation of sterols was a DB-5 (5%-phenyl)-methyl polysiloxane with 30 m length, 0.25 μm film thickness and 0.25 mm i.d. (J & W Scientific USA). The carrier gas was helium with a column pressure of 14 psi. The injected volume was 1 μL in splitless mode with an inlet temperature of 290°C. The temperature program was 80°C for 1 min followed by ramping to 200°C at 50°C/min, then increasing to 305°C at 4°C/min, and holding for 5 min. The mass detector was operated in electron ionization (EI) mode with electron beam energy of 70 eV and a mass range of 50-550 m/z. The scan rate was 2.94 scans/sec. Sterols were identified using the NIST MS library, and by comparing retention times of TMS sterol peaks with those of standards and using published spectral data [17].

GC-MS Single Ion Monitoring (GC-MS-SIM) was used to quantify co-eluting peaks and those sterols with low abundances. A GC-FID (Trace Ultra Thermo Scientific, USA) was used to quantify other sterols. The column used for GC-FID was an Equity TM-5 with 30 m length, 0.25 μm film thickness and 0.25 mm i.d. The temperature program was 80°C for 1 min, then ramping to 260°C at 25°C/min followed by heating to

300°C at 2°C/min, and holding for 5 min. The carrier gas pressure was 14 psi. The temperatures of the injector and the detector were 315°C and 310°C, respectively.

2.2.7 Statistical analysis

One-way ANOVA and *t*-tests were carried out, using SigmaStat 2.03 (SPSS Inc), to compare levels of phytosterols in mussels fed different diets. All data are expressed as mean ± SD.

2.3 Results and Discussion

2.3.1 Lipid content

In this study, the lipid classes were identified in cultivated mussels included hydrocarbons, steryl/wax esters, ethyl ketones, triacylglycerols, free fatty acids, sterols, acetone mobile polar lipids and phospholipids. The most abundant lipid class was phospholipids (58.4% of total lipid). Other lipid classes were present as follows: sterols (10.6%), triacylglycerols (10.4%), hydrocarbons (9.5%), free fatty acids (7.1%), acetone mobile polar lipids (2.9%), steryl/wax esters (0.8%) and ethyl ketones (0.63%). There are many factors that affect the chemical composition of lipids in mussels; some of these factors were investigated by Jeong et al. [18, 19]. These factors include the temperature and the duration of mussel storage; it was revealed that as temperature and storage time increase, the possibility of lipid oxidation significantly increases. This causes depletion in total lipids and polar lipids and increasing nonpolar lipids and free fatty acids. They attributed the depletion to lipases and lipolytic enzymes that decompose polar lipids by a hydrolysis mechanism. Some studies revealed that the major source of some of the lipid classes is from environmental contamination. Motorboat fuel, fuel oil and crude oil were reported by Perry [20] as the main sources of hydrocarbons in molluscs. Li et al [21]

showed that the total lipid content in *Perna viridis* mussels differs depending on season. Mussels had the highest total lipid content in spring compared to other seasons. They also observed that the phospholipid class was dominant in summer, autumn and winter, while triacylglycerol was the major lipid class in spring. Copeman et al. [5] reported that the major lipid class in blue mussels was phospholipids (37.8%) but at a lower proportion than in the present study. However, Alkanani et al. [13] found cultured blue mussels having a phospholipid proportion (76.0%) higher than the present study. The level of free fatty acids (FFA) found in this study was 7.1% which is in agreement with its level in Tasmanian blue mussels reported by Murphy et al. [7]. FFA reported by Copeman et al. [5] was slightly higher (9.0%). The sterol proportions found in this study are higher than reported by Copeman et al. [5] and Murphy et al [7]. These studies revealed that sterol proportions were (8.6%) and (5.0%), respectively.

2.3.2 Free sterol composition of cultivated mussels

Sterols were identified in cultivated blue mussels harvested in August 2009 using GC-MS (Figure 2.1). The most abundant of these sterols were cholesterol, desmosterol, brassicasterol, 24-methylenecholesterol, *trans*-22-dehydrocholesterol, 24-nordehydrocholesterol and occelasterol (24-methyl-27-norcholesta-5,22(E)-dien-3β-ol). Six phytosterols were quantified using GC-MS-SIM. The concentration of cholesterol was the highest (321 mg/kg wet weight). The concentrations of other sterols were as follows: desmosterol (148 mg/kg), brassicasterol (104 mg/kg), β-sitosterol (28 mg/kg), ergosterol (26 mg/kg) and campesterol (13 mg/kg). King et al. [22] reported cholesterol as the major sterol in blue mussels with a concentration of 370 mg/kg wet weight, followed by brassicasterol (180 mg/kg), campesterol (80 mg/kg), and β-sitosterol (40

mg/kg). These results are higher than those in our study and most likely can be attributed to diet. Furthermore, the concentrations and composition of sterols vary in a wide range depending on the season in which the mussels are harvested, the diet, and the sex of the mussel [8]. The composition of identified sterols in the present study were similar to those identified in blue mussels in New Zealand by Murphy et al. [7, 23] and in Labrador, Canada by Copeman et al. [4] with a few differences which can be attributed to differences in diets or in the season in which the mussels were sampled. Li et al. [21] found that mussels had the highest level of cholesterol in the autumn (36.9% of total sterols), and 24-methylenecholesterol was highest in the spring (15.4%).

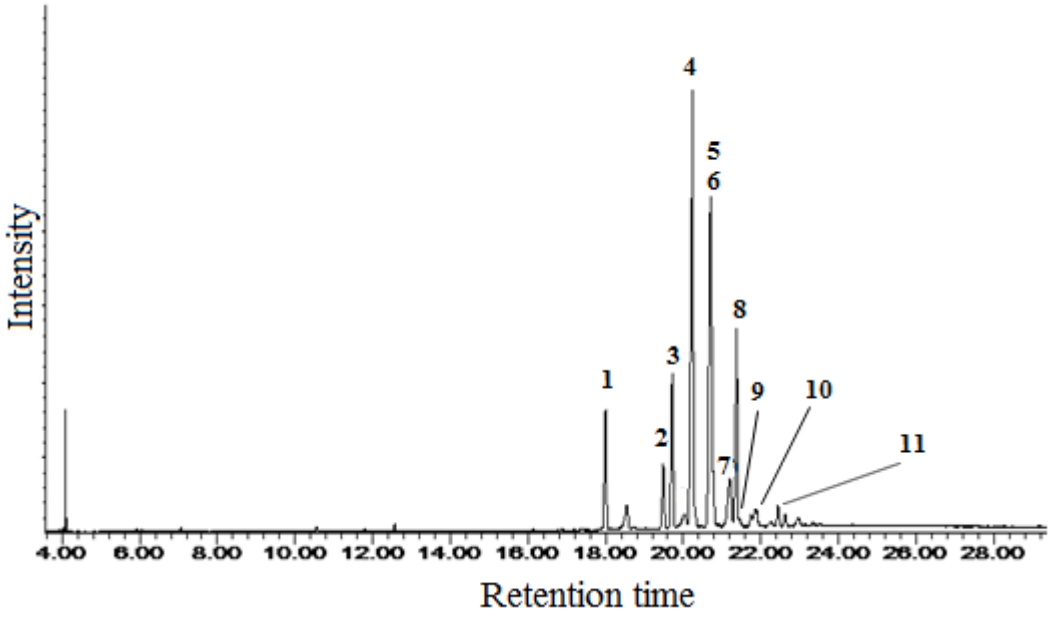

Figure 2.1. Total ion chromatogram of sterols found in cultivated mussels using GC/MS. Peaks: 1. 24-*nor*-dehydrocholesterol, 2. occelasterol, 3. *trans*-22-dehydrocholesterol, 4. cholesterol, 5.desmosterol, 6.brassicasterol, 7. ergosterol, 8. 24- methylenecholesterol, 9. campesterol, 10. stigmasterol, 11. β-sitosterol.

2.3.3 GC-MS selected-ion monitoring (GC-MS-SIM)

In contrast to the full-scan spectrum, GC-MS-SIM is a technique in which one or more ions (m/z) are chosen to be analyzed for quantitation purposes. It is particularly useful for co-eluting peaks and in trace analysis. The co-eluting peaks at 20.70 min, labelled 5 and 6, contain desmosterol and brassicasterol (Figure 2.1). They were not separated although different chromatographic conditions were attempted. These compounds were clearly identified and then quantified using GC-MS-SIM (Figure 2.2). The same sample is injected twice, the mass spectrometer first monitoring at m/z 470 (Figure 2.2(A)), in the second run it was monitoring at m/z 456 (Figure 2.2(B)). Brassicasterol and desmosterol were each quantified separately using standards of both compounds.

2.3.4. The concentrations of sterols in the diets used in this study

Only five sterols were identified in the algal diet (Table 2.1). The results showed that campesterol was predominant with a concentration of 298 mg/kg wet weight, followed by β-sitosterol (216 mg/kg), brassicasterol (213 mg/kg), cholesterol (181 mg/kg) and desmosterol (84 mg/kg). Veron et al. [11] found that brassicasterol was the highest (49% of total sterols) in *Isochrysis aff. galbana* and the lowest was β-sitosterol (1%). In contrast, the highest proportion in *Tetraselmis suecica* was campesterol (47% of total sterols) and the lowest was stigmasterol (0.5 %).

The results revealed that cholesterol was the major sterol in fish waste (2086 mg/kg dry weight), followed by β-sitosterol (1441 mg/kg) and campesterol (852 mg/kg). No other phytosterols were detected in this diet (Table 2.1).

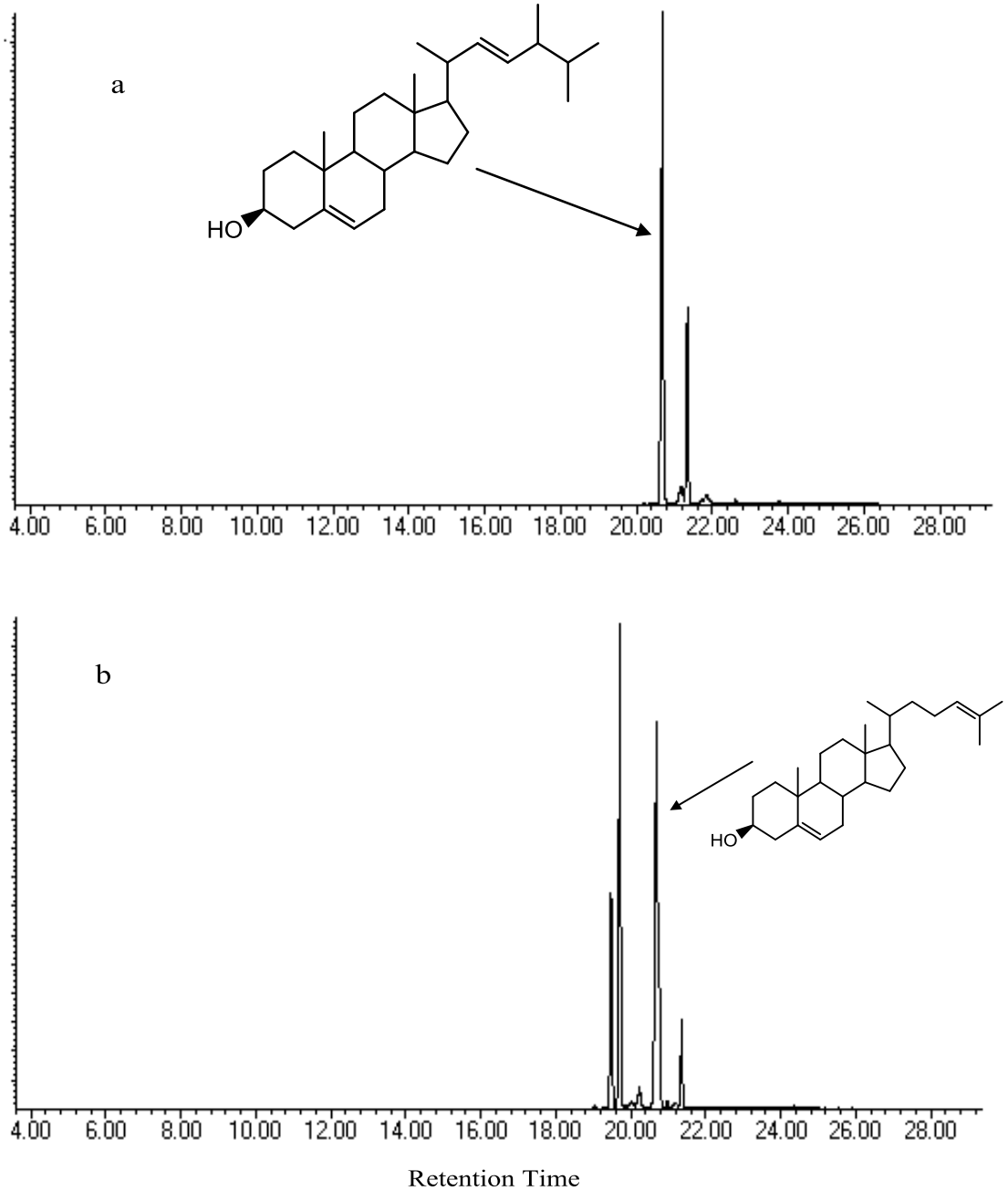

Figure 2.2. (a) Selected-ion monitoring at m/z 470 (brassicasterol) using GC/MS. The retention time was 20.69 min, and (b) single ion monitoring at m/z 456 (desmosterol) using GC/MS. The retention time was 20.70 min.

Table 2.1. Concentration of sterols in various diets (mg/kg wet weight; n=3*)

	Algae	% of total sterols (algae)	Fish waste	% of total sterols (fish waste)
cholesterol	181±53	19.2±2.0	2086±293	49±7
brassicasterol	213±81	22±1	ND	ND
desmosterol	84±13	10.7±2.0	ND	ND
sitosterol	216±105	22±3	1441±586	32±5
campesterol	298±143	30±4	852±365	19±3
ergrosterol	ND	ND	ND	ND
24-methylenecholesterol	ND	ND	ND	ND
occelasterol	ND	ND	ND	ND
trans-22-dehydrocholesterol	ND	ND	ND	ND
24-nordehydrocholeterol	ND	ND	ND	ND
Total Sterols	992		4379	

Data are mean of 3 diet samples ± SD
ND: not detected

2.3.5 Sterols in unfed mussels and those fed on algae or culture finfish effluent (fish waste)

Ten sterols were quantified using GC-FID and GC-MS-SIM in unfed mussels and those fed algae or fish waste (Table 2.2). The content of sterols were compared at the beginning and at the end of the feeding experiment.

In unfed mussels, it was found that the concentrations of all sterols decreased significantly by the end of the experiment; the concentration of cholesterol was 216

mg/kg at the beginning and at the end of experiment it was 59 mg/kg. For desmosterol, it decreased from 77 to 23 mg/kg. The results revealed that total sterols in unfed mussels also decreased from 756 to 184 mg/kg at the end of the experiment.

In algae-fed mussels, the concentration of cholesterol increased significantly by the end of experiment. Campesterol also increased by the end of the experiment, but this increase was not statistically significant. The concentrations of occelasterol and 24-methylenecholesterol decreased but were not significant. There were no significant differences between the beginning and the end of the experiment for brassicasterol, desmosterol, β-sitosterol and *trans*-22-dehydrocholesterol. However, 24-nor-dehydrocholesterol decreased significantly. The results showed that the total sterols almost stayed constant over the experiment, at the beginning it was 1140, and at the end 1090 mg/kg (Table 2.2). The main reason for the decrease in the concentrations of some sterols in algae-fed mussels is that compounds such as 24-nor-dehydrocholesterol are not present in the algal diets (Table 2.1). The concentrations of brassicasterol and desmosterol in mussels were almost constant over the experiment, although they were detected in algal diets. This may be attributed to the fact that some phytosterols were biologically converted to cholesterol and other phytosterols by mussels. A previous study by Teshima and Kanazawa [24] revealed that blue mussels (*Mytilus edulis*) are capable of converting desmosterol to cholesterol, 22-dehydrocholesterol and 24-methylenecholesterol. Danton et al. [25] observed that the concentration of brassicasterol in oysters fed algal diets was low although its concentration in the diet was high and they attributed this to the bioconversion of brassicasterol to other sterols.

In mussels fed fish waste, there was a statistically significant difference in the concentration of cholesterol between the beginning and the end of the experiment. It was observed that the concentration of cholesterol increased significantly from 231 to 710 mg/kg wet weight and 24-methylenecholesterol decreased significantly from 187 to 92 mg/kg. In contrast, there were no significant differences observed for other sterols (Table 2.2). The concentration of total sterols increased from 1020 to 1370 mg/kg.

The concentration of cholesterol found in fish waste-fed mussels was high as a result of its high level in the diet (fish waste). However, in spite of β-sitosterol being relatively high in fish waste, its concentration stayed almost constant in mussels fed fish waste compared to cholesterol over the experiment. This may be due to the bioconversion of some dietary phytosterols to other sterols, as mentioned previously, and/ or to the selective uptake of cholesterol by mussels. Another reason for the elevated levels of cholesterol in mussels fed fish waste may be that bivalves do not have the ability to alter the structure of dietary cholesterol leading to cholesterol being selectively retained [26]. Berenberg et al. [27] observed that the concentration of cholesterol in oysters was higher than that in its algal diets and it was the highest compared to other phytosterols. They attributed this to the selective uptake of cholesterol by the oyster and/or to the bioconversion of other dietary sterols to cholesterol. This observation is consistent with our results.

By comparing the results obtained from feeding experiments with those observed in cultivated mussels, we found that the fish waste-fed mussels contained significantly higher cholesterol concentration compared to the cultivated and algae-fed mussels. The

concentration of campesterol in algae-fed mussels reflected its level in algal diets where it was observed that the algae-fed mussels had significantly higher campesterol concentration compared to the cultivated and fish waste-fed mussels. There were no significant differences in the concentrations of cholesterol, desmosterol and brassicasterol between cultivated and algae-fed mussels.

Table 2.2. Concentrations of sterols in mussels fed different diets (mg/kg wet weight)*

	Unfed		Fed on algae		Fed on fish wast	
	Start	End	Start	End	Start	End
cholesterol	216±67[a]	59±18[a]	211±54[a]	**409±139[a]**	231±56[a]	**710±171[a]**
brassicasterol	63±12[a]	18±8[a]	126±10	100±40	92±27	85±19
desmosterol	77±17[a]	22±17[a]	249±75	202±107	215±90	170±65
sitosterol	16.2±1.7[a]	4.0±1.1[a]	38±7	34±16	28±5	40±11
Campesterol	5.4±1.5[a]	2.1±0.76[a]	29±11	**62±28**	21±4	**24±3**
ergrosterol	8.6±2.5[a]	2.8±0.8[a]	ND	ND	ND	ND
24methylenecholesterol	62±27[a]	17.5±7.5[a]	237±81	107±56	187±45[a]	92±15[a]
occelasterol	54±10[a]	10.1±9.5[a]	67±16	48±27	38±15	69±23
trans-22-dehydrocholesterol	134±27[a]	24±14[a]	78±41	78±33	77±25	92±21
24-nordehydrocholeterol	120±62[a]	25±15[a]	107±23[a]	49±21[a]	130±16	88±40
Total sterols	756	184	1140	1090	1020	1370

*Data are mean of four individual animals ± SD.

[a] Significantly different between the start and the end of experiment for each sterol.

Bold face numbers indicate a significant difference in sterol concentration between algae-fed and fish waste-fed mussels at the end of experiment.

Sterol proportions (% of total sterols) are presented in Table 2.3 by using sterol concentration data in Table 2.2 and converting them to % of total sterols (Table 2.3), more significant differences are then observed among mussel groups. The proportion of cholesterol increased significantly from 19% to 37% in algae-fed mussels and from 25% to 51% in fish waste-fed mussels. 24-Methylenecholesterol decreased significantly from 21% to 9% in algae-fed mussels and from 20% to 7% in fish waste-fed mussels. In mussels fed fish waste, brassicasterol and campesterol decreased significantly. *Trans*-22-dehydrocholesterol, and 24-nor-dehydrocholesterol decreased significantly in unfed and algae-fed mussels, respectively.

By comparing sterol proportion (% of total sterols) in algae-fed mussel and in fish waste-fed mussels, it was found that mussels fed algae had significantly higher proportions of brassicasterol, campesterol and 24-methylenecholesterol than those found in fish waste-fed mussels. Also, it has been found that algae-fed mussels contained significantly lower cholesterol proportions than observed in fish waste-fed mussels.

In mussels fed fish waste, the cholesterol proportion was 51% of total sterols, while in mussels fed on algae; cholesterol was 37% and other sterols 63% (Table 2.3). Murphy et al. [7] reported cholesterol as the main sterol in blue mussels (30.0%) followed by desmosterol (10.0%), *trans*-22-dehydrocholesterol (9.8%), brassicasterol (9.6%), campesterol (9.6%), and 24-methylenecholesterol (5.2%). Copeman et al. [5] found that cholesterol (39.4%), brassicasterol (8.8%), *trans*-22-dehydrocholesterol (8.0%) and 24-methylenecholesterol (7.6%) were the major sterols in blue mussels. We have demonstrated that the concentration and composition of sterols in mussels depended

mainly on their diet. In addition, the content of sterols in mussels might be influenced by the bioconversion of some dietary sterols to other sterols; mainly to cholesterol.

Table 2.3. Sterols proportions (% of total sterols) in mussels fed different diets*

	Unfed		Fed on algae		Fed on fish waste	
	Start	End	Start	End	Start	End
cholesterol	29±6	34±7	19.3±1.4[a]	**37±3**[a]	25±3[a]	**51±6**[a]
brassicasterol	8.6±2.0	9.9±1.2	11.9±2.7	**8.9±1.1**	9.7±2.1[a]	**6.1±0.8**[a]
desmosterol	10.3±1.6	11.0±3.9	23±3	18.3±4.2	22.1±7.0	12.3±4.6
sitosterol	2.3±0.2	2.5±1.0	3.8±0.8	3.2±1.4	2.9±0.30	2.9±0.3
campesterol	0.70±0.2	1.2±0.2	3.2±1.4	**5.6±1.8**	2.3±0.1[a]	**1.8±0.1**[a]
ergosterol	1.2±0.6	1.6±0.4	ND	ND	ND	ND
24-methylenecholesterol	8.8±5.1	9.7±1.6	21.4±4.9[a]	**8.9±0.8**[a]	20±3[a]	**6.5±1.0**[a]
occelasterol	7.2±1.3	4.9±2.6	5.5±1.0	4.6±2.7	4.2±2.3	4.8±1.1
trans-22-dehydrocholesterol	17.9±2.7[a]	12.6±2.1[a]	7.2±3.3	7.4±1.4	8.6±4.1	6.6±1.4
24-nordehydrocholeterol	15.6±6.5	12.9±2.1	10.2±3.7[a]	4.7±1.4[a]	13.3±3.6	6.5±3.9

*Data are mean of four individual animals ± SD.

[a] Significantly different between the start and the end of experiment for each sterol.

Bold face numbers indicate a significant difference in sterol content between algae-fed and fish waste-fed mussels at the end of experiment.

2.4 Conclusion

The most abundant sterols in cultivated mussels were cholesterol, desmosterol, brassicasterol, 24-methylenecholesterol, *trans*-22-dehydrocholesterol, occelasterol, and 24-nordehydrocholesterol. The concentrations of all dietary sterols decreased significantly in unfed mussels over the experiment. However, fish waste-fed mussels had significantly higher cholesterol concentration compared to local cultivated and algae-fed mussels. In algae-fed mussels, 24-nordehydrocholesterol decreased significantly. Mussels fed algae had significantly higher campesterol compared to local cultivated, and fish waste-fed mussels.

The concentration of cholesterol found in fish waste-fed mussels was high as a result of its high level in the diet (fish waste). Algae-fed mussels had higher proportions of beneficial phytosterols (non-cholesterol sterol) than fish waste-fed mussels. We demonstrated that the content and composition of phytosterols in mussels fed different diets reflected their diets.

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Chapter Three: Direct analysis of derivatized sterols using mass spectrometry and tandem mass spectrometry

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3.1 Introduction

Sterols are natural substances present in plants, animals and fungi. β-sitosterol, stigmasterol and 24-methylenecholesterol occur abundantly in plants [1]; in animals, it is cholesterol and, in fungi, ergosterol [2]. Plant-derived sterols are often called phytosterols [3, 4] and are important constituents of plant membranes where they assist in maintaining the phospholipid bilayers in a stable form [5]. This function is similar to that of cholesterol in animal cell membranes [3, 5]. The chemical structures of phytosterols are similar to that of cholesterol, but differ in that phytosterols contain an additional methyl or ethyl group at C-24 of the side chain.

Phytosterols have many positive impacts on human health. It has been found that their consumption can reduce levels of cholesterol in blood [6, 7] via inhibition of the absorption of cholesterol in the small intestine [8], and protect against cardiovascular and heart disease [9]. Therefore, phytosterols have recently gained an important role in the nutraceutical and pharmaceutical industries where they are extracted from plants and added to prepared food as a health ingredient [9].

Mussels are considered a good source of dietary phytosterols. The diversity in content and composition of phytosterols found in mussels depends mainly on their diets which usually consist of microscopic marine plants containingan array of phytosterols [10]. In addition, the season and the life cycle of mussels affect the levels and composition of phytosterols. Although the percentage of lipids in mussels is low (2% wet weight), they contain a considerable proportion of beneficial phytosterols [10].

Matrix-assisted laser desorption/ionization (MALDI), electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are the most common soft ionization techniques used for analysis of biological compounds such as lipids.

MALDI-time-of-flight-MS has been used for analysis of proteins [11, 12], oligosaccharides [13], synthetic polymers [14, 15], lipids [16-18], and unstable or non-volatile compounds [19]. One of the most important characteristics of this technique is the fact that sample preparation can be done easily and quickly. In addition, MALDI-TOF spectra are not as adversely affected by the presence of small amounts of buffer or contaminants from sample workup [20].

A variety of different techniques have been used for the analysis of free sterols including gas chromatography mass spectrometry (GC/MS) [21, 22], GC-flame ionization detection (FID) [23], liquid chromatography/ultraviolet detection (LC-UV) (24), LC/atmospheric pressure chemical ionization detection (APCI-MS) [24, 25], LC/APCI-MS/MS [26], LC/atmospheric pressure photoionization detection (APPI)-MS/MS [27], and high-performance liquid chromatography/electrospray ionization detection (HPLC-ESI-MS) [28]. These techniques usually require extensive sample handling and chromatography.

Another approach to sterol analysis which is suitable for rapid analyte profiling is MALDI-TOFMS. Recently, it has been used for the analysis of lipids [29]. Lipids and most matrices used in MALDI easily dissolve in common solvents resulting in a homogeneous mixture with a high degree of crystallization. This leads to improved

reproducibility of the method and better signal-to-noise (S/N) ratio for the analysis of conjugated sterols [20].

APCI is suitable for analysis of non-polar and low molecular weight compounds such as sterols. However, ESI and MALDI can be used for sterol analysis but the sensitivity is lower than that of APCI [30]. Therefore, sterols can be derivatized to enhance their ionization efficiency and selectivity [31]. Besides derivatization of sterols by TMS followed by GC analysis, other studies have used various other derivatization techniques producing more polar derivatives. These include the analysis of sterols in human serum by LC/ESI-MS/MS as their picolinyl esters [31], determination of cholesterol by nano-ESI-MS/MS after derivatization to cholesterol sulfate [32], and determination of cholesterol by ESI-MS as its *N*-methylpyridyl ether [33]. These techniques require sample cleanup. However, analysis of these sterol derivatives using MALDI-TOFMS should not require this since the method is more tolerant to sample complexity and contaminants. For example, Wang et al. [34] described a MALDI-TOF/TOF method for direct analysis of oxosteroids after their derivatization using Girard P (GP) hydrazine to give GP hydrazones without the need of sample cleanup.

The coupling of a soft ionization technique like ESI with quadrupole ion trap mass spectrometry ((ESI-QIT MS^n) along with direct flow injection can be a very efficient way for rapid analysis of mixtures, even those containing isomers, as the technique allows for more detailed structural elucidation using collision-induced dissociation (CID) tandem MS. Contrary to triple quadrupole MS, QIT MS can also perform MS^3 and higher CID experiments thus giving additional structural information [35].

Lipid samples are complex mixtures ranging from nonpolar compounds like sterols and triacylglycerols (TAG) to very polar compounds like phospholipids. Analysis of sterols by GC/MS and LC/MS requires preparative chromatography i.e., solid-phase extraction (SPE) and thin-layer chromatography (TLC)) that takes a long time. However, the present study will show that the analysis of sterols by derivatization MALDI-TOFMS and ESI/APCI-QITMS will not require sample cleanup, one reason being that derivatization targets only the sterol alcohols thus lessening interference from other lipid classes.

The first part of this chapter describes for the first time, the analysis of sterols by MALDI-TOF and MALDI-TOF/TOF and their quantitation after derivatization to picolinyl esters, *N*-methylpyridyl ethers and sulphated esters. The influence of the derivative on sensitivity, the effect of additives and the selection of a proper matrix will be discussed. The derivatization MALDI-TOF approach was further investigated for analysis of sterols extracted from mussels. The objectives of the second part of this chapter were to examine the applicability of directly analyzing picolinyl ester derivatives using flow injection ESI-quadrupole ion trap (QIT) MS and APCI-QIT MS and to investigate their fragmentation behavior using low energy CID-tandem mass spectrometry. This study also examined the possibility of distinguishing among sterol isomers using ESI-QIT MS^3 and APCI-QIT MS^3. To the best of our knowledge, this is the first study that demonstrates the analysis of picolinyl esters of sterols using ESI/APCI-QIT MS^n and to demonstrate its ability to differentiate among sterol isomers.

3.2 Experimental

3.2.1 Chemicals and reagents

2,5-Dihydroxybenzoic acid (DHB), 2,4,6-trihydroxyacetophenone monohydrate (THAP), α-cyano-4-hydroxycinnamic acid (CHCA), dithranol, *p*-nitroaniline, picolinic acid, 2-methyl-6-nitrobenzoic anhydride, 4-dimethylaminopyridine, triethylamine, pyridine, sodium acetate, 2-flouro-1-methyl pyridinium *p*-toulenesulfonate, sulfur trioxide pyridine and barium acetate were obtained from Sigma Chemical Co. (St. Louis, Mo, USA). Cholesterol, β-sitosterol, ergosterol, cholestanol, brassicasterol, fucosterol, desmosterol, stigmasterol and 7-dehydrocholesterol were obtained from Steraloids, Inc. (New Port, RI, USA). All other chemicals and solvents were of analytical grade.

3.2.2 Derivatization of sterols

3.2.2.1 Picolinyl esters

Picolinyl ester derivatization was carried out using a procedure reported by Yamshita et al. [36] with minor modifications. A 200 μL aliquot of a reagent mixture consisting of picolinic acid (80 mg), 4-dimethylaminopyridine (30 mg), 2-methyl-6-nitrobenzoic anhydride (100 mg), pyridine (1.5 mL), and triethylamine (250 μL) was added immediately to 2.0 mg of sterol standard or dried mussel extract. The reaction mixture was heated at 60°C for 20 min followed by addition of 750 μL of hexane and vortexed for 1 min. The reaction mixture was then centrifuged for 5 min. The supernatant was transferred to a vial and evaporated to dryness under nitrogen flow. The residue was re-dissolved in 2 mL of dichloromethane and analyzed immediately.

3.2.2.2 *N*-Methylpyridyl ethers

N-Methylpyridyl ether derivatives were prepared according to Mukiyama et al. [37] with minor modifications: 9.4 mg of 2-fluro-1 methylpyridinium *p*-toluenesulfonate was added to a solution of 13.0 mg of sterol standard in 1 mL of dichloromethane and 10 μL of triethylamine were then added to the reaction. The solution was allowed to stir at room temperature for 1 h. The derivative was diluted with dichloromethane to100 μg/mL for analysis.

3.2.2.3 Sulphated esters

The sulphated ester derivatization was performed using a modified Duff procedure [38]: 12.5 mg of sulphur trioxide pyridine complex were dissolved in 2.5 mL of pyridine and the solution sonicated for 30 s. A 200 μL aliquot of the sulphur trioxide pyridine solution was immediately added to 2.0 mg of sterol standard or to dried mussel extract. The vials were sonicated for 30 s, and then left to sit at room temperature for 10 min followed by addition of 20 μL of barium acetate solution (314.0 mM) as a base catalyst. The sample was vortexed for 30 s and sonicated for 1 min, then left for 10 min at room temperature followed by 1 h at 4° C in order to allow hydrolysis and precipitation of barium sulphate. Dichloromethane (500 μL) was added and the mixture was centrifuged for 10 min. The supernatant was stored in the freezer until analyzed. Figure 3.1 shows the chemical structures of each type of derivative based on cholesterol.

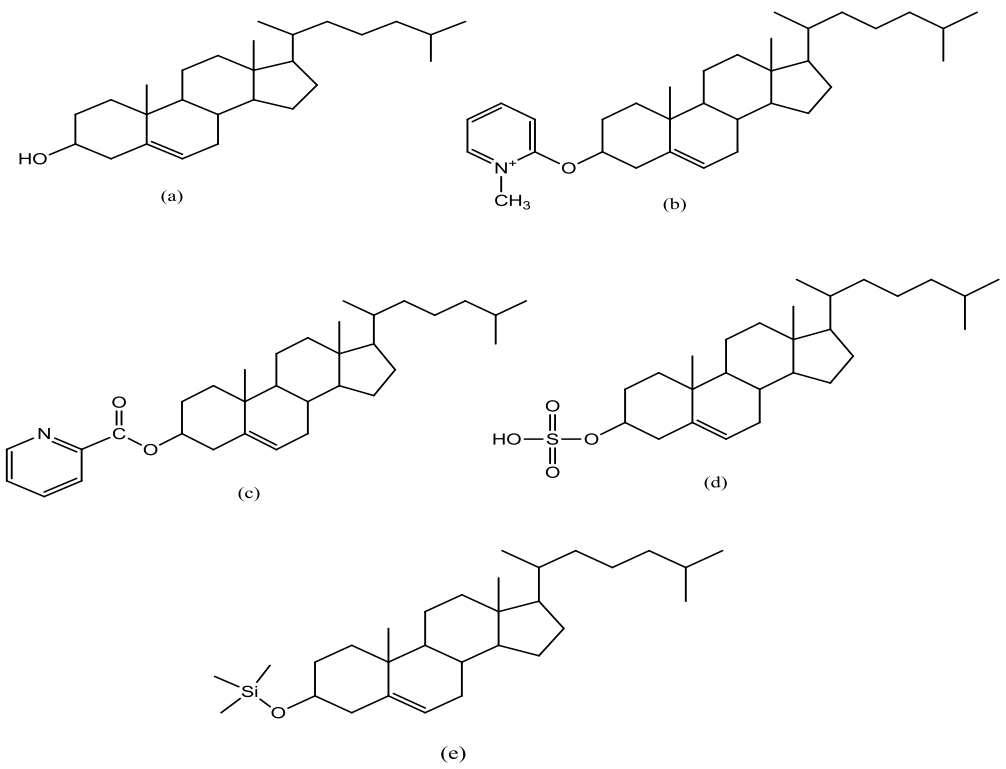


Figure 3.1. Chemical structures of (a) cholesterol, (b) cholesterol *N*-methylpyridyl ether, (c) cholesterol picolinyl ester, (d) cholesterol sulphate ester, and (e) cholesterol trimethylsilyl ether.

3.2.3 Sample preparation for MALDI-TOF analysis

3.2.3.1 Free sterols

Cholesterol, cholestanol and β-sitosterol were separately prepared in a mixture of chloroform/ methanol (1:1). The matrix (DHB) was prepared in the same solvent at a concentration of 10 mg/mL followed by addition of trifluoroacetic acid to give a final concentration of 0.1%. Each sterol standard was mixed with the matrix at a ratio of 1:1.

3.2.3.2 Sterol picolinyl esters

Picolinyl esters dissolved in dichloromethane were mixed with THAP (10 mg/mL in acetonitrile) at a ratio of 1:1 followed by addition of sodium acetate to yield a final concentration of 20 mM.

3.2.3.3 Sterol *N*-methylpyridyl ethers

N-methylpyridyl ethers dissolved in dichloromethane were mixed with THAP (10 mg/mL in dichloromethane/acetone (1:1)) at a ratio of 1:1.

3.2.3.4 Sterol sulphates

Sterol sulphates in dichloromethane were mixed with dithranol (2.5 mg/mL in dichloromethane) at a ratio of 1:1.

3.2.4 Quantification of derivatized sterols

Cholesterol and β-sitosterol were measured in mussel extracts by MALDI-TOFMS as their picolinyl and sterol sulphate esters. Calibration curves for cholesterol and β-sitosterol picolinyl esters, and cholesterol and β-sitosterol sulphates, were constructed using cholestanol picolinyl and cholestanol sulphate esters as internal standards, respectively. A 100 μg sample of cholestanol picolinyl and cholestanol sulphate ester were added to 500 μL of picolinyl and sulphated ester standards prepared at different concentrations (1000, 500, 250, 125, 62.5 μg/mL), respectively. The same amounts of internal standards were added to dried mussel samples after sample derivatization.

3.2.5 MALDI-TOF Instrumentation

A 1 μL sample of sterol derivative/matrix mixture was applied to the MALDI target plate and dried slowly before insertion into a 4800 MALDI TOF/TOF analyzer (AB Sciex). The machine was equipped with a Nd: YAG laser of 355 nm with a firing rate of 200 Hz and a camera to help direct the laser beam. For MS/MS studies, 1 kV positive or negative ion mode was used. Air at medium pressure was used for collision-induced dissociation.

3.2.6 ESI-QIT MS and APCI-QIT MS of picolinyl ester of sterols

Picolinyl esters of sterols were detected as protonated ions $[M+H]^+$ using a flow injection-ESI/APCI-MS system (Agilent 1100, SL LC/MSD (Trap) CA.USA) equipped with an ion trap mass selective detector operating in positive mode. 10 μL of picolinyl sterol esters in dichloromethane were injected into an acetonitrile eluent at a flow rate of 0.4 mL/min. The ESI operating parameters were as follow: nebulizer gas was nitrogen at a flow rate of 9 L/min and pressure of 60 psi, drying temperature 350 ºC, capillary voltage 3500 volts, capillary exit 109.4 volts and skimmer potential 40 volts. Full scan ESI mass spectra were acquired in positive mode and the mass range was set at m/z 50-600. In addition, picolinyl sterol esters were analyzed using an APCI source operating in positive mode. 10 μL of picolinyl sterol esters in dichloromethane were injected into a methanol eluent at a flow rate of 0.4 mL/min. The operating parameters were as follows: nebulizer gas was nitrogen with flow a rate of 7 L/min and with pressure of 60 psi, drying

gas temperature was 350 ºC, APCI source temperature was 250 ºC, capillary voltage 3500 volts and corona discharge current was 5000 nA.

ESI/APCI-QIT MS^2 and ESI/APCI-QIT MS^3 spectra of picolinyl ester of sterols were acquired in positive mode using helium as the collision gas with a fragmentation amplitude voltage of 1 volt and a mass window of 1.5 Da.

3.3 Results and Discussions

3.3.1 Direct analysis of sterols by derivatization MALDI-TOF mass spectrometry and tandem mass spectrometry

3.3.1.1 MALDI-TOF analysis

3.3.1.1.1 Free sterols

Sterol standards, cholesterol, cholestanol and β-sitosterol were analyzed by MALDI-TOFMS to observe their ionization behavior. Figure 3.2(a) shows the MALDI-TOF mass spectrum of cholesterol. The sterol fragment ion $[M+H-H_2O]^+$ at m/z 369.24 wasonly observed with a low signal intensity. Wang et al. [34] and Schiller et al. [39] reported that the molecular ion of cholesterol cannot be detected by MALDI-TOFMS and that free sterols readily lose a water molecule. Cholestanol and β-sitosterol were also detected as $[M+H-H_2O]^+$ ions at m/z 371.28 and 397.29, respectively (spectra not shown).

3.3.1.1.2 Picolinyl esters

In order to study the enhanced ionization efficiency of sterols by derivatization, cholesterol, β-sitosterol, ergosterol and cholestanol were derivatized to their picolinyl esters. The picolinyl esters were easily detected as their sodiated adducts ($[M+Na]^+$) and their ion intensities were significantly enhanced after addition of sodium acetate (final concentration = 20 mM). The most suitable matrix was either THAP or DHB. By illustration, the picolinyl ester of cholesterol is shown in Figure 3.2(b) showing a strong ion at m/z 514.36 ($[M+Na]^+$) using THAP. In additional experiments, the picolinyl esters of cholestanol, β-sitosterol and ergosterol showed strong $[M+Na]^+$ signals at m/z 516.36, 542.37 and 524.30, respectively (spectra are shown in Appendix. 1). When sodium acetate dopant was added, the intensity of $[M+Na]^+$ increased by 90%. Dopants ammonium acetate (20 mM) and trifluroacetic acid (0.1%) were attempted but only weak $[M+Na]^+$ signals were observed. In another study, Honda et al. [31] described the analysis of serum sterols as their picolinyl esters; however, the esters were analyzed by LC-ESI/MS and observed as $[M+Na+CH_3CN]^+$.

3.3.1.1.3 *N*-Methylpyridyl ethers

Sterols were also converted into their *N*-methypyridyl ethers. These derivatives were easily detected in positive mode using THAP since the derivative is positively charged. Cholesterol *N*-methylpyridyl ether was detected at m/z 478.40 $[M]^+$ (Fig. 3.2(c)). *N*-Methylpyridyl ethers of cholestanol and β-sitosterol were observed at m/z 480.41 and 506.41, respectively (spectra not shown). Ions related to incomplete derivatization of the

sterol standard, i.e., [sterol+H-H_2O]$^+$, were observed at m/z 369.33 for cholesterol (Fig. 3.2(c)) and m/z 397.34 for β-sitosterol (spectra not shown). *N*-Methylpyridyl ethers exhibited significant fragmentation to give an ion at m/z 110.00 (*N*-methylpyridyl fragment). Attempts to improve the conversion yield of this ether derivative were unsuccessful.

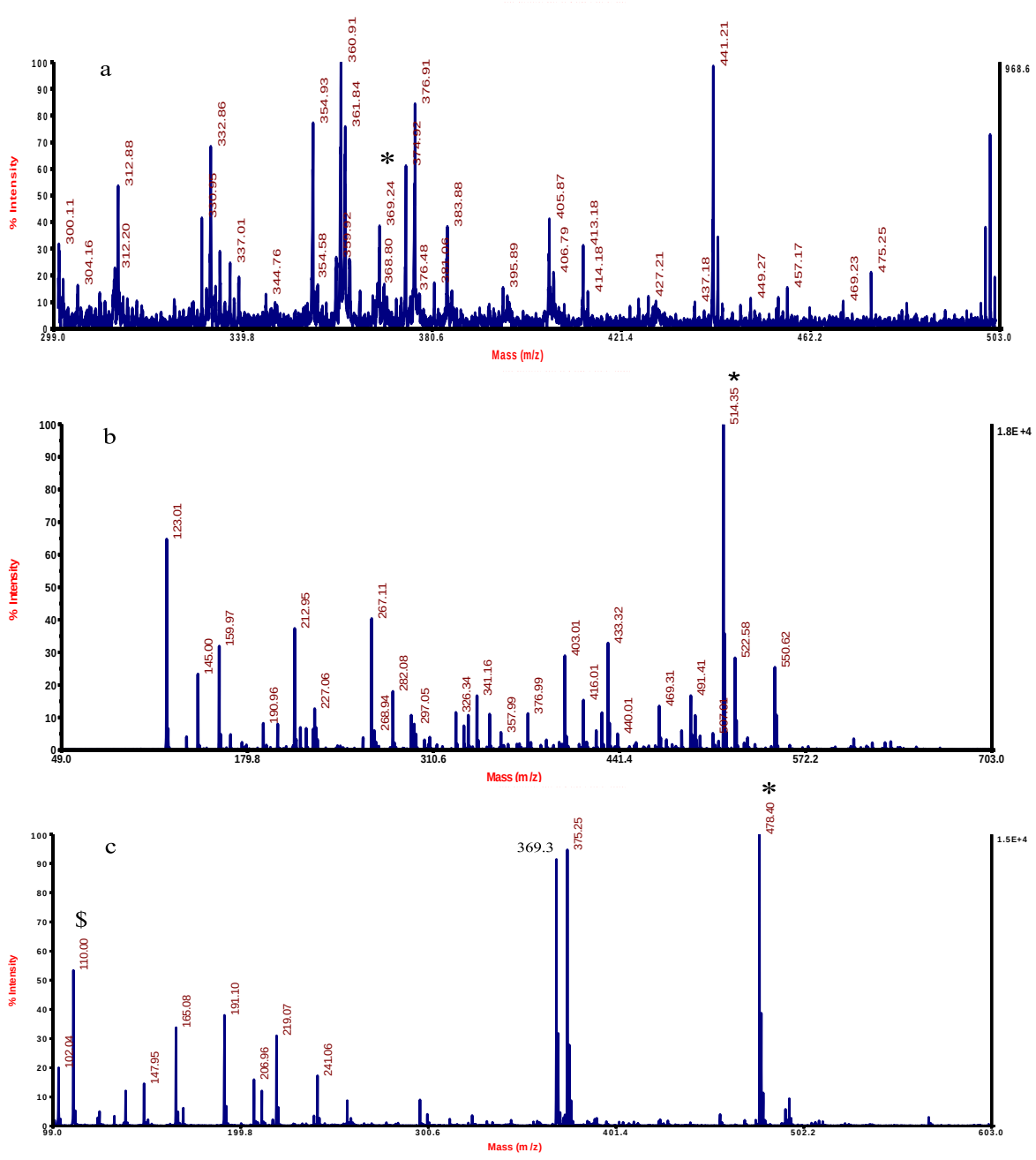


Figure 3.2. MALDI-TOF mass spectra of (a) cholesterol detected at m/z 369.24 $[M+H-H_2O]^+$, (b) cholesterol picolinyl ester at 514.36 $[M+Na]^+$, and (c) cholesterol *N*-methylpyridyl ether at 478.40 $[M]^+$. * signifies the target analyte; $ = *N*-methylpyridyl fragment.

3.3.1.1.4 Sterol sulphates

Sterol standards were derivatized to their sulphated esters and analyzed by MALDI-TOFMS. Finding the best MALDI matrix proved difficult and DHB, CHCA, THAP, dithranol and *p*-nitroaniline were tested. *p*-Nitroaniline or dithranol was the most suitable resulting in strong analyte ion intensity with the lowest matrix ion interference. As expected sulphated esters were observed best in negative mode $[M-H]^-$ and the use of base additives was not required to observe strong ion signals. The MALDI-TOF mass spectrum of cholesterol sulphate is shown in Figure 3.3(a) with $[M-H]^-$ at m/z 465.32. $[M-H]^-$ signals for other sterol sulphate esters were as follows: cholestanol (m/z 467.32), β-sitosterol (m/z 493.33) and ergosterol (m/z 475.28) (spectra are shown in appendix. 2).

Fragmentation for sterol sulphates was observed by the presence of ions HSO_3^- (m/z 79.95) and HSO_4^- (m/z 96.92) in Figures. 3.3(a) and 3.3(b). A higher abundance of these fragment ions was noted using *p*-nitroaniline (Fig. 3.3(b)) than with the dithranol matrix (Fig. 3.3(a)). Neto et al. [40] demonstrated the usefulness of sulphate esters for sterol analysis in tissue using LC/ESI-MS. It was found that steryl sulphates were best detected as $[M-H]^-$ which is in agreement with the present method. Sandhoff et al. [32] reported that sulphate ester derivatives easily lose a proton and thus result in high ionization efficiency.

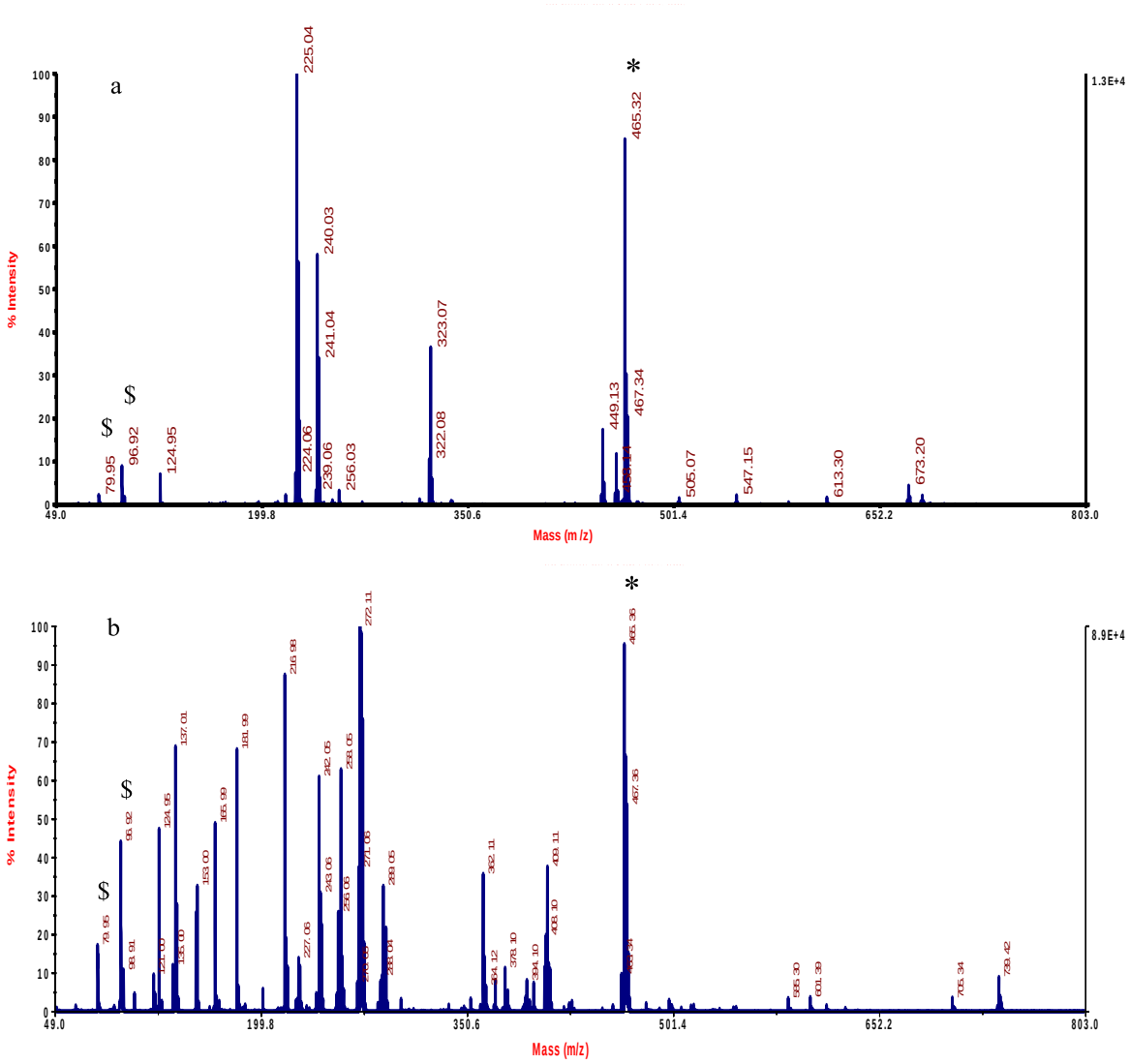


Figure 3.3. MALDI-TOF mass spectra of cholesterol sulphated ester detected at m/z 465.3 $[M-H]^-$ using (a) dithranol and (b) *p*-nitroaniline. * signifies the target analyte; $ = fragments.

3.3.1.2 MALDI-TOF/TOF analysis

MALDI-TOF/TOF experiments were performed on the derivatized sterols in order to confirm the identity of these derivatives and to study their fragmentation. Product ion scanning was carried out on the picolinyl ester of cholesterol with the precursor ion m/z 514.34 (Fig. 3.4(a)). A strong product ion was observed at m/z 146.03 representing [picolinate + Na]$^+$ and indicates facile ester bond cleavage. No product ion associated with the steryl moiety was observed. By subtracting the mass of picolinyl ion from the precursor ion, the molar mass of steryl moiety can be determined, i.e., 369 Da for cholesterol's carbon skeleton.

The MALDI-TOF/TOF mass spectrum of cholesterol *N*-methylpyridyl ether (precursor ion m/z 478.40) is shown in Figure 3.4(b). CID results in ether cleavage to give the charged *N*-methylpyridyl moiety at m/z 110.04.

The MALDI-TOF/TOF mass spectrum of cholesterol sulphated ester (precursor ion at m/z 465.32) is given in Figure 3.4(c). The ion $[HSO_4]^-$ at m/z 96.89 is the result of ester bond cleavage. An unexplained ion at m/z 458.95 is observed, likely an artifact from the derivative preparation.

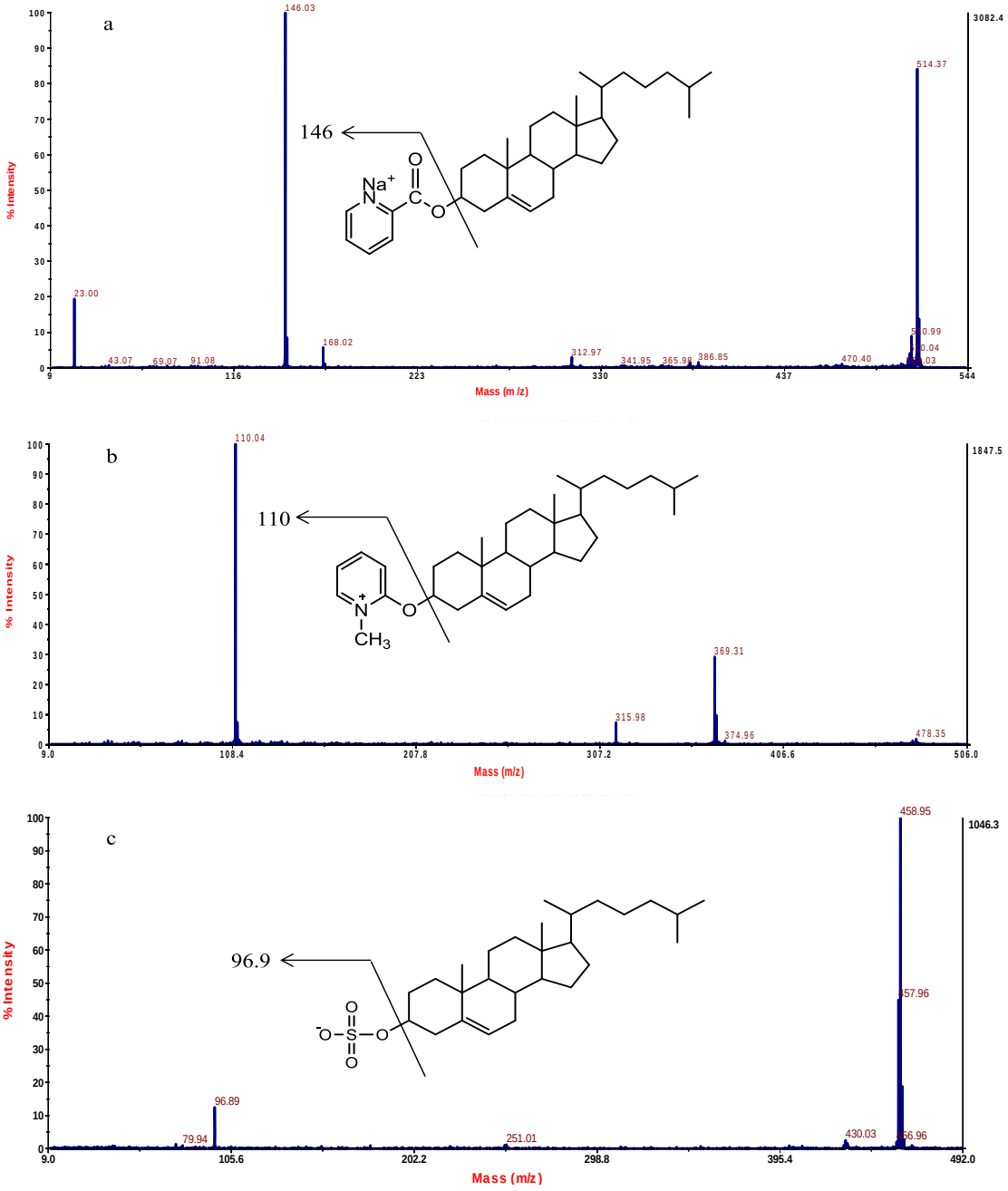


Figure 3.4. MALDI-TOF/TOF product ion spectra of (a) cholesterol picolinyl ester, selected precursor ion of m/z of 514.36, (b) cholesterol *N*-methylpyridyl ether, precursor ion of m/z of 478.40, and (c) cholesterol sulphated ester, precursor ion of m/z 465.32.

3.3.1.3 Reproducibility and sensitivity

The picolinyl and sulphate ester derivatives were selected for statistical analysis. Cholesterol at a concentration of 200 μg/mL was derivatized as its picolinyl and sulphated esters in replicates (n=3) and their molecular ion intensities measured by MALDI-TOFMS. Sources of variability include the preparation of derivatives and MALDI-TOFMS measurements which can be expressed as relative standard deviation (RSD %). Variability resulting from derivative preparation was found to be 8.3 % for cholesterol picolinyl and 8.5% cholesterol sulphated esters (Table 3.1). The same two derivatives were tested for reproducibility in measurement by MALDI-TOFMS where variability can be divided into within-spot and among-spots analyses. Variability within spots was calculated to be 3.7 % and 5.9 % for cholesterol picolinyl and cholesterol sulphated esters, respectively. Variability among sample spots was 5.3 % (picolinyl esters) and 6.6 % (sulphated esters) (Table 3.1).

The limit of detection for cholesterol was 12.0 μg/mL, lower for the picolinyl ester at 1.5 μg/mL and lowest for the sulphate ester at 0.2 μg/mL (Table 3.1). The limits of quantitation values are also given. The derivatization MALDI-TOFMS approach using the sterol picolinyl ester (LOD 1.5 μg/mL) shows a LOD similar to that reported for cholesterol trimethylsilyl ether by GC-MS single ion monitoring (1.2 μg/mL) [41]. The use of sulphated ester derivatives with MALDI-TOF analysis gives significantly better LODs than GC/MS.

Sensitivity was calculated based on the S/N ratio at an analyte concentration of 60 µg/mL. Sulphated ester derivatives had the highest S/N ratios (4028). MALDI-TOFMS analysis of sterols as their sulphated esters was 5 times more sensitive than picolinyl esters and 75 times more sensitive than free sterols.

Table 3.1. Comparison of figures of merit among derivatized sterols (cholesterol as standard) versus free sterols by MALDI-TOFMS.

	S/N At 60 µg/mL	LOD (µg/mL) at S/N=3	LOQ (µg/mL)	Reproducibility (RSD %) Preparation the derivative (n=3)	measurements within spot (n=3)	among spots (n=3)
Free sterols	54	12.0	40.0	-	-	-
Cholesterol picolinyl ester	780	1.5	5.0	8.3 %	3.7 %	5.3 %
Cholesterol sulphated ester	4028	0.2	0.67	8.5 %	5.9 %	6.6 %

3.3.1.4 Identification of sterols in blue mussels

Application of MALDI-TOFMS of sterol derivatives was applied to sterols extracted from cultivated mussels. GC/MS analysis was used alongside MALDI-TOFMS, as a comparative method. Mussel sterols were converted into their sulphated and picolinyl esters for MALDI-TOFMS sterol profiling and quantification. Figure 3.5 shows the resulting MALDI-TOF mass spectra of mussel sterols as their sulphated esters using dithranol (Fig. 3.5(a)) and as their picolinyl esters using DHB (Fig. 3.5(b)). Confirmation of the sterols labeled in Fig. 3.5 was performed by MS/MS experiments as shown in

Table 3.2 whereby sulphate esters were screened for fragment ion m/z 97 and sterol picolinyl esters using fragment ion m/z 146.

Sterols identified in mussels are listed in Table 3.2 based on MALDI-TOFMS data and confirmed by GC-MS analyses. The chemical structures of these sterols are given in Fig. 3.6. The data obtained from the analysis of sterols using GC/MS, as shown in Chapter two (Fig. (2.1)), revealed that the two groups of sterol isomers could not be resolved by the MALDI-TOFMS. The three sterol isomers occelasterol, *trans*-22-dehydrocholesterol and desmosterol (Table 3.2) for example, show up at m/z 512.30 as their picolinyl ester derivatives while two other sterols isomers, brassicasterol and 24-methylenecholesterol show up as their picolinyl esters at m/z 526.31.

A comparison of the MALDI-TOFMS spectra of the two derivatives for sterol analysis (Fig. 3.5), shows that the sulphate ester derivative profile has a significantly better S/N and has a lower matrix ion interference.

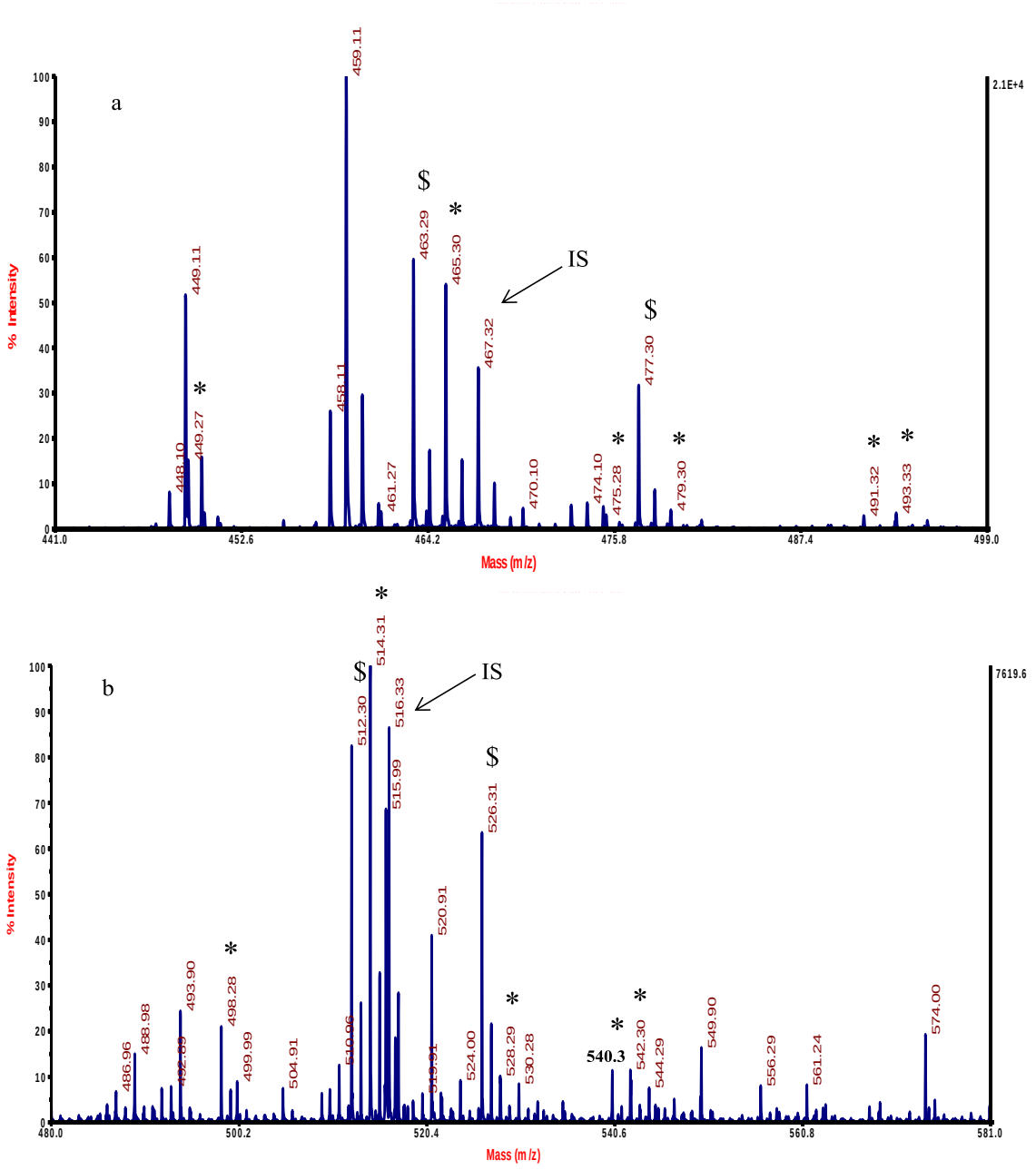


Figure 3.5. MALDI-TOF mass spectra of sterols profile in mussels as (a) sulphated esters and (b) picolinyl esters. * signifies derivatized sterols, $ signifies sterol isomers, IS = internal standard.

Table 3.2. Sterols identified in mussels as their picolinyl esters and sulphated ester derivatives, and their characteristic MS/MS fragmentation.

Sterols	Picolinyl esters		Sulphated esters	
	m/z $[M + Na]^+$	MS/MS data	m/z $[M - H]^-$	MS/MS data
24-nordehydrocholesterol	498.28	498.28 →145.98	449.27	449.27 → 96.91
occelasterol or trans-22-dehydrocholesterol or desmosterol	512.30	512.30 →145.97	463.29	463.29 → 96.89
cholesterol	514.31	514.31 →146.01	465.30	465.30 → 96.90
ergosterol	524.30	524.30 →146.01	475.28	475.28 → 96.90
brassicasterol or 24-methylenecholesterol	526.31	526.31 →145.97	477.30	477.30 → 96.91
campesterol	528.29	528.29 →145.99	479.30	479.30 → 96.89
stigmasterol	540.30	540.30 →146.01	491.32	491.32 → 96.90
β-sitosterol	542.30	542.30 →146.01	493.33	493.33 → 96.91

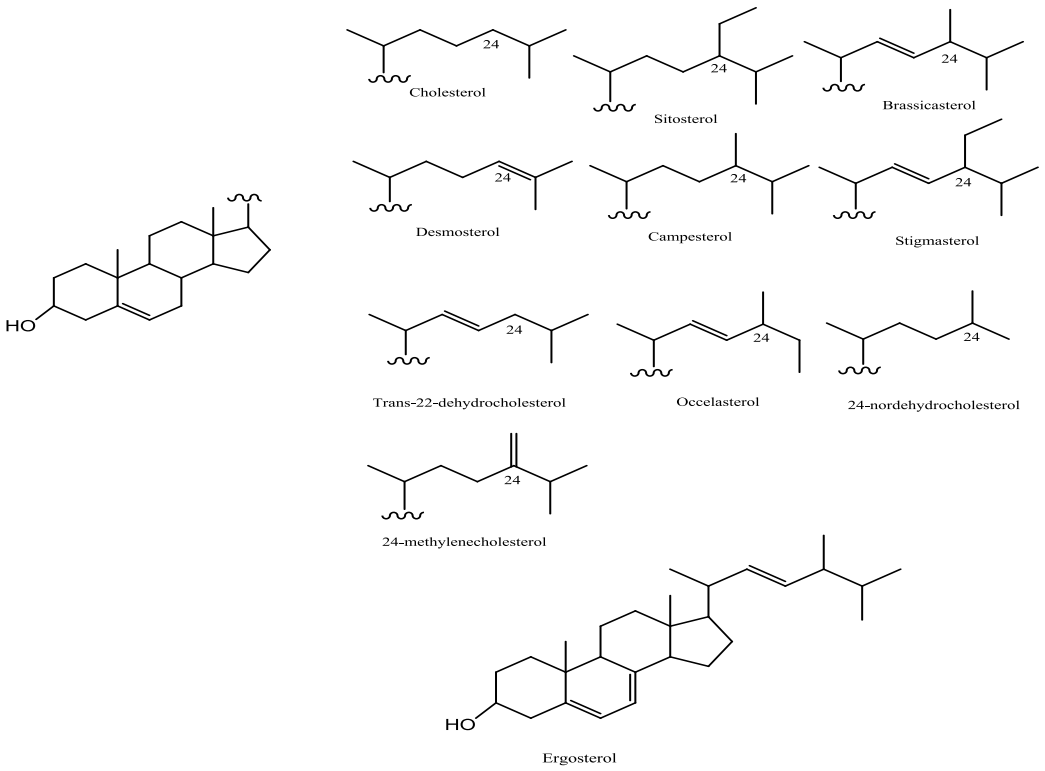


Figure 3.6. Chemical structures of sterols found in mussels.

3.3.1.5 Calibration for derivatives and sterol quantification in mussels

As a trial, two sterols (cholesterol and β-sitosterol) were quantified in the mussel extract. Calibration curves for cholesterol and β-sitosterol picolinyl esters, and cholesterol and β-sitosterol sulphate esters, were constructed using cholestanol picolinyl and cholestanol sulphate ester as internal standards, respectively (Appendix. 3). The derivatized sterol standard curves gave excellent correlation coefficients (R^2), and good reproducibility (Table 3.3).

The concentrations of cholesterol and β-sitosterol in mussels measured by MALDI-TOFMS as their picolinyl and sulphated esters and by GC/MS as their TMS derivatives are given in Table 3.4. The results of the two methods are reasonably comparable noting that the comparison was not between the same mussel extract, but from different mussel extracts from the same mussel supplier. Although these results represent only one biological sample, the results are very promising for the use of derivatization MALDI-TOFMS for rapid analysis of sterols extracted from biological samples.

Table 3.3. Calibration curve equations of derivatized sterol standards

	Calibration curve equation	R^2	Reproducibility (RSD %) (n=3)
cholesterol picolinyl ester	$y = 0.0027x - 0.0183$	0.9995	6.3
cholesterol sulphated ester	$y = 0.0031x - 0.0317$	0.9973	7.2
β-sitosterol picolinyl ester	$y = 0.0019x - 0.0385$	0.9987	7.5
β-sitosterol sulphated ester	$y = 0.0025x - 0.0609$	0.9993	8.6

Table 3.4. Sterol amounts (mg/kg ± SD wet wt) in mussel samples obtained by MALDI-TOFMS and GC/MS (n=3).

	MALDI-TOFMS		GC-MS
	Picolinyl ester	Sulphated ester	Trimethylsilyl ether
cholesterol	157.8 ± 8.4	232.9 ± 12.3	211.0 ± 10.2
β-sitosterol	32.7 ± 1.8	35.9 ± 1.9	38.2 ± 2.1

3.3.2 Analysis of sterols as their picolinyl esters using both flow-injection ESI-QIT MS^n and APCI-QIT MS^n

3.3.2.1 ESI-QIT MS and APCI-QIT MS

Sterol standards were derivatized to their picolinyl esters to investigate their ionization behavior and fragmentation pathways using ESI/APCI-QIT MS. The chemical structures of sterol standards as their picolinyl esters are shown in Figure 3.7. Picolinyl esters of sterols were found to be easily detected as the protonated molecular ion $[M+H]^+$. Fig. 3.8(a) shows flow injection ESI-QITMS analysis of cholesterol picolinyl ester which was detected at m/z 492.4. In addition to the protonated molecular ion, an abundant fragment ion $[M+H-C_6H_5NO_2]^+$ at m/z 369.4 was observed indicating that picolinyl esters can lose picolinic acid under ESI conditions. The analysis of this derivative by APCI-QITMS showed similar ionization and fragmentation behaviors. However, sterols containing an additional double bond in the B ring of the sterol skeleton such as 7-dehydrocholesterol behaved differently under both ESI and APCI conditions. Its molecular ion cannot be observed by ESI-QIT MS and facile cleavage occurred, giving an abundant fragment ion ($[M+H-C_6H_5NO_2]^+$) at m/z 367.4. However, in APCI-QITMS, the sterol derivative could be detected as a radical cation $[M]^{\cdot+}$ (Fig. 3.9). An abundant fragment ion corresponding to $[M+H-C_6H_5NO_2]^+$ at m/z 367.4 was observed (Fig. 3.9(a)). This observation is in agreement with the results reported by Herrera et al. [42] as they observed benzothiophene formed radical cations when analyzed by APCI. The analysis of other picolinyl sterol esters, using ESI-MS and ESI-MS^2, showed similar ionization and fragmentation behaviors to the picolinyl ester of cholesterol (Appendices 4-7).

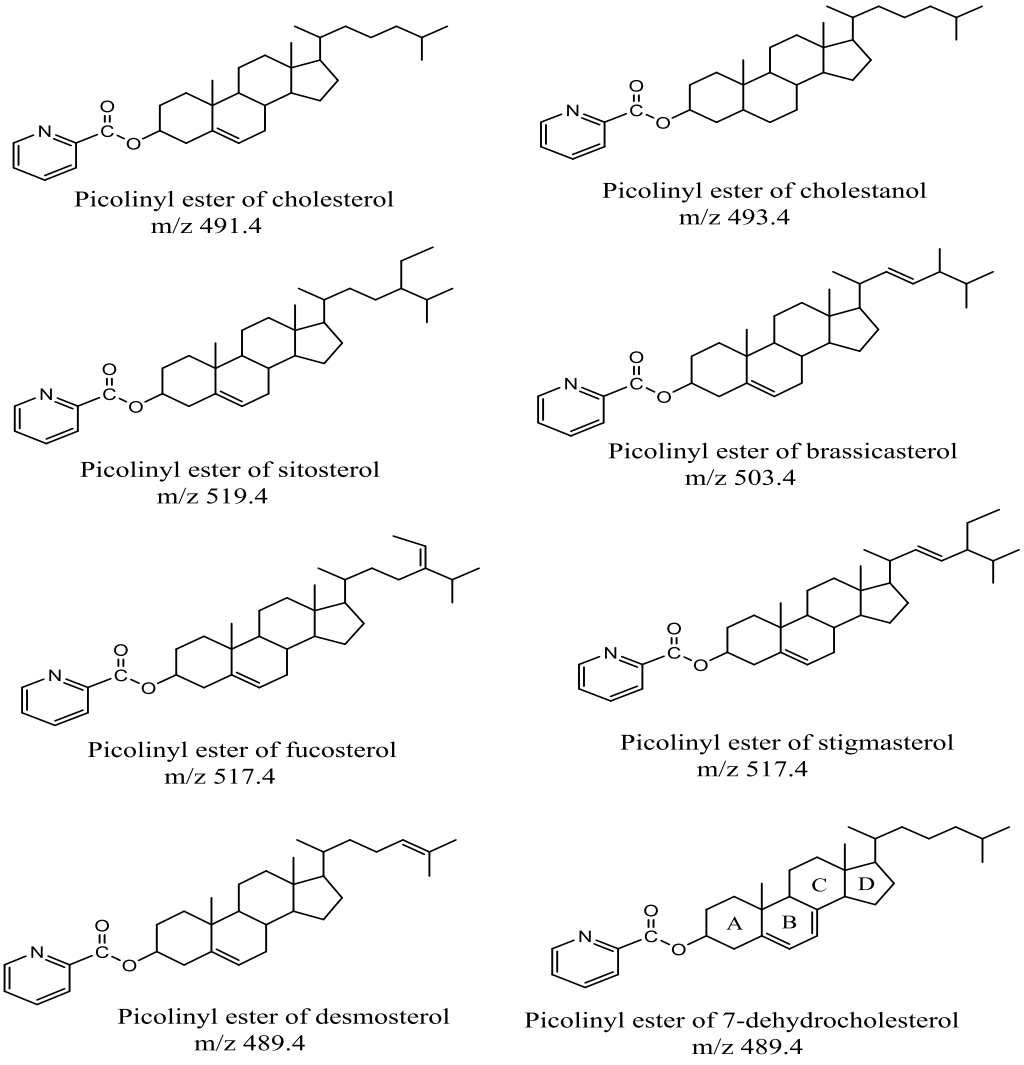


Figure 3.7. Chemical structures of picolinyl ester of sterols used in this study

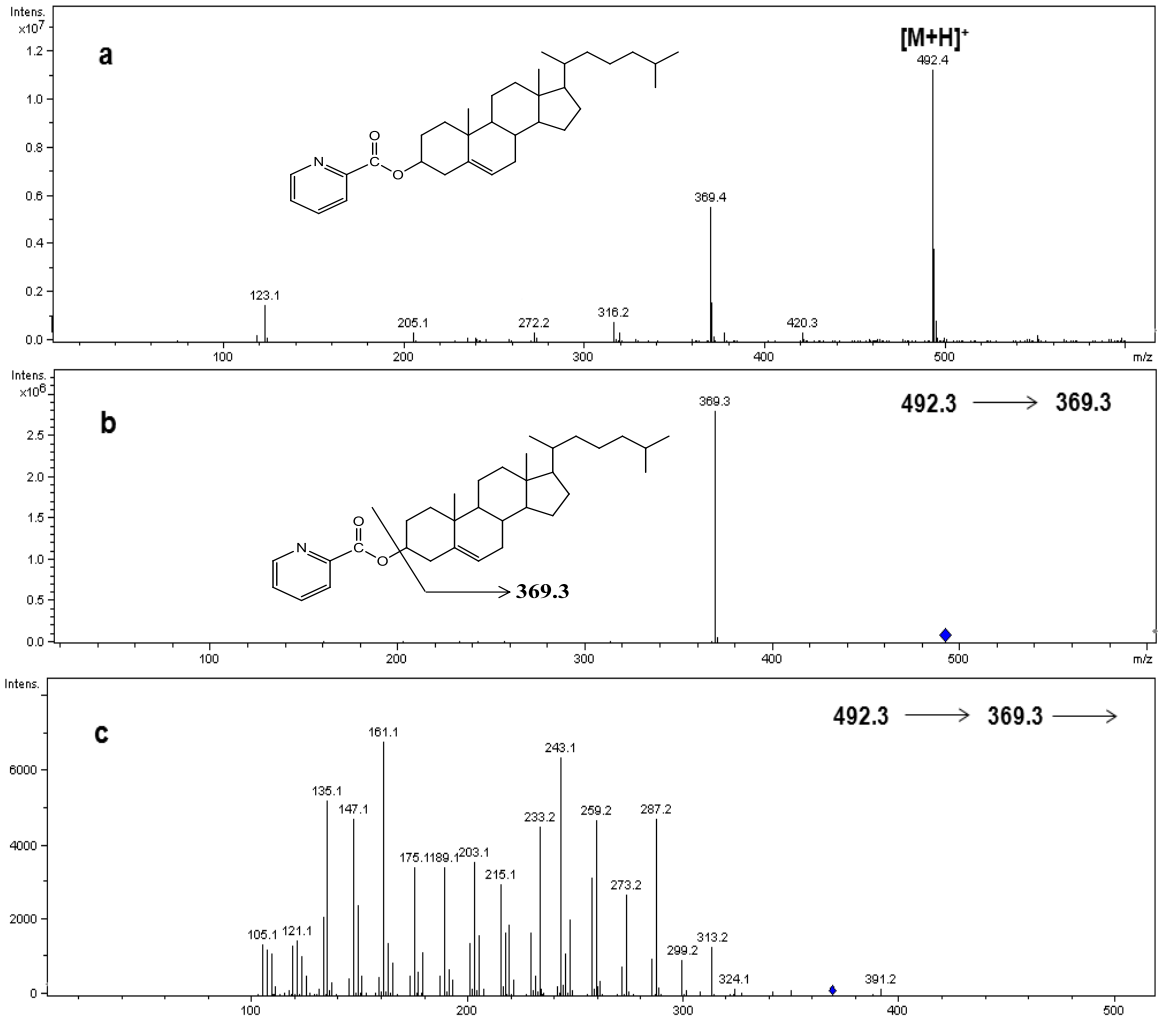
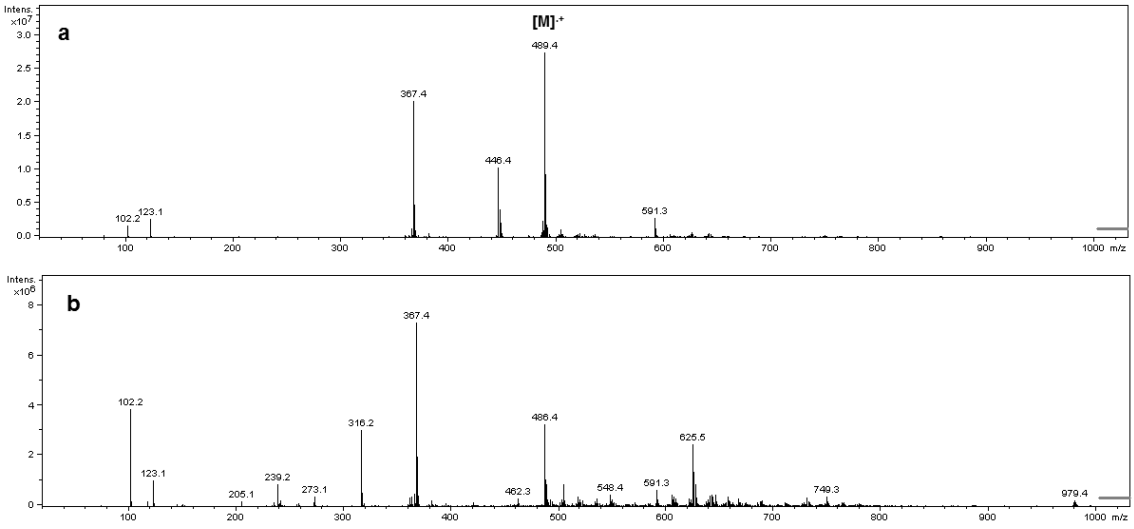


Figure 3.8. (a) Flow injection ESI-QIT MS spectrum of cholesteryl picolinyl ester detected as $[M+H]^+$, (b) The CID MS^2 spectrum of the precursor ion m/z 492.3, and (c) the CID MS^3 spectrum of the precursor ion m/z 369.3 which is the cholesteryl cation.



3.9. (a) Flow injection APCI-QIT MS spectrum of picolinyl ester of 7-dehydrocholesterol detected as radical cation $[M]^{\cdot+}$, (b) flow injection ESI-QIT MS spectrum of picolinyl ester of 7-dehydrocholesterol with no molecular ion observed.

3.3.2.2 ESI-CID MS^2 and APCI-CID MS^3 of picolinyl sterol esters

ESI-CID MS^2 and APCI-CID MS^2 of picolinyl esters were carried out to investigate the fragmentation pathways and to confirm the identity of the sterols. CID MS^2 spectra were obtained for cholesterol picolinyl ester using both ESI-QIT and APCI-QIT. As shown in Fig. 3.8(b), the cholesterol picolinyl ester cleaves at the ester bond, during CID MS^2, exhibiting product ion $[M+H-C_6H_5NO_2]^+$ (steryl cation moiety) at m/z 369.3. ESI-QIT MS^3 and APCI-QIT MS^3 were performed on picolinyl esters. in the results showed that both r ESI-CIDMS^3 and APCI-CID MS^3 of $[M+H]^+ \rightarrow [M+H-C_6H_5NO_2]^+$ gave informative, unique fragments that can be utilized for structural elucidation of the sterol carbon skeleton and for distinguishing among sterol isomers as will be discussed in the following section.

MALDI-TOF MS of derivatized sterols as discussed above showed different ionization and fragmentation behaviors since picolinyl sterol esters showed very weak protonated molecular ion signals. However, sodiated adducts, with excellent intensity, were formed after addition of sodium acetate (20 mM). Product ion scanning high energy CID MALDI-TOF/TOF resulted in the sodiated picolinyl moiety ($[C_6H_5NO_2+Na]^+$), m/z 146.1, while product ion scanning low energy ESI/APCI QIT MS^2 resulted in the steryl cation as mentioned previously.

A method for analysis of sterols in serum using liquid chromatography tandem mass spectrometry (LC-ESI-triple quadruple-MS/MS) after derivatization to picolinyl esters was developed by Honda et al. [31]. It was found that picolinyl sterol esters formed adduct ions with sodium and acetonitrile $[M+Na+CH_3CN]^+$ and the CID-MS/MS spectra of these adducts gave a base peak ($[M+Na]^+$) and a sodiated picolinyl moiety with a low intensity at m/z 146.

3.3.2.3 ESI-MS^3 of sterol picolinyl esters

ESI-CID MS^3 mass spectra of four sterol picolinyl ester derivatives were obtained in positive ion mode as shown in Fig. (3.10) those being cholesterol (Fig. 3.10(a)), cholestanol (Fig. 3.10(b)), β-sitosterol (Fig, 3.10(c)) and brassicasterol (Fig. 3.10(d)). The CID of $[M+H]^+ \rightarrow [M+H-C_6H_5NO_2]^+$ showed unique and multi-pathway fragmentation. Figure 3.11 shows a proposed fragmentation pathway of the β-sitosteryl moiety using ESI-QIT MS^3. The most abundant ions are at m/z of 161.1, 147.1, 135.1, 243.2, 257.2, 315.3, and 297.2. Fragment ions at m/z of 315.3, 327.3, 341.2, 355.3 and 369.3 are

diagnostic of the side chain cleavage, and other fragment ions arise from multi carbon ring cleavage. Fragment ions at m/z 147.1 and 161.1 are diagnostic of free sterols that have a double bond in the B ring (i.e. β-sitosterol and cholesterol). However, most of fragment ions observed in the saturated sterol (cholestanol) are shifted by 2 mass units; i.e. in cholestanol, fragment ions at m/z 163.1 and 149.1 were observed instead of ions at m/z 161.1 and 147.1 in cholesterol (Fig. 3.10 (a)). The peak at m/z 257.2 probably arises from losing the side chain of the steryl cation. However, the CID spectrum of brassicasteryl showed a different fragmentation pathway where the fragment ion at m/z 255.1 was observed as a base peak which is diagnostic of sterols that have a double bond between carbons 22 and 23. However, the fragment ion at m/z 255.1 could result either from loss of the side chain or the cleavage in the A ring and the side chain as shown in Figure 3.11 (b)

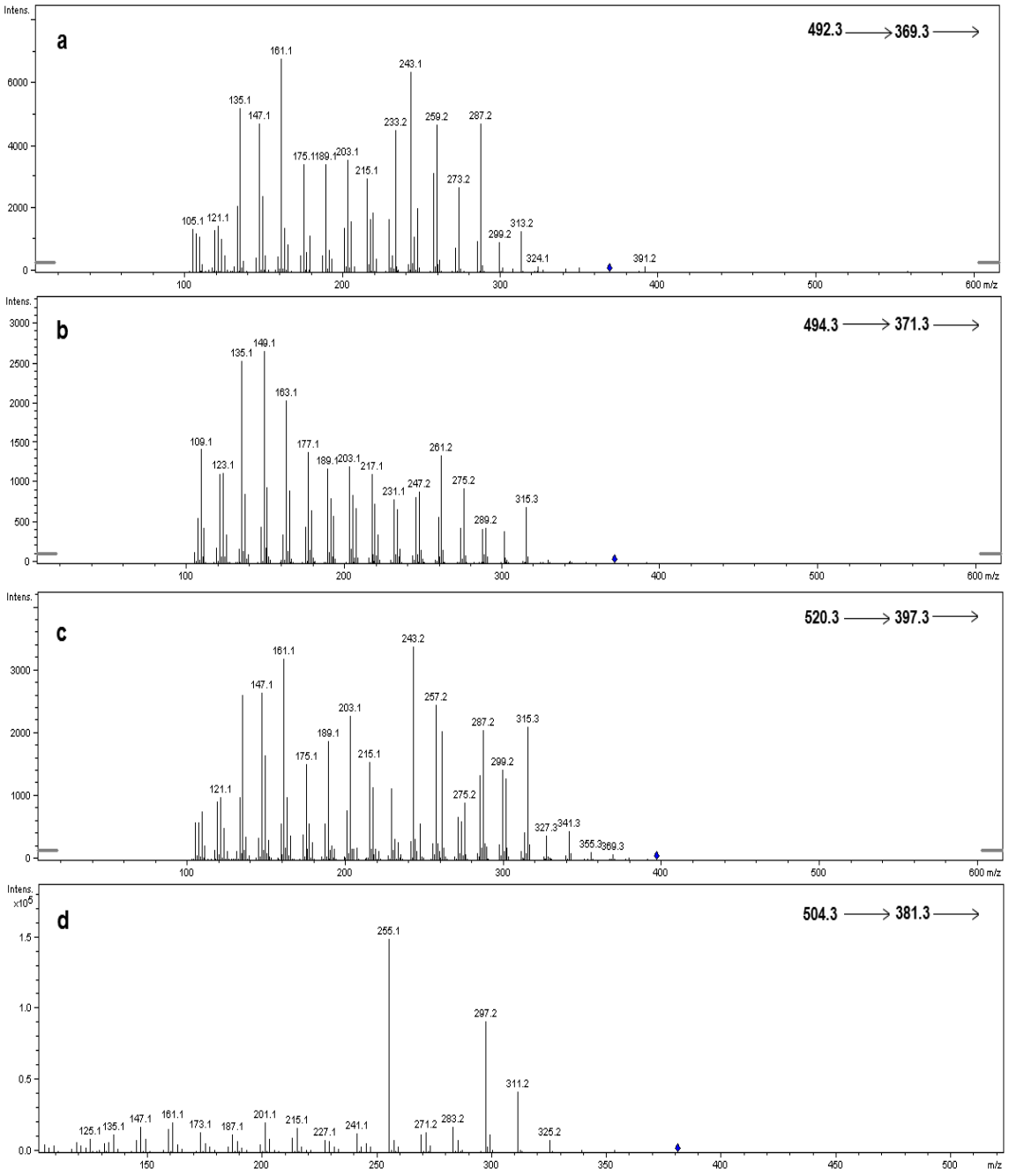


Figure 3.10. ESI MS^3 spectra of precursor ions of the steryl cationic fragment moieties of the picolinyl ester of sterols. (a) picolinyl ester of cholesterol, (b) picolinyl ester of cholestanol, (c) picolinyl ester of β-sitosterol, and (d) picolinyl ester of brassicasterol.

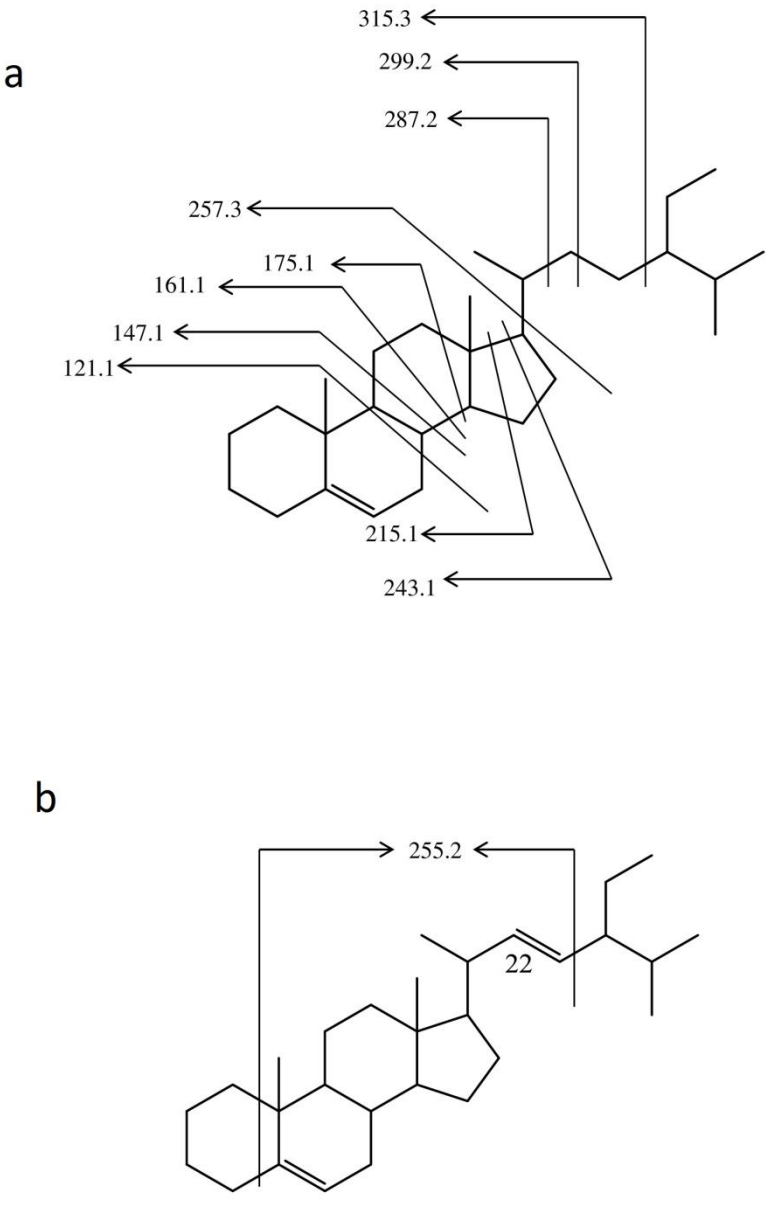


Figure 3.11. (a) Proposed CID fragmentation pathway of t β-sitosterol cationic moiety resulting from QIT MS^n as revealed in spectra of Figure 3.10 (c), (b) fragmentation pathway for the generation of the base ion m/z 255.2 from stigmasteryl cationic moiety as revealed in the spectra of Figure 3.12(a`)

3.3.2.4 Distinguishing among isomers using CID-ESI/APCI MS^3

CID-ESI/APCI MS^3 experiments resulted in unique fragmentation patterns that allow structural elucidation and differentiation among sterol picolinyl ester isomers. Figure 3.12 shows CID-ESI MS^3 spectra of two sets of sterol picolinyl ester isomers. The first set consisted of the picolinyl esters of fucosterol (Fig. 3.12(a)), and stigmasterol (Fig. 3.12(a`)), m/z 517.4, and the second set included picolinyl esters of desmosterol (Fig. 3.12(b)), and 7-dehydrocholesterol (Fig. 3.12(b`)), m/z 489.4. Significant differences in ion intensities were observed in the CID-MS^3 spectra of the picolinyl ester of fucosterol and stigmasterol. In the spectrum of the picolinyl ester of stigmasterol, the fragment ion at m/z 255.2 was found to be the base peak while fragment ions at m/z 161.1, 255.2, 297.2 and 311.2 were found to be significant fragments in the CID spectrum of the picolinyl ester of fucosterol (Fig. 3.12(a)).

The picolinyl esters of desmosterol and 7-dehydrocholesterol can also be distinguished. The CID spectrum of the picolinyl ester of desmosterol showed major fragment ions at m/z 257.2, 161.1, 147.1, 203.1 and 285.2 (Fig. 3.12(b)), while, the picolinyl ester of 7-dehydrocholesterol showed only two major fragment ions at m/z 159.1 and 145.1 (Fig. 3.12(b`)). The fragment ions at m/z 161.1 and 147.1 in the CID-MS^3 spectrum of the picolinyl ester of desmosterol and those at m/z 159.1 and 145.1 in the CID-MS^3 spectrum of the picolinyl ester of 7-dehydrocholesterol arise from the cleavage of the C ring. However, the shifting by 2 mass units in the CID-MS^3 spectrum of picolinyl ester of 7-dehydrocholesterol confirm the presence of a second double bond in the C ring of 7-dehydrocholesterol (Fig. 3.12(b`)).

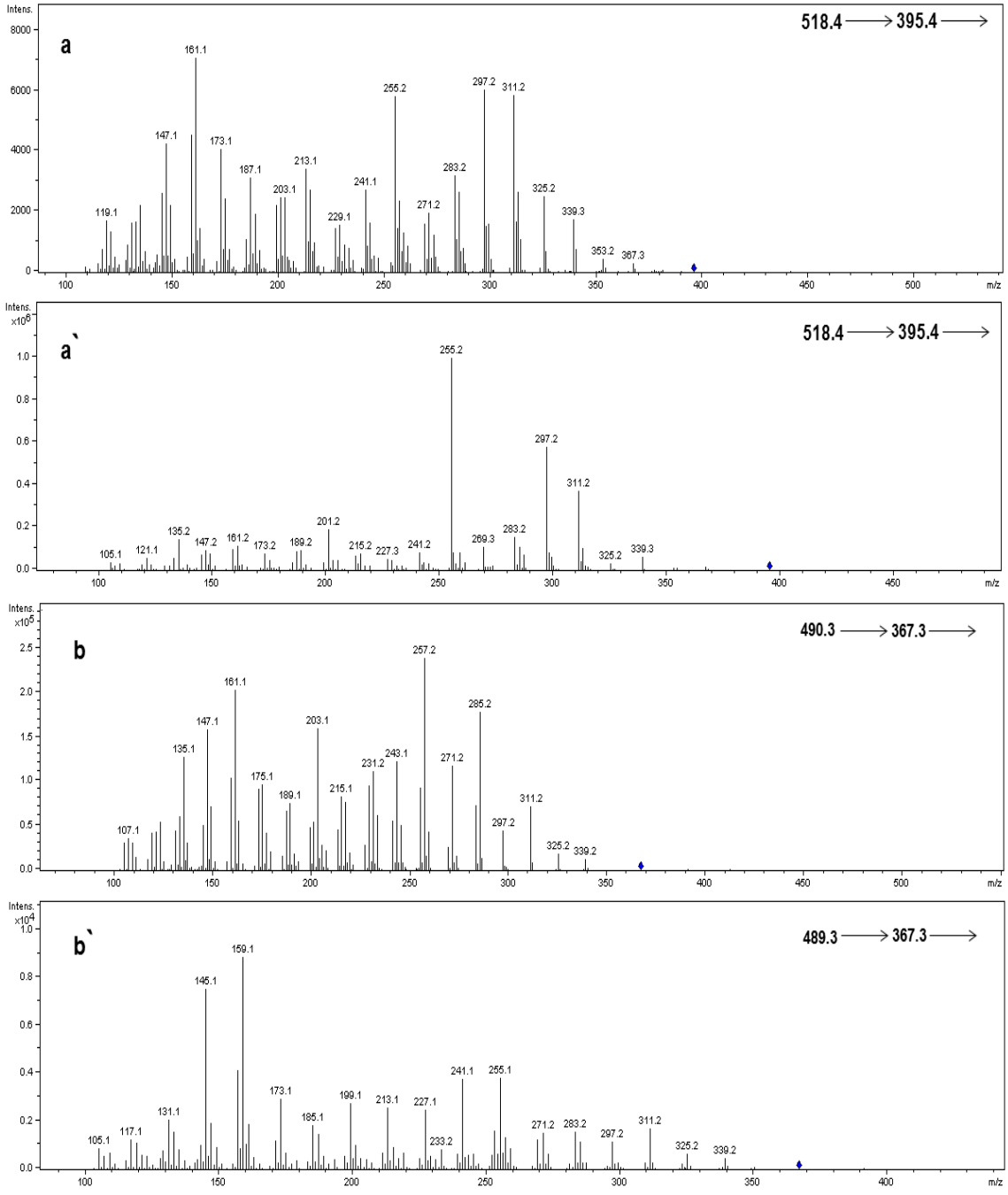


Figure 3.12. ESI MS^3 spectra of precursor ions of the steryl cationic fragment moieties of the picolinyl ester of sterol isomers. (a) picolinyl ester of fucosterol, (a`) picolinyl ester of stigmasterol, (b) picolinyl ester of desmosterol, and (b`) APCI MS^3 of picolinyl ester of 7-dehydrocholesterol.

3.4 Conclusion

A new and rapid method has been developed for analysis of free sterols using MALDI-TOFMS, MALDI-TOF/TOFMS and ESI/APCI-QIT MS^n. Free sterols are poorly suited for direct MS analysis and were converted to their corresponding picolinyl esters, *N*-methylpyridyl ethers and sulphated esters. Using MALDI-MS, picolinyl esters were detected as sodiated adducts $[M+Na]^+$ whose signal were enhanced after the addition of sodium acetate using either DHB or THAP. *N*-Methylpyridyl ethers were detected as $[M]^+$ using THAP. Sulphated esters were detected as $[M-H]^-$ using either *p*-nitroaniline or dithranol. The ester bonds of picolinyl and sulphated esters showed facile cleavage during MALDI-CID MS/MS resulting in diagnostic fragments at m/z 146 and 97, respectively. Based on MALDI-TOFMS and GC/MS data, the most abundant sterols in mussels were cholesterol, desmosterol, brassicasterol, 24-nor-dehydrocholesterol, 24-methylenecholesterol and *trans*-22-dehydrocholesterol. The sterol profile of mussels obtained by MALDI-TOF is in good agreement with those obtained by GC/MS. Cholesterol and β-sitosterol were successfully quantified using MALDI-TOFMS. Sulphated esters showed the highest sensitivity; 5 times more sensitive than picolinyl esters and with a better detection limit for sterols than GC/MS. Calibration curves exhibited excellent linear regression which indicates that MALDI-TOF can be used for quantitation of sterols.

Picolinyl esters of sterols were analyzed also using direct injection ESI-QIT MS^n and by APCI-QIT MS^n as well. The picolinyl esters readily formed protonated molecular ions ($[M+H]^+$) in ESI and APCI sources except for the picolinyl ester of 7-

dehydrocholesterol which was detected as the radical cation ion $[M]^{\cdot+}$ using APCI-QIT MS. The ester bonds of picolinyl esters cleaved during CID MS^2 resulting in diagnostic fragments corresponding to steryl cation moieties $[M+H-C_6H_5NO_2]^+$. The CID MS^3 of $[M+H]^+ \rightarrow [M+H-C_6H_5NO_2]^+$ of picolinyl esters spectra were found to be useful for structural elucidation and to distinguish among steryl isomers.

3.5 References

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Chapter Four: Identification of fatty acid steryl esters in margarine and corn using direct flow injection ESI-MS^n Ion Trap-Mass Spectrometry

A version of this chapter has been published. Iyad Hailat and Robert J. Helleur. Identification of fatty acid steryl esters in margarine and corn using direct flow injection ESI-MS^n Ion Trap-Mass Spectrometry. *Int. J. Mass Spectrom.* **2014**, 362, 24-31.

4.1. Introduction

It is known that increased levels of cholesterol in human blood can cause coronary heart disease and scientists are interested in studying the factors that can lead to its decrease. Several studies have demonstrated that a diet containing plant-derived steryl esters can inhibit the absorption of cholesterol inside the small intestine and thus decrease the level of cholesterol [1-3]. In one study [1], plant steryl esters and non-esterified stanols, two-thirds of which were incorporated into low-fat foods, contributed effectively to LDL cholesterol lowering (13.6 % for esters and 8.3 % for non-esterified sterols). The LDL cholesterol-raising effect of butter fat could be countered by including steryl esters. Therefore, there is growing interest in phytosterols and steryl esters as healthy ingredients in various foods within the scientific community and by commercial organizations [4-8]. Sterols occur in plants as free sterols, esterified to fatty acids (steryl esters), as steryl glucosides (sterols attached to monosaccharides) or as acylated steryl glucosides (attached to acylated monosaccharides) [4, 9]. Cereals, seeds and their derived vegetable oils are considered important sources that contain a significant amount of phytosterols [10]. Recently, the food sector has begun esterifying free phytosterols (extracted from vegetable oils and plants) with fatty acids and adding them to food products like margarine and marketing them as healthy phytosterol ingredients [11]. The U.S. Food and Drug Administration has permitted the marketing of enriched food products that contain a considerable proportion of sterols and steryl esters [12].

The diversity of steryl esters is due to the variety in the chemical structures of both steryl and fatty acyl moieties. The fatty acyl moieties can be saturated or mono- or polyunsaturated and can have variable chain lengths. The steryl moieties also have different chemical structures where they differ from each other in the carbon side chain and by the degree of unsaturation. Several techniques have been used for determining steryl esters in biological samples. Typically, the analysis of steryl esters using GC requires alkaline hydrolysis to obtain the free sterol. The free sterol fraction is then isolated by solvent extraction, then solid phase extraction or preparative thin layer chromatography [14-16]. However, these techniques are time consuming and identification of the fatty acid moiety is missing. Reversed phase HPLC using different detectors has been used for the analysis of intact steryl esters. UV detection was used for determining cholesterol esters [17] while evaporative light scattering detection (ELSD) has been used for analysis of mixed steryl esters [18]. In both methods, the triacylglycerol fraction which can interfere with the analysis has to be removed using preparative chromatography.

Mezine et al. [12] have used HPLC with APCI-MS for identification of intact steryl and stanyl esters in cholesterol-lowering spreads and beverages. HPLC coupled to electrospray ionization (ESI)-MS and ELSD has been used for the identification of fatty acid steryl esters in wheat samples [19]. A C_{18} column was used and the order of elution of steryl esters was found to depend on the length of the fatty acyl moiety and the number of double bonds. For steryl esters with the same steryl moiety and the same length of fatty acids but differing in the unsaturation degree of the fatty acyl moiety, the higher degree of

unsaturation eluted first. Nanospray ionization quadrupole-time-of-flight tandem mass spectrometry (Q-TOF MS/MS) has been used for identification of steryl esters in plants [20].

Since steryl esters are nonpolar and resist electrospray ionization, dopants may be added to facilitate their ionization. Ammonium acetate is the most common dopant used for the analysis of steryl esters using ESI-MS forming ammonium adducts $[M+NH_4]^+$ which are easily detected in positive mode. However, steryl esters cannot be identified in atmospheric pressure chemical ionization (APCI) as intact molecules since they easily fragmented and form $[M+H-HOOCR]^+$ [12].

The coupling of a soft ionization technique like ESI with quadrupole ion trap mass spectrometry ((ESI-QIT MS^n) along with direct flow injection can be a very efficient way for rapid analysis of mixtures even those containing isomers as the technique allows for more detailed structural elucidation using collision-induced dissociation (CID) tandem MS. In contrast to triple quadrupole MS, QIT MS can also perform MS^3 and higher CID experiments thus giving additional structural information [13]. In tandem mass spectrometry, a precursor ion of the target analyte is selected by the first mass analyzer which is then transmitted to the collision cell in which the precursor ion is subjected to fragmentation as a result of collision with a neutral gas and, by scanning the second mass analyzer, the product ions can be identified.

This study demonstrates for the first time a comprehensive identification of fatty acid steryl esters in margarine and corn using flow injection-ESI-QIT MS along with

CID tandem MS (MS^2 and MS^3) without the need for chromatographic separation. Many of the fatty acid steryl ester isomers were identified using ESI-QIT MS^2 while ESI-QIT MS^3 assisted in confirming the chemical structures of the steryl moieties.

4.2. Materials and methods

4.2.1. Chemicals and materials

Cholesterol, β-sitosterol, stigmasterol and brassicasterol were purchased from Steraloids Co. (New Port, RI, USA) and cholesteryl oleate and ammonium acetate were obtained from Sigma Chemicals (Oakville, ON, Canada). All solvents were HPLC grade and purchased from Fisher Scientific Ltd (Ottawa, ON, Canada). The DSC-NH_2 solid phase cartridges (6 mL tube, 1 g) were obtained from Supelco (Bellefonte, PA, USA) and silica cartridges (6 mL tube, 0.5 g) from UCT Environ (Bristol, PA, USA).

4.2.2. Samples

A calorie-reduced margarine enriched in plant sterols (extracted from soybeans and other legumes) was purchased from a local supermarket and stored in the freezer. Corn cobs were obtained fresh from a local supermarket. The corn kernels were removed with a knife and stored at 4 °C.

4.2.3. Lipid extraction

Two gram samples of margarine and 3 g of corn kernel samples (previously mashed using a mortar and pestle) were transferred to an extraction vessel containing a magnetic stir bar. Lipids were extracted using 20 mL (for margarine) and 30 mL (for corn) of

hexane, with continuous stirring for 1 h at room temperature. Other, more polar, extraction solvents were tested but hexane was found to be suitable for steryl ester extraction. Samples were filtered and the residue washed 3 times with hexane. Hexane extracts were then evaporated to dryness under nitrogen. These lipid extracts were re-dissolved in 5 mL hexane and stored in a N_2 filled vial in a fridge at 4 ^{0}C.

4.2.4. Solid phase extraction

A solid phase extraction (SPE) cartridge (DSC- NH_2, 6 mL) was used for extraction and cleanup of steryl esters. The cartridge was conditioned with 10 mL of hexane. 1 mL of solvent-extracted lipids (in hexane) solution was loaded and the triglycerides removed with 15 mL of hexane/ethyl acetate (95:5, v/v). Steryl esters were then eluted with 10 mL of hexane: diethyl ether (98:2, v/v), and evaporated to dryness under nitrogen. The crude steryl ester samples were further purified on a silica cartridge. Samples were dissolved in 1 mL of toluene and loaded on to the cartridge (which was first preconditioned with10 mL of hexane). Steryl esters were eluted with 15 mL of hexane. Samples were evaporated to dryness and stored under nitrogen in the freezer.

4.2.5. Instrumentation and analysis

Steryl esters were identified as ammonium adducts using flow injection-ESI-MS system (Agilent 1100, SL LC/MSD (Trap) CA, USA) equipped with an ion trap mass selective detector operating in positive mode. The steryl ester fraction was dissolved in 1 mL of chloroform: methanol (2:1) containing 20 mM ammonium acetate. The steryl standard solutions (100 µg/mL) were made up in the same solvent.

A 10 μL sample was injected into a methanol eluent flow of 0.3 mL/min. The ESI-MS^n operating parameters were adjusted to give intense $[M + NH_4]^+$ ions and useful and reproducible fragmentation spectra and are as follows: nebulizer gas was nitrogen at a flow rate of 8 L/min and pressure of 40 psi, drying temperature 350 °C, capillary voltage 3500 volts, capillary exit 109 volts and skimmer potential 40 volts. Full scan ESI mass spectra were acquired at m/z 200-1000. ESI-QIT MS^2 and ESI-QIT MS^3 spectra of steryl esters and their fragment ions were recorded in positive mode using helium as the collision gas with a fragmentation amplitude voltage of 1 volt and a mass window of 1.7 Da.

Free sterol standards were analyzed in positive mode APCI-QIT MS (Agilent 1100, SL LC/MSD (Trap) CA, USA). 10 μL of 100 μg/mL of each sterol, (dissolved in chloroform: methanol (2:1, v/v)), was injected into a methanol eluent with a flow rate of 0.5 mL/min. The APCI MS operating parameters were as follows: drying gas temperature 220 °C, APCI source temperature 250 °C, nebulizer gas was nitrogen at a flow rate of 5L/min and pressure 50 psi, capillary voltage 3500 volts and corona discharge current was 4000 nA. APCI-QIT MS^2 spectra of free sterols were acquired in positive mode using helium as the collision gas with a fragmentation amplitude voltage of 1 volt and a mass window of 1.5 Da.

4.3. Results and discussion

Flow injection ESI-QIT MS^2 and ESI-QIT MS^3 was shown to be an effective tool for structure identification of the steryl moieties and the molecular weights of fatty acid moieties of steryl esters present in a lipid mixture. However, in order to overcome ion suppression in ESI by the presence of other lipid components, lipid extracts were subjected to cleanup as described in the experimental. Steryl esters are nonpolar and resist electrospray ionization so addition of ammonium acetate dopant facilitates ionization as ammonium adducts $[M+NH_4]^+$.

4.3.1. ESI- QIT MS^n analysis of cholesteryl oleate standard

Figure 4.1(a) shows the ESI-QIT MS spectrum of cholesteryl oleate standard as its ammoniated adduct ($[M+NH_4]^+$) at m/z 668.5. Further CID analysis of the precursor ion m/z 668.5 by ESI-QIT MS^2 shows that cholesteryl oleate cleaves at the ester bond giving a fragment ion at m/z 369.3 (the steryl carbon skeleton) and the loss of the neutral fatty acyl moiety as shown in Figure 4.1(b). The ESI-$CIDMS^3$ of $[M+NH_4]^+ \rightarrow$ cholesteryl moiety (m/z 369.3), showed unique and multi-pathway fragmentation which can be useful for structural elucidation (Figure 4.1(c)).

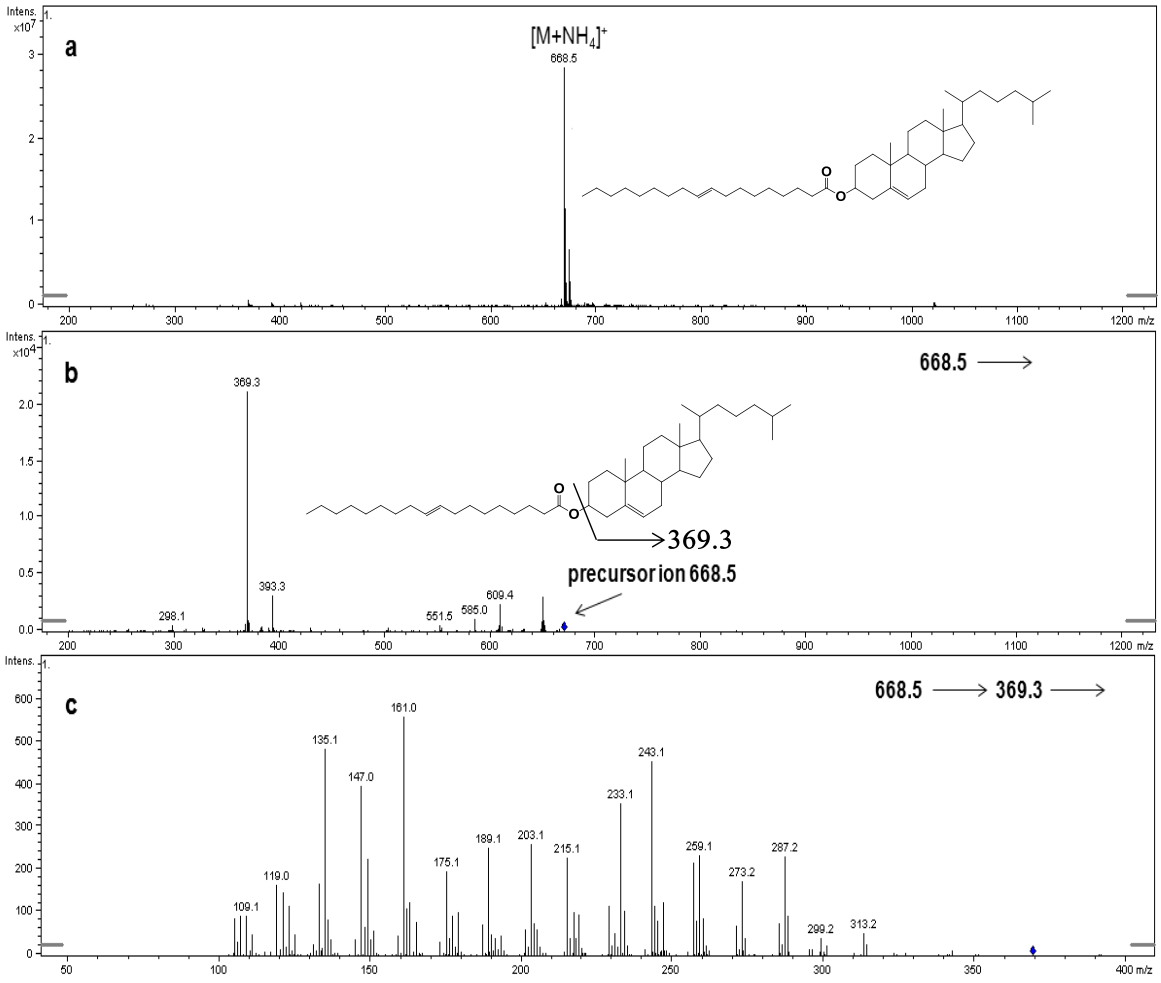

Figure 4.1. (a) ESI-QIT MS spectrum of cholesteryl oleate standard as its ammoniated adduct at m/z 668.5, (b) the MS^2 CID spectrum of the prescursor ion m/z 668.5, (c) the MS^3 CID spectrum of the precursor ion m/z 369.3 which is the cholesteryl cation

4.3.2. ESI-MS profile of steryl esters in margarine and corn

Figure 4.2 (a, b) illustrates the flow injection ESI-MS spectra of the steryl ester profile identified in margarine and corn, respectively. The numbers shown in Figure 4.2 are the identity of the steryl esters as listed in Table 4.1, their structural elucidation being described in the subsequent sections. Research into lipid analysis by MS [20] indicates that the molecular weight range of steryl esters in these types of samples should be between m/z 600 and 800. Ions in this m/z window which showed good abundance were further analyzed by ESI-QIT MS^2 to screen for ammoniated steryl esters ions.

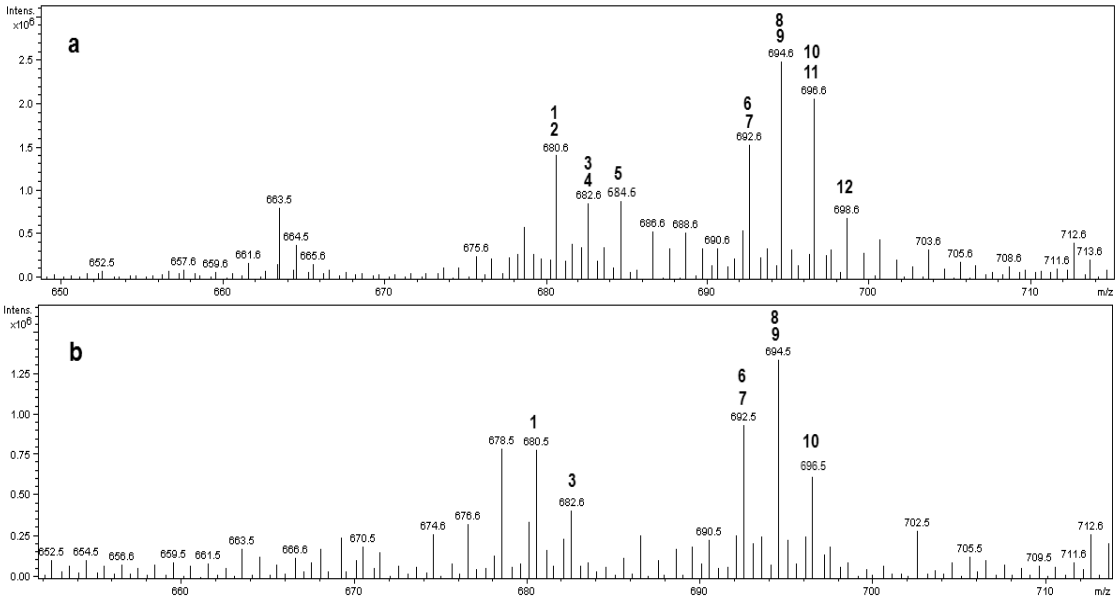

Figure 4.2. Full scan ESI-QIT MS of the steryl ester extract of (a) margarine and (b) corn. The numbers indicate various steryl ester compounds (as their ammonium adducts), their identifies are listed in Table 4.1.

4.3.3. Positive identification of steryl esters in samples

The first $[M+NH_4]^+$ ion in Figure 4.2 to be selected as a precursor ion for MS^2 CID was the m/z 698.6 ion present in the ESI-MS spectrum of the margarine sample. The resulting spectrum (Figure 4.3(a)) shows a strong fragment ion at m/z 397.4. Based on the results of the MS^2 CID of standard cholesteryl oleate, subtraction of 397.4 from 698.6 should represent the ammonium salt of a saturated fatty acid consisting of 18 carbon atoms (stearic acid). Furthermore, the m/z 397.4 ion should represent the steryl cation of the steryl ester. From the APCI-MS^2 CID analysis of β-sitosterol (Figure 4.4(b)) and further MS^3 CID of $[M+NH_4]^+ \rightarrow$ fragment ion at m/z 397.4 of the ion at m/z 698.6 (Figure 4.3(b)), this moiety (fragment ion at m/z 397.4) was identified to be β-sitosterol. Therefore the ion at m/z 698.6 is β-sitosteryl stearate. Other steryl esters in the samples were identified in the same fashion (as shown in Appendices 8-10).

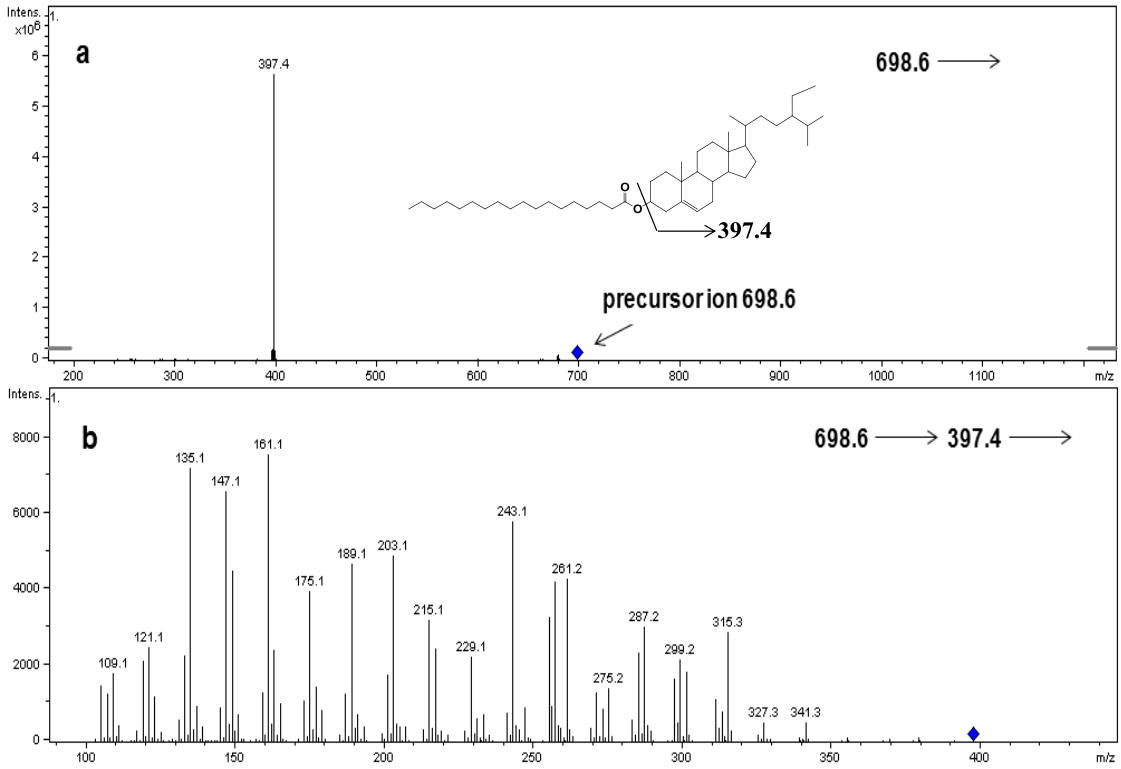

Figure 4.3. (a) the MS^2 CID spectrum of the precursor ion m/z 698.6, the ammonium adduct of β-sitosteryl stearate, a sterol found in margarine and (b) the MS^3 CID spectrum of the precursor ion m/z 397.4 representing a CID fragmentation fingerprint of the β-sitosteryl cation.

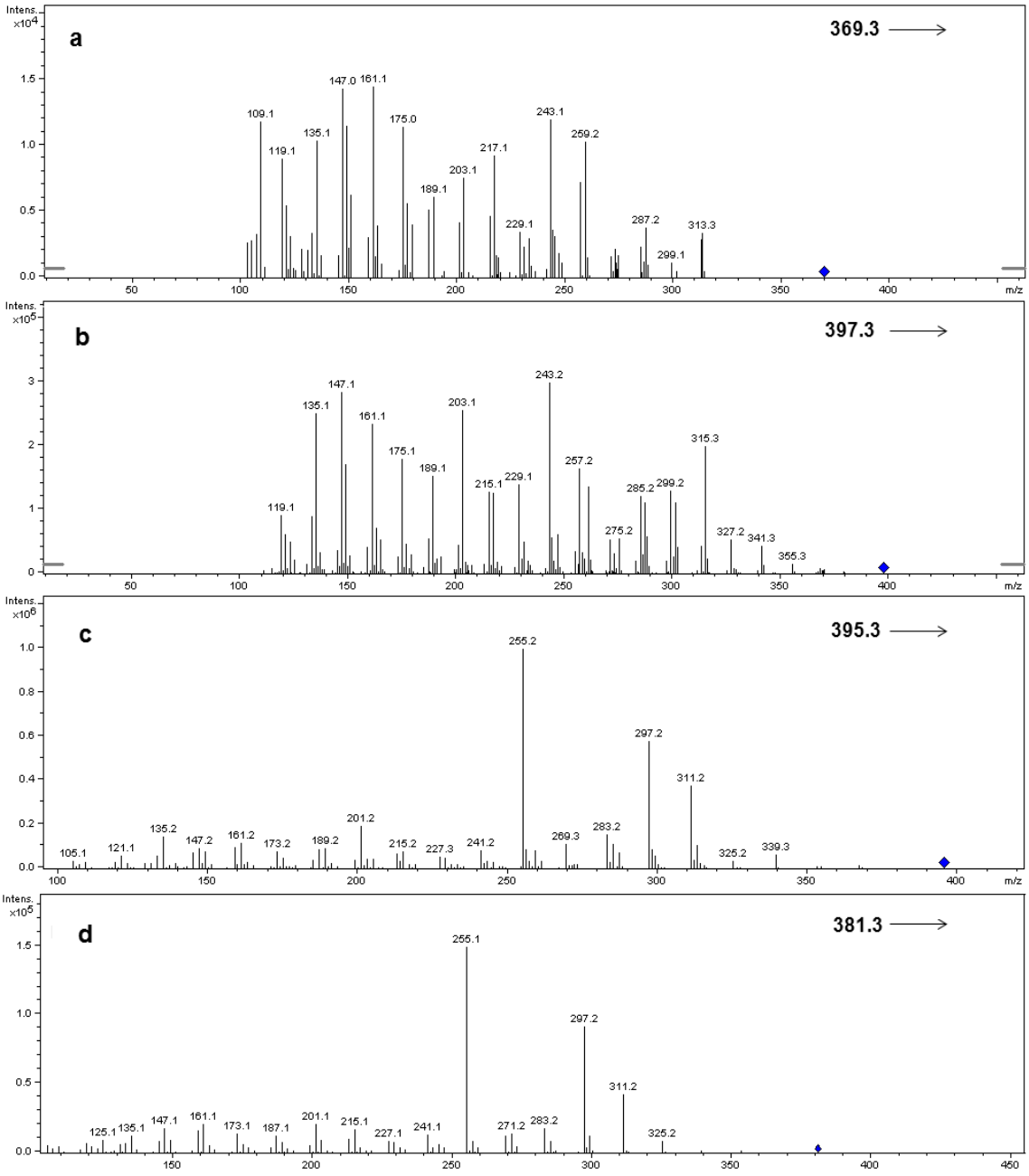

Figure 4.4. Flow injection analysis of free sterols. The APCI-MS^2 CID spectra of (a) the chlolosterol cation precursor m/z 369.3, (b) the β-sitosterol cation precursor m/z 397.4, (c) the stigmasterol cation precursor m/z 395.3 and (d) the brassicasterol cation precursor m/z 381.3.

4.3.3.1. Identification of steryl ester isomers

In Figure 4.2, it can be seen that there are numerous molecular isomers (i.e., two numbers with one m/z ion) present in the samples, particularly in the margarine sample. Positive identification of these isomeric pairs can be easily achieved by MS^n CID experiments. As an example, Figure 4.5(a) shows the ESI-MS^2 CID spectrum of a precursor ion at m/z 694.6 which is suspected to represent two steryl ester isomers. Two steryl fragment ions at m/z 397.4 and 395.4 were observed indicating that the ion m/z 694.6 contains two different fatty acyl moieties, linoleate (associated with steryl m/z 397.4) and oleate (associated with the steryl m/z 395.4), Further, ESI-MS^3 CID of precursor ion of m/z 694.6 , i.e., $[M+NH_4]^+ \rightarrow$ fragment ion at m/z 397.4 and $[M+NH_4]^+ \rightarrow$ fragment ion at m/z 395.4 gave CID fragmentation spectra unique to β-sitosteryl (Fig. 4.5(b)) and stigmasteryl (Fig. 4.5(c)), respectively. The identity of these steryl moieties were confirmed by investigating the fragmentation pathways of free sterols using APCI-MS^2 CID (Figure 4). Our MS^n results indicate that the parent ion m/z 694.6 was found to be a mixture of β-sitosteryl linoleate and stigmasteryl oleate isomers. Other steryl ester isomers in samples were identified in the same fashion.

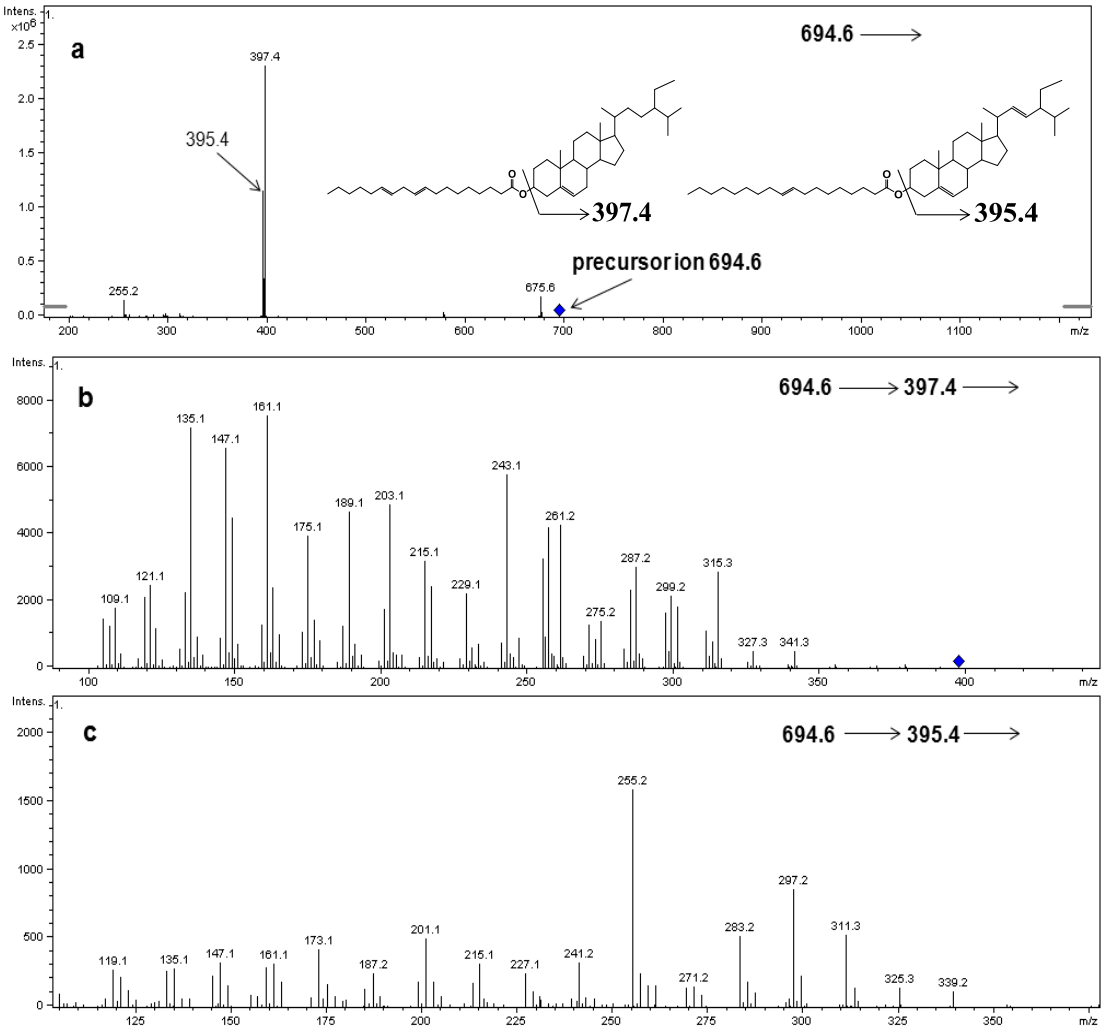

Figure 4.5. (a) the MS^2 CID spectrum of the prescursor ion m/z 694.6 representing the ammonium adduct of two steryl ester isomers found in margarine i.e., β-sitosteryl linoleate and stigmasteryl oleate. (b) the MS^3 CID spectrum of the precursor ion m/z 397.4 representing a CID fragmentation fingerprint of the β-sitosteryl cationic fragment of β-sitosteryl linoleate and (c) the MS^3 CID spectrum of the precursor ion m/z 395.4 representing a CID fragmentation fingerprint of the stigmasteryl cationic fragment of stigmasteryl oleate.

4.3.3.2. Use of MS^3 CID spectra to identify the steryl moieties

The CID MS^3 of $[M+NH_4]^+ \rightarrow$ steryl cations of all steryl esters in this study showed unique CID spectra. Figure 6 illustrates this by showing the QIT MS^3 mass spectra of all 4 steryl moieties identified in the samples; β-sitosteryl from two different steryl esters (Fig. 4.6(a) and (b)), campesteryl (Fig. 4.6(c)), stigmasteryl (Fig. 4.6(d)) and brassicasteryl (Fig 4.6(e)). Figures (4.6a and b) CID spectra were shown to indicate ease of steryl moiety identification from different steryl esters, in this case, β-sitosteryl stearate and oleate.

The CID fragmentation spectra of steryl cations are complex. Figure 4.7(a) demonstrates a proposed CID fragmentation pathway of the β-sitosteryl moiety. The most abundant ions are m/z 161.1, 147.1, 135.1, 243.2, 257.2, and 297.2. Other fragment ions m/z of 315.2, 327.2 and 341.2 are diagnostic for side chain cleavage, while the other listed fragment ions arise from multi carbon ring cleavage. In particular, fragment ions m/z 147 and 161 are diagnostic of sterols that have a double bond in the B ring (Figure 4.7(a)). The ion m/z 257.2 likely arises from complete loss of the side chain. According to Neto et al. [21], the proposed fragmentation pathway (shown in Figure 4.7b) that produces the ion at m/z 255 in the CID spectra of stigmasteryl, the base peak in Figure 4.6(d).

ESI-QIT MS^3 CID spectra of steryl moieties of steryl esters observed by this study exhibited more unique fragmentation than those obtained for free sterols analyzed by GC-EIMS [22] and are similar to those for steryl sulphates as seen in positive APCI-QqQ MS^2

[21]. Interestingly, Kalo et al. [23] reported the ESI-CID MS^2 analysis of steryl moieties with 381, 383, 395 and 397 Da of steryl esters which were similar to those of free sterols and which is consistent with our results.

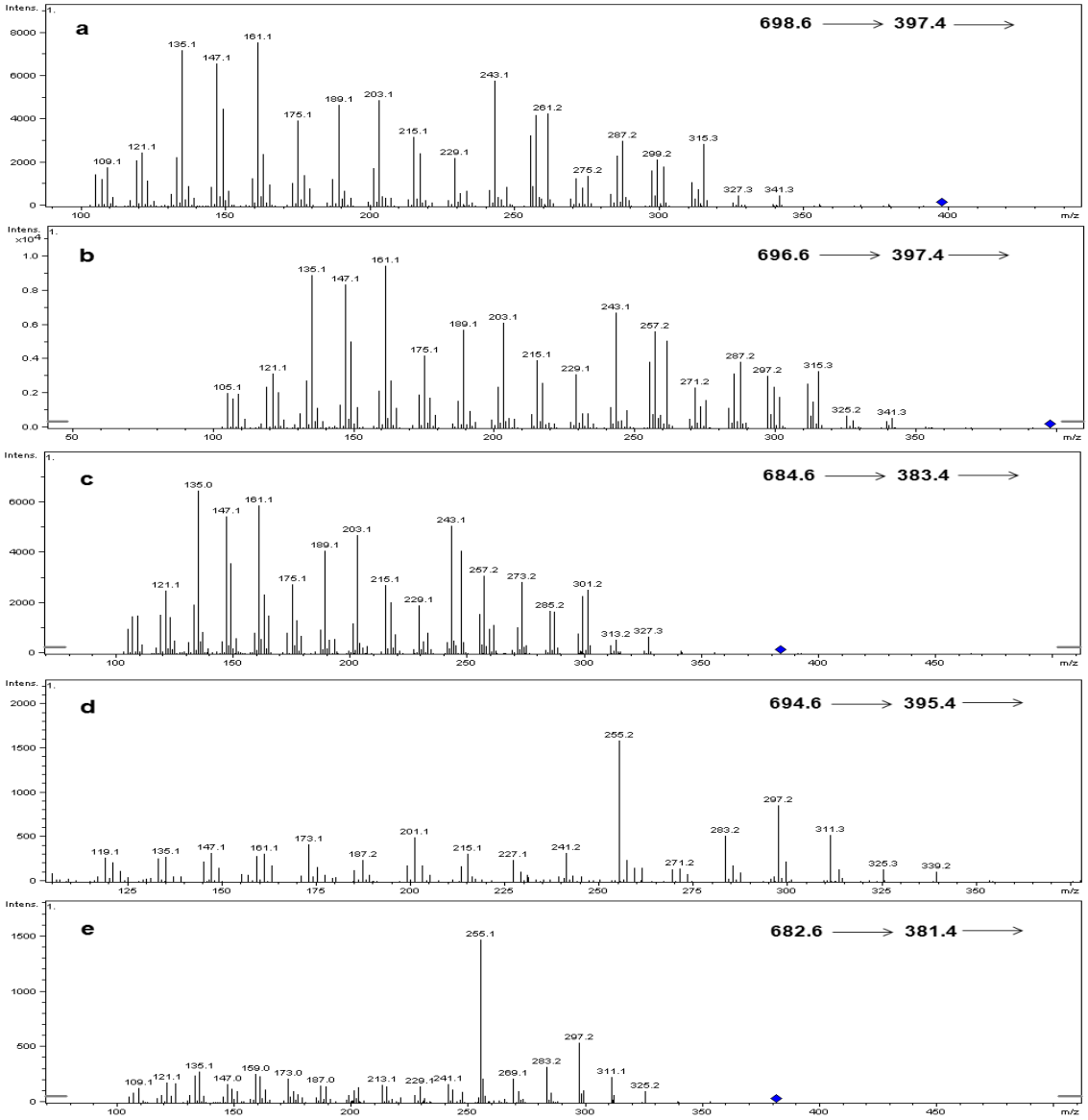

Figure 4.6. ESI MS^3 spectrum of precursor ions of the steryl cationic fragment moieties of all the identified steryl esters found in the samples. (a) β-sitosteryl stearate (b), β-sitosteryl oleate (c), campesteryl stearate (d), stigmasteryl oleate (e), brassicasteryl stearate

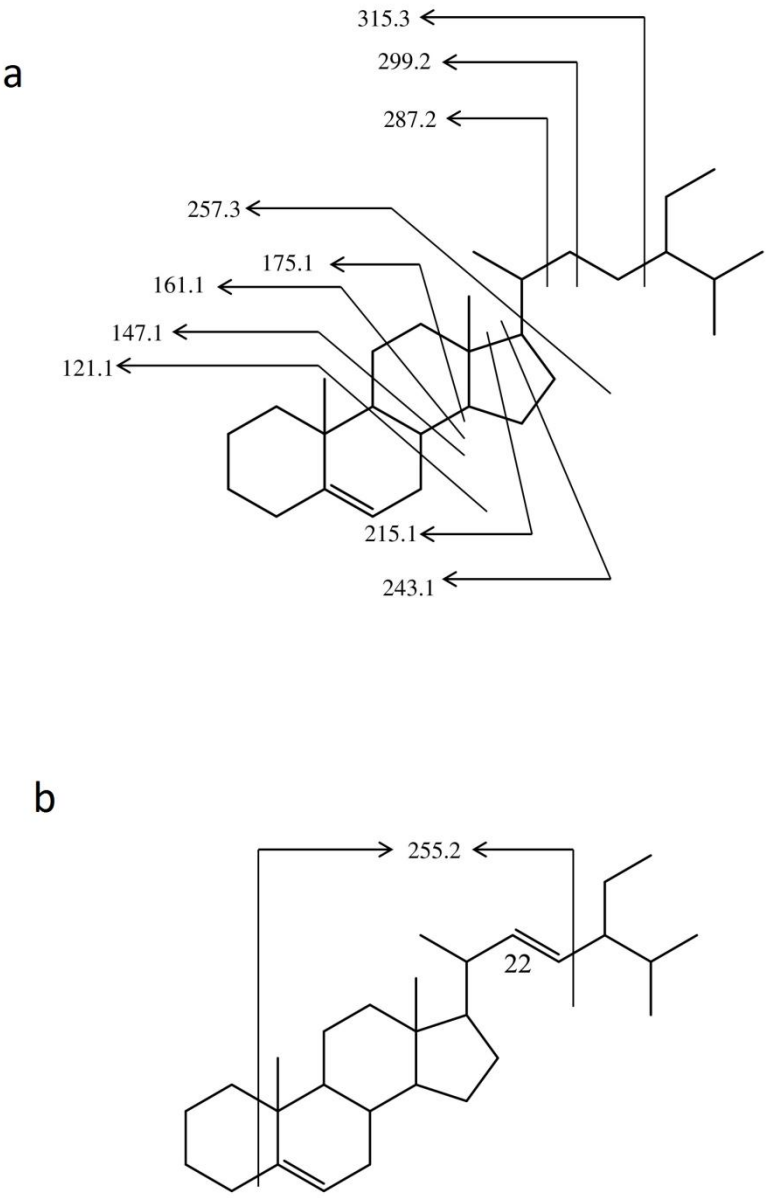

Figure 4.7. (a) Proposed CID fragmentation pathway of the β-sitosteryl cationic moiety resulting from QIT MS^3 as revealed in spectra of figure 4.6 (a, b) fragmentation pathway for the generation of the base ion m/z 255.2 from stigmasteryl cationic moiety as revealed in the spectra of figure 4.6 (d).

4.3.4. Steryl esters in samples

Table 4.1 lists the steryl esters identified in margarine and corn as shown in the ESI-MS profile in Figure 4.2 along with the m/z values for the steryl esters' molecular ammonium adducts and the m/z values of their steryl moieties. Based on the results of ESI-QIT MS, ESI-QIT CID MS^2 and ESI-QIT CID MS^3 experiments, the major steryl esters in margarine were found to be: β-sitosteryl stearate, β-sitosteryl oleate, β-sitosterol linoleate, campesteryl stearate and campesteryl linoleate; in corn: β-sitosteryl oleate, campesteryl linoleate, campesteryl oleate, stigmasteryl linoleate and β-sitosterol linoleate.

In summary, ESI-QIT MS^n is a versatile and rapid technique for the identification and structural elucidation of steryl esters in complex lipid-containing samples without the need for chromatography. Moreover, this technique can be used to differentiate among steryl moieties and between steryl ester molecular isomers. It is expected that flow injection ESI- QIT MS^n will also be useful for identification of other classes of conjugated sterols such as sterol glycosides.

Table 4.1. Steryl esters identified in margarine and corn using ESI-QIT MS^n

Steryl esters in *margarine*	Steryl esters in *corn*	molecular ion $[M+NH_4]^+$	m/z of steryl moiety MS/MS data
β-sitosteryl linoleate	β-sitosteryl linoleate	694.6	397.4 (β-sitosteryl)
stigmasteryl oleate	stigmasteryl oleate	694.6	395.4 (stigmasteryl)
β-sitosteryl oleate	β-sitosteryl oleate	696.6	397.4 (β-sitosteryl)
stigmasteryl stearate	-	696.6	395.4 (stigmasteryl)
β-sitosteryl stearate	-	698.6	397.4 (β-sitosteryl)
stigmasteryl linoleate	stigmasteryl linoleate	692.6	395.4 (stigmasteryl)
β-sitosteryl linolenate	β-sitosteryl linolenate	692.6	397.4 (β-sitosteryl)
campesteryl linoleate	campesteryl linoleate	680.6	383.4 (campesteryl)
brassicasterol oleate	-	680.6	381.4 (brassicasteryl)
campesterol oleate	campesteryl oleate	682.6	383.4 (campesteryl)
brassicasteryl stearate	-	682.6	381.4 (brassicasteryl)
campesteryl stearate	-	684.6	383.4 (campesteryl)

4.4. Conclusion

ESI-QIT MS^n along with proper sample cleanup was successfully used for the comprehensive profiling of steryl esters in margarine and corn. Steryl esters were identified as their ammonium adducts $[M+NH_4]^+$ after addition of ammonium acetate. Flow injection ESI-MS^2 CID of ammoniated steryl esters resulted in the neutral loss of the fatty acyl moiety giving a steryl cation. Steryl ester molecular isomers can be distinguished using ESI-QIT MS^2. ESI-QIT MS^3 was carried out on the intact steryl fragmentation cation. The resulting CID spectra of the steryl cation were found to be unique, allowing for structural elucidation of the steryl moieties including isomers. Based on the results of ESI-QIT MS, ESI-QIT CID MS^2 and ESI-QIT CID MS^3 experiments, the major steryl esters in margarine were found to be: β-sitosteryl stearate, β-sitosteryl oleate, β-sitosterol linoleate, campesteryl stearate and campesteryl linoleate; in corn: β-sitosteryl oleate, campesteryl linoleate, campesteryl oleate, stigmasteryl linoleate and β-sitosterol linoleate.

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Chapter Five: Conclusions and future work

5.1 Conclusions

In this thesis, several mass spectrometric techniques were used for identification and quantification of sterols and steryl esters in different sample matrices.

The composition of free sterols in cultivated blue mussels obtained commercially and from laboratory feeding experiments has been investigated. Total lipids were extracted from mussels using a modified Folch procedure followed by isolation of free sterols from total lipid extracts using column chromatography. Mussels were fed on algae or fish waste for six months as well as unfed for two months. Sterols were identified as their TMS ethers using GC/MS and quantified using GC/MS-SIM and GC/FID. The concentrations of sterols in mussels were compared at the beginning and at the end of the experiment in those fed algae and fish waste. The most abundant free sterols in commercially cultivated mussels were cholesterol, desmosterol, brassicasterol and 24-methylenecholesterol. The major sterol at the end of the experiment was cholesterol in all dietary treatments. In fish waste-fed mussels, cholesterol had increased significantly by the end of experiment, while 24-methylenecholesterol decreased significantly. Also, fish waste-fed mussels had significantly higher cholesterol concentrations compared to locally cultivated and algae-fed mussels. In algae-fed mussels, 24-nordehydrocholesterol decreased significantly. Mussels fed algae had significantly higher campesterol compared to locally cultivated, and fish waste-fed mussels. In unfed mussels, the concentrations of all dietary sterols decreased significantly over the duration of the experiment.

The levels of some sterols (i.e., brassicasterol and desmosterol in mussels fed algae) were almost constant during the experiment, although they were detected in algal

diets. This may be attributed to the fact that some phytosterols were biologically converted to cholesterol and other phytosterols by mussels. The concentration of cholesterol found in fish waste-fed mussels was high as a result of its high level in the diet (fish waste). However, in spite of β-sitosterol being relatively high in fish waste, its concentration stayed almost constant in mussels fed fish waste compared to cholesterol during the experiment. This may be due to the bioconversion of some dietary sterols to other sterols, and/ or to the selective uptake of cholesterol by mussels. Another reason for the elevated concentration of cholesterol in mussels fed fish waste may be due to the fact that bivalves do not have the ability to alter the structure of dietary cholesterol leading to cholesterol being selectively retained in bivalves. It can be concluded that the content and composition of sterols in mussels fed different diets reflected their diets.

In the second part, new and rapid MS techniques have been developed for analysis of free sterols in biological samples by MALDI-TOFMS, MALDI-TOF/TOFMS, and ESI/APCI MS^n. In order to enhance their ionization efficiency, sterols were converted into their corresponding picolinyl esters, *N*-methylpyridyl ethers and sulphated esters. Using MALDI-MS picolinyl esters were detected as sodiated adducts $[M+Na]^+$ and the signal enhanced after addition of sodium acetate using either DHB or THAP. Due to *N*-methylpyridyl ethers being positively charged, they were detected as $[M]^+$ using THAP while sulphated esters were detected as $[M-H]^-$ using either *p*-nitroaniline or dithranol. MALDI-TOF/TOF experiments were carried out to study the fragmentation pathways of the derivatives and their use in structural elucidation. The ester bonds of picolinyl and sulphated esters easily cleaved during MALDI-CID MS/MS resulting in diagnostic

fragments at m/z 146 and 97, respectively. Cleavage of the ether bond of *N*-methylpyridyl ethers gave a diagnostic fragment ion at m/z 110. Lipid extracts from mussels were used as a test sample for MALDI-TOF analysis of sterols in biological samples. It has been found that sterol profiles in mussels obtained by MALDI-TOFMS were in close agreement with those obtained by GC/MS. Cholesterol and β-sitosterol were successfully quantified using MALDI-TOFMS. The calibration curves gave excellent correlation coefficients which indicate that MALDI-TOF can be used for quantitation of sterols. Sulphated esters showed the highest sensitivity; 5 times more sensitive than picolinyl esters and 75 times more sensitive than free sterols and with a better detection limit for sterols than GC/MS.

Picolinyl esters of sterols were analyzed also using flow injection ESI-QIT MS^n and by APCI-QIT MS^n as well. The picolinyl esters readily formed protonated molecular ions ($[M+H]^+$) in ESI and APCI sources except for picolinyl ester of 7-dehydrocholesterol where it was detected as radical cation ion $[M]^{\cdot+}$ using APCI-QIT MS. Its molecular ion cannot be observed by ESI-QIT MS and was easily cleaved, giving an abundant fragment ion ($[M+H-C_6H_5NO_2]^+$) at m/z 367.4. The ester bonds of picolinyl esters cleaved during CID MS^2 resulting in diagnostic fragments corresponding to steryl cation moieties $[M+H-C_6H_5NO_2]^+$. The CID MS^3 of $[M+H]^+ \rightarrow [M+H-C_6H_5NO_2]^+$ of picolinyl esters spectra were found to be useful for structural elucidation and for distinguishing among steryl isomers. For example, picolinyl isomers (i.e., fucosterol and stigmasterol) showed significantly differences in ion intensities in their ESI QIT MS^3 mass spectra.

In the third part of this thesis, a MS method was developed for identification and structural elucidation of steryl esters in margarine and corn using flow injection ESI-QIT MS^n without the need for chromatography. Moreover, this method can be used to differentiate among steryl moieties and between steryl ester molecular isomers. The use of ESI-QIT MS^n along with solid phase extraction fractionation was successfully used for comprehensive profiling of steryl esters in margarine and corn. Steryl esters were identified as their ammonium adducts $[M+NH_4]^+$ after addition of ammonium acetate as dopant. Flow injection ESI-MS^2 CID of ammoniated steryl esters resulted in the neutral loss of the fatty acyl moiety giving a steryl cation and thus steryl ester isomers can be distinguished. ESI-QIT MS^3 was carried out on the intact steryl fragmentation cation. The resulting CID spectra of the steryl cations were found to be unique, allowing for the structural elucidation of the steryl moieties including isomers. Based on the results of ESI-QIT MS, ESI-QIT CID MS^2 and ESI-QIT CID MS^3 experiments, the major steryl esters in margarine were found to be: β-sitosteryl stearate, β-sitosteryl oleate, β-sitosterol linoleate, campesteryl stearate and campesteryl linoleate; in corn: β-sitosteryl oleate, campesteryl linoleate, campesteryl oleate, stigmasteryl linoleate and β-sitosterol linoleate.

5.2 Future work

Based on this thesis' findings the following research is proposed for future work in the area of analytical chemistry of sterols:

1. The utilization of ESI-QIT MS^n for other classes of conjugated sterols such as steryl glycosides and acylated steryl glycosides and the applicationthis method to food and plant samples. This would be complimentary to steryl ester analysis and could lead to full sterol conjugate profiling in samples without the need for chromatography.
2. The application of MALDI-TOF/TOFMS for the analysis of the steryl conjugate classes in real samples since it is expected that these conjugates can easily be detected as $[M+Na]^+$ and $[M+K]^+$ adducts.
3. Examine the use of Desorption Electrospray Ionization-Mass Spectrometry (DESI-MS) for the rapid analysis of picolinyl and sulphated esters of sterols as a means of developing a more practical way of analyzing samples without the need for lipid extraction. DESI is an ambient ionization technique that can be used for analysis of liquid, gaseous and solid samples [1]. Sulphate esters for free sterols present in human plasma [2] with the use of DESI-MS might be useful for sterol identification and quantification. This technique also can be used for analysis of steroids and lipids in biological matrices [3].

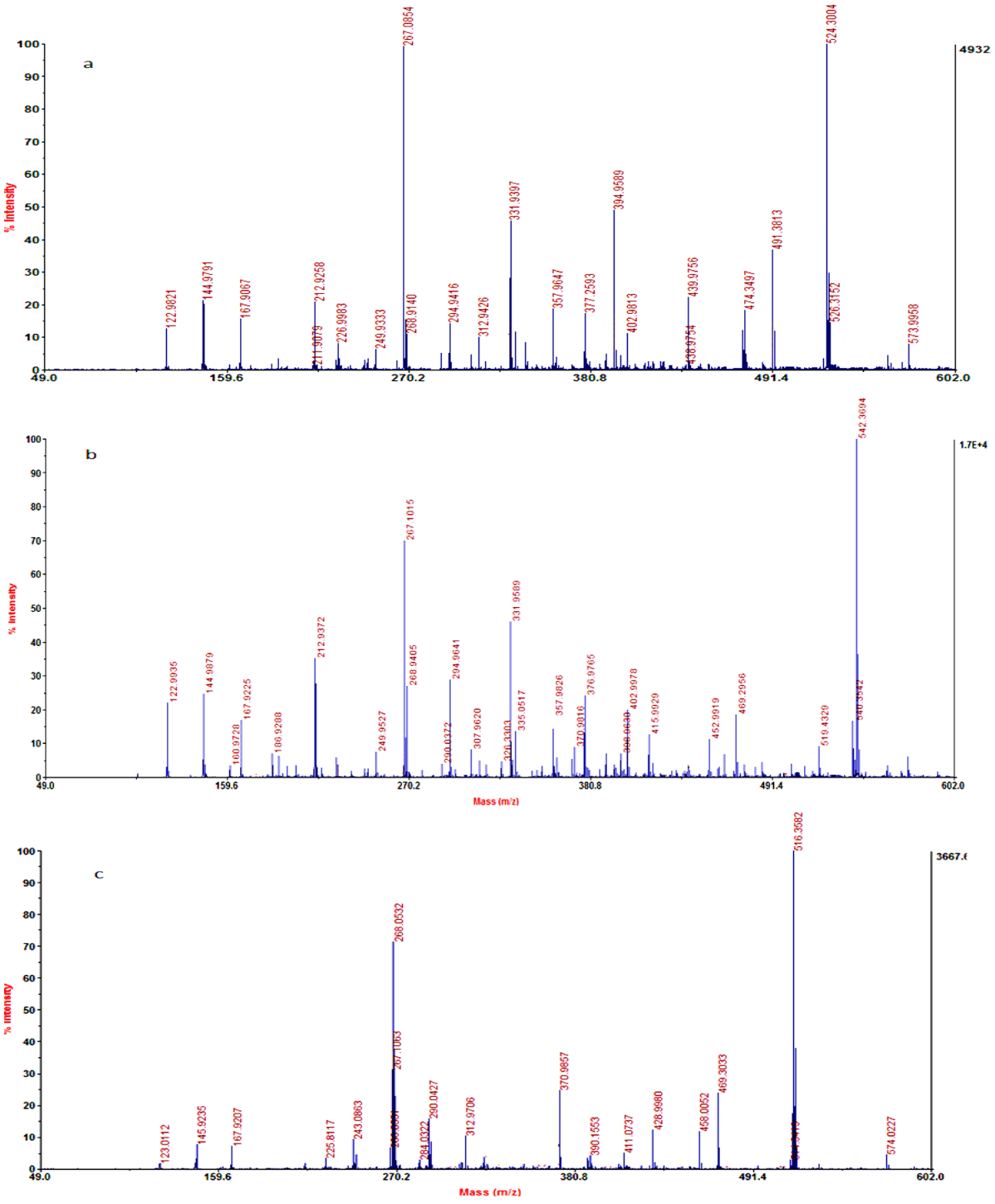
5.3 References

1. http://en.wikipedia.org/wiki/Desorption_electrospray_ionization.

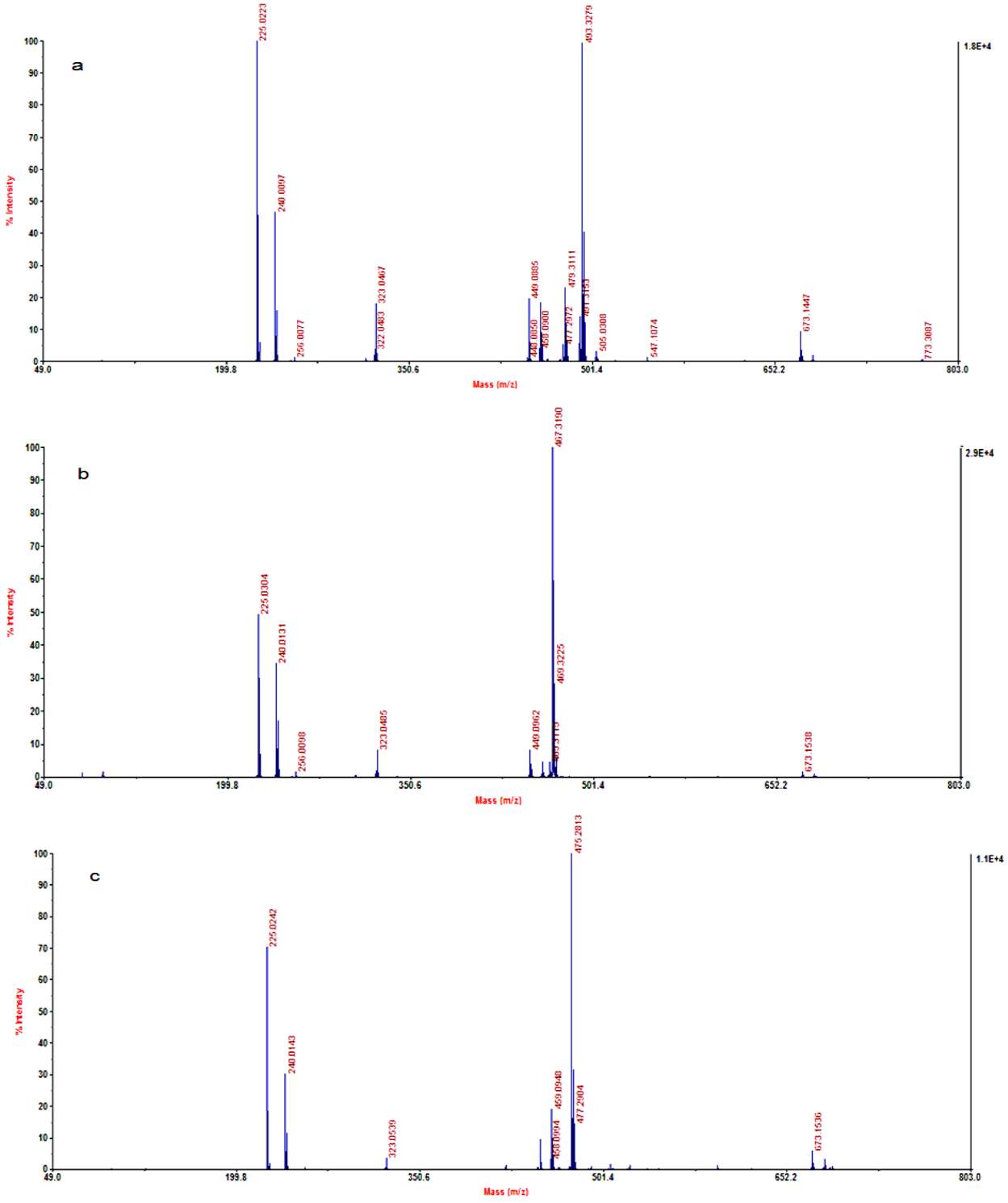
2. Strott CA, Higashi Y. Cholesterol sulfate in human physiology: what's it all about? *J. Lipid. Res*. **2003**, 44, 1268.

3. Gonzàlez-Serrano AF, Pirro V, Ferreira CR, Oliveri P, Eberlin LS, Heinzmann J, Lucas-Hahn A, Niemann H, Cooks RG. Desorption electrospray ionization mass spectrometry reveals lipid metabolism individual Oocytes and Embryos. *Plos One*, **2013**, 8(9), 1.

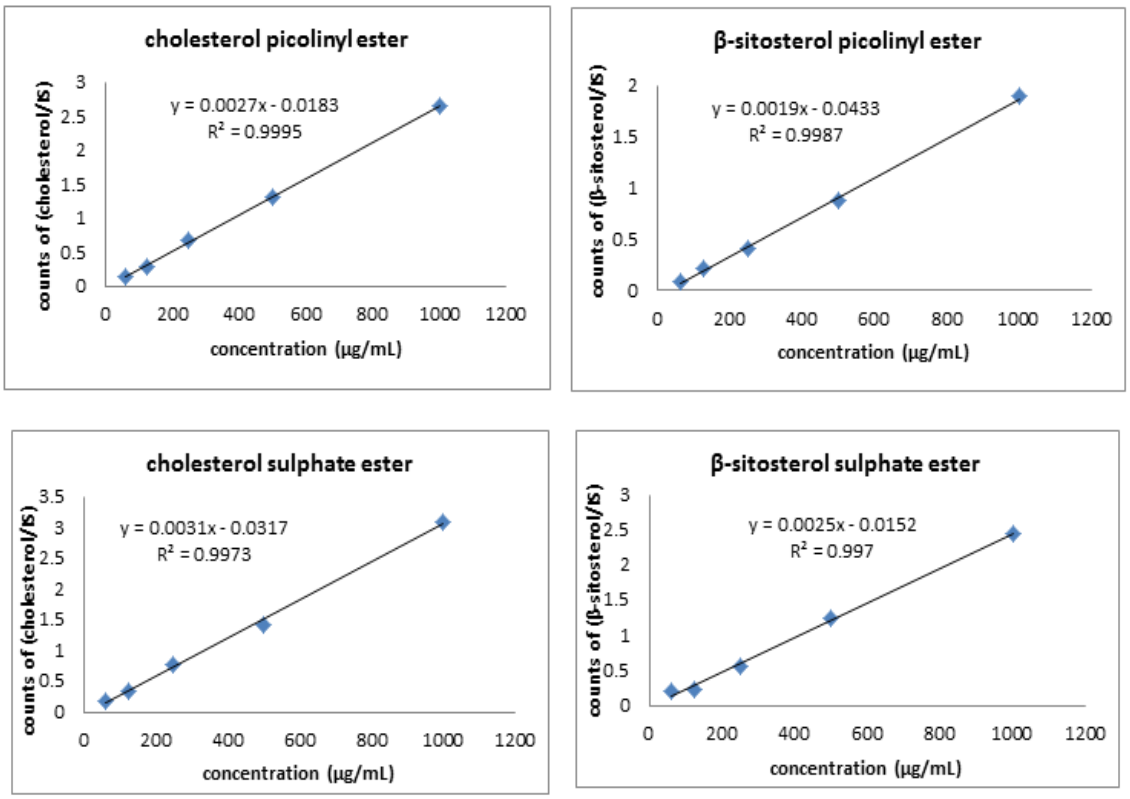
6. Appendices



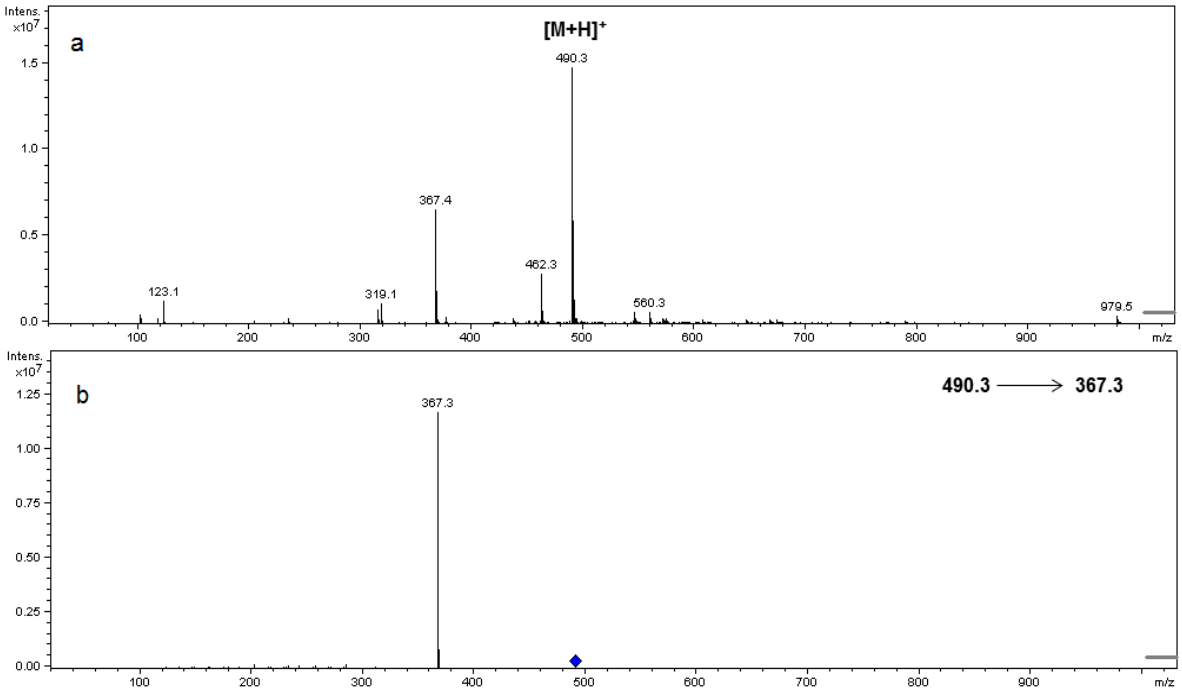
Appendix 1. MALDI-TOF mass spectra of (a) ergosterol picolinyl ester at m/z 524.3 $[M+Na]^+$, (b) β-sittosterol picolinyl ester at m/z 542.3 $[M+Na]^+$, and (c) Cholestanol picolinyl ester at m/z 516.3 $[M+Na]^+$.



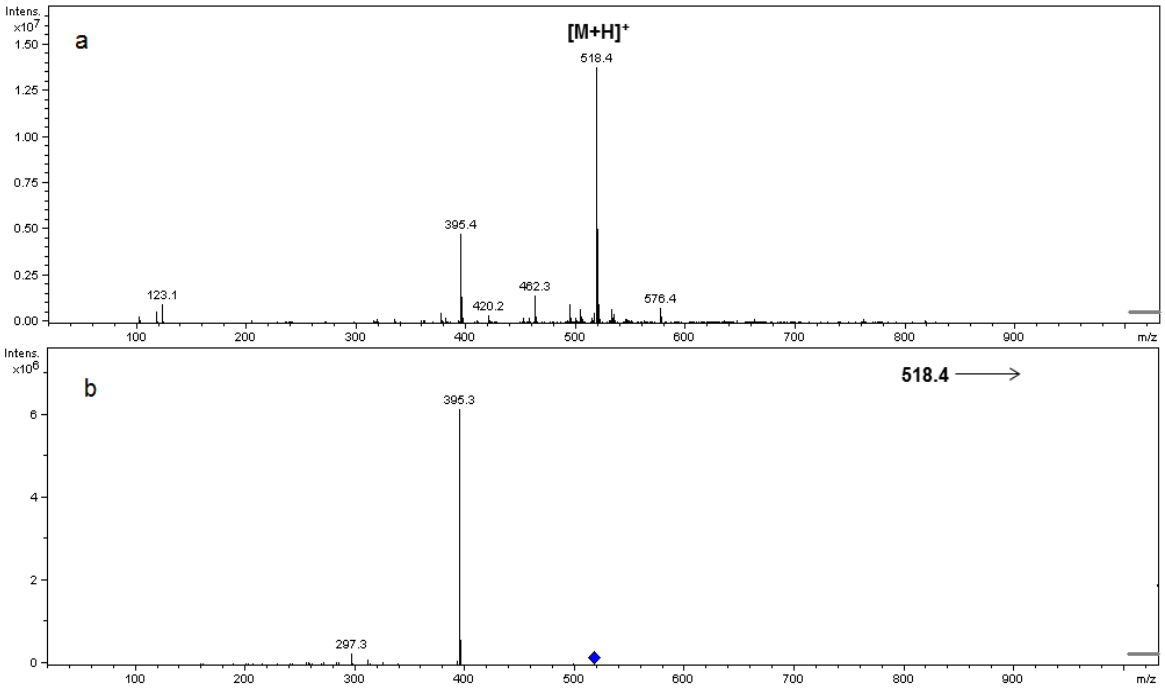
Appendix 2. MALDI-TOF mass spectra of (a) β-sitosterol sulphate at m/z 493.3 $[M-H]^-$, (b) cholestanol sulphate at m/z 467.3 $[M-H]^-$, and (c) ergosterol sulphate at m/z 475.3 $[M-H]^-$.



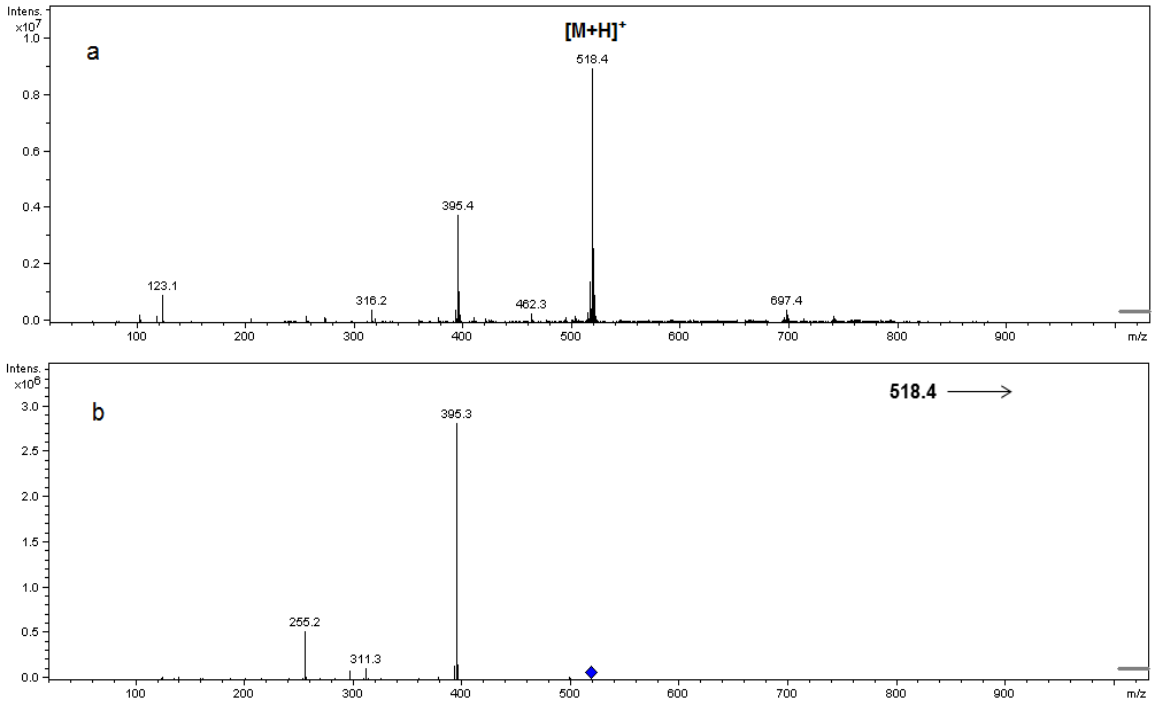
Appendix 3. Calibration curves of derivatized sterols standards



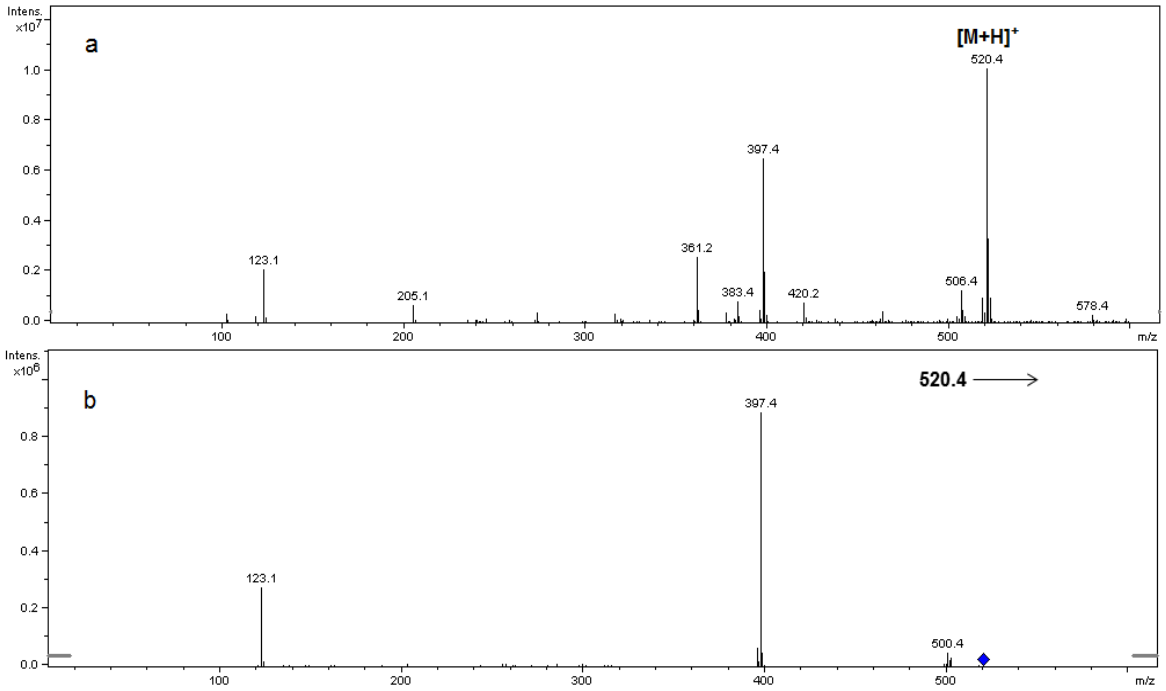
Appendix 4. (a) Flow injection ESI-QIT MS spectrum of desmosteryl picolinyl ester detected as $[M+H]^+$ at m/z 490.3, (b) The CID MS^2 spectrum of the precursor ion m/z 490.3.



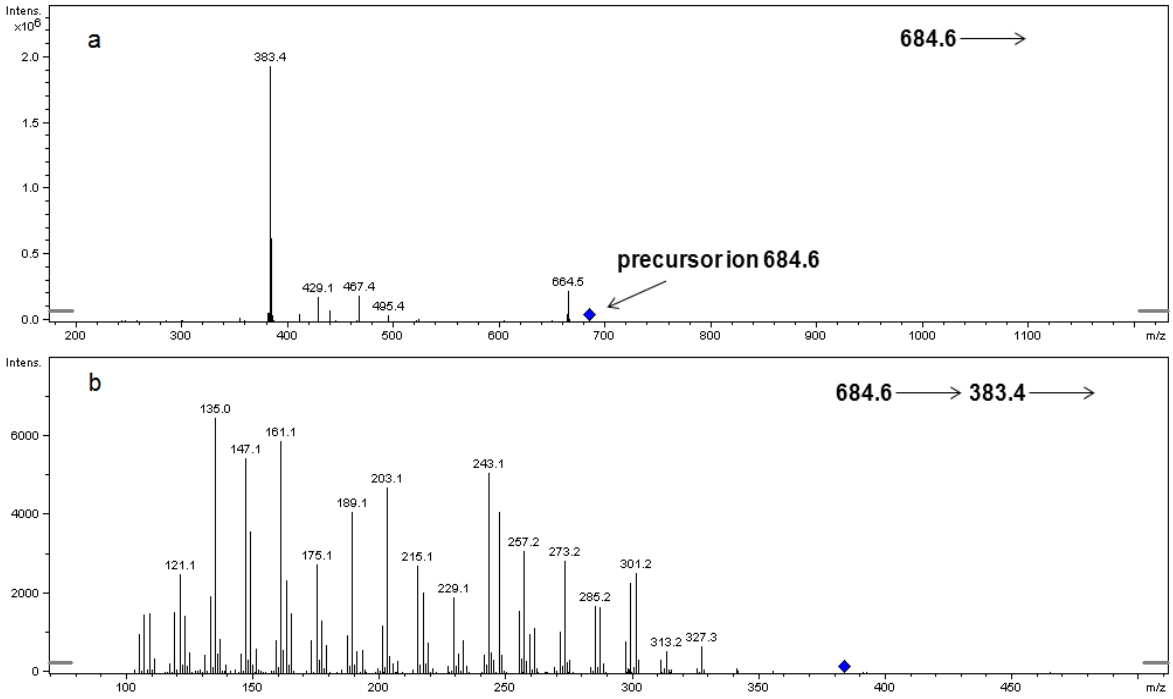
Appendix 5. (a) Flow injection ESI-QIT MS spectrum of fucosteryl picolinyl ester detected as $[M+H]^+$ at m/z 518.4 and (b) CID MS^2 spectrum of the precursor ion m/z 518.4.



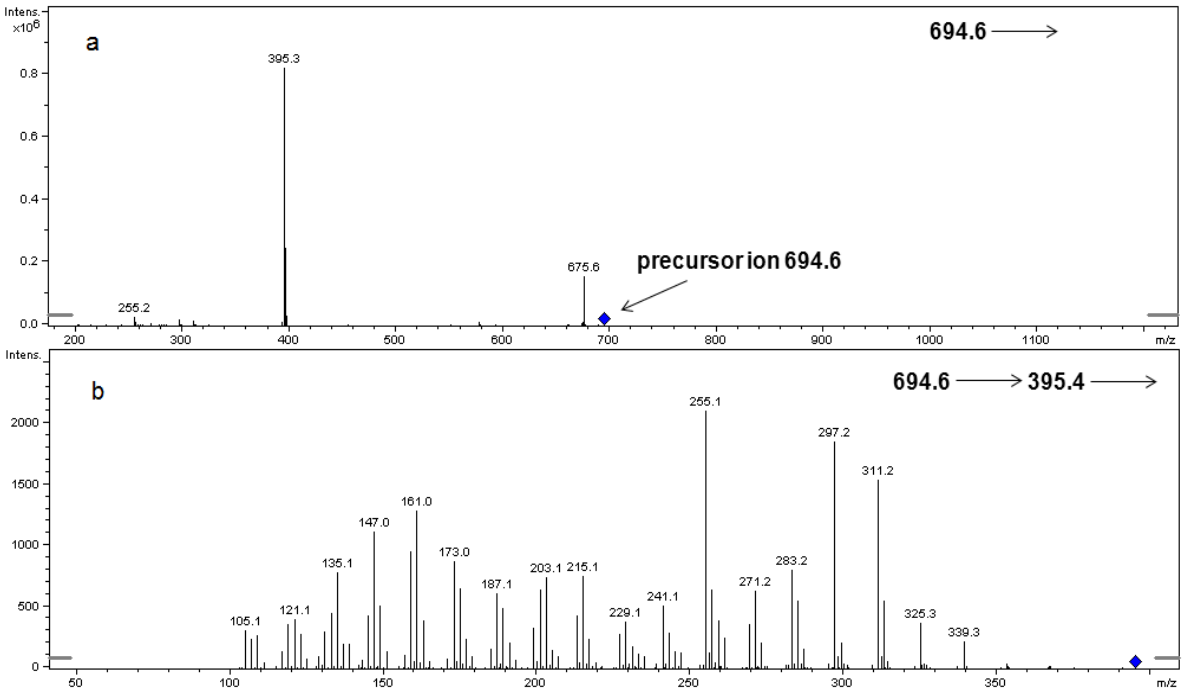
Appendix 6. (a) Flow injection ESI-QIT MS spectrum of stigmasteryl picolinyl ester detected as $[M+H]^+$ at m/z 518.4 amd (b) CID MS^2 spectrum of the precursor ion m/z 518.4.



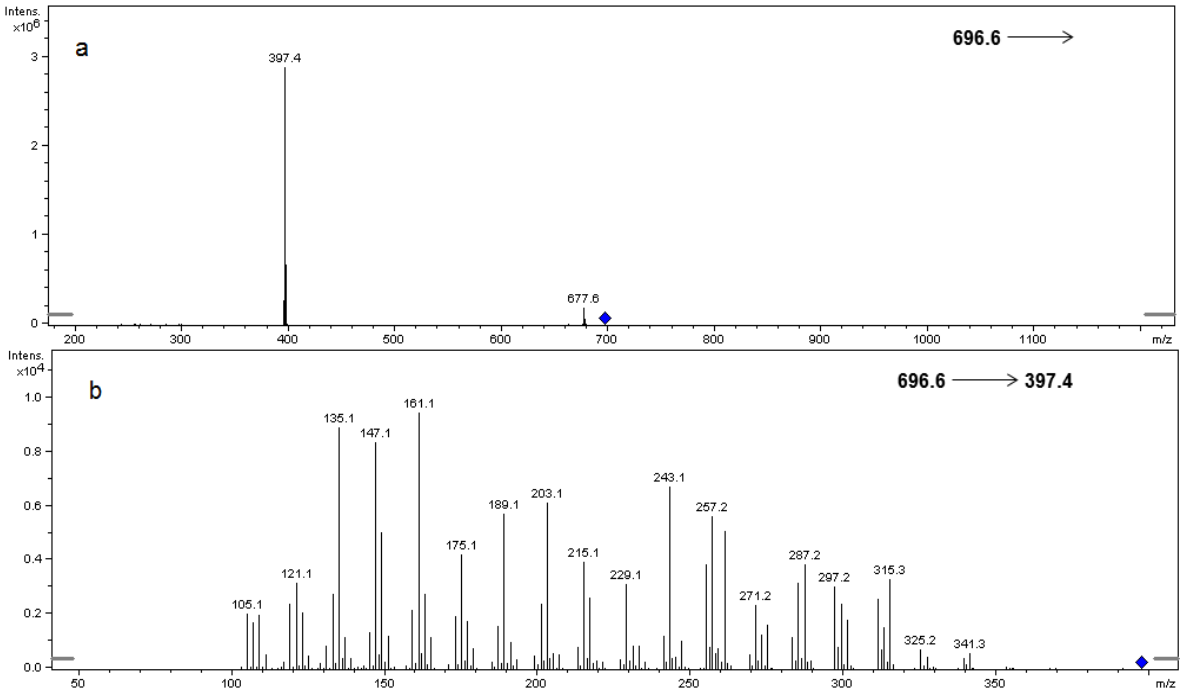
Appendix 7. (a) Flow injection ESI-QIT MS spectrum of β-sitosteryl picolinyl ester detected as $[M+H]^+$ at m/z 520.4 and (b) CID MS^2 spectrum of the precursor ion m/z 520.4.



Appendix 8. (a) the MS^2 CID spectrum of the prescursor ion m/z 684.6, the ammonium adduct of campesteryl stearate, a sterol found in margarine and (b) the MS^3 CID spectrum of the precursor ion m/z 397.4 representing a CID fragmentation fingerprint of the β-sitosteryl cation.



Appendix 9. (a) the MS^2 CID spectrum of the prescursor ion m/z 694.6, the ammonium adduct of stigmasteryl oleate, a sterol found in corn and (b) the MS^3 CID spectrum of the precursor ion m/z 395.4 representing a CID fragmentation fingerprint of the stigmasteryl cation.



Appendix 10. (a) the MS^2 CID spectrum of the prescursor ion m/z 696.6, the ammonium adduct of β-sitosteryl oleate, a sterol found in corn and (b) the MS^3 CID spectrum of the precursor ion m/z 397.4 representing a CID fragmentation fingerprint of the β-sitosteryl cation.