MASS SPECTROMETRIC ANALYSIS OF PHYTOSTEROLS AND TOCOPHEROLS IN PLANT OILS

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By

Kang Jiang

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For tocopherols analysis, advice was provided by co-authors, Asmita Poudel. The extract of phytosterols and tocopherols in CODD was conducted by co-author, George Gachumin. As first author, I wrote all draft versions of the manuscript and revised based on comments from the co-authors. This work was fully supervised by Dr. Anas El-Aneed.

ABSTRACT

Phytosterols and tocopherols are bioactives that have numerous health claims. Seeds, oils, legumes, cereals, and nuts are the main sources of phytosterols and tocopherols. Daily consumption of phytosterols and tocopherols is diverse in various countries where food intake habits are significantly different. Plant oils, a source of phytosterols, are commonly consumed in most countries and they are also a rich source of tocopherols. Beside dietary sources, consumption of functional foods and nutraceuticals are increasing rapidly in recent years. Plant oil deodorizer distillate is generated during the oil refining process offers an ideal source of these compounds. Canola is a major source for edible vegetable oils, and the most abundant oilseed crop in Canada. With growing consumer preference on natural bioactives, canola oil deodorizer distillate (CODD) has great economic values to be utilized as natural source of phytosterols and tocopherols.

Evaluation of the tandem mass spectrometric (MS/MS) behavior of phytosterols and tocopherols is needed for the development of qualitative and quantitative methods for these biologically active plant metabolites. Herein, the MS/MS dissociation behavior of phytosterols and tocopherols is elucidated to establish generalized MS/MS fingerprints. MS/MS and multistage (MS^3) analysis revealed common fragmentation behavior among the four tested phytosterols, namely β-sitosterol, stigmasterol, campesterol and brassicasterol. Similar analysis was conducted for the tested tocopherols (i.e. α-tocopherols, β- tocopherols, γ- tocopherols and δ-tocopherols). As such, universal MS/MS fragmentation pathway for both phytosterols and tocopherols were successfully established for the first time. Based on the generalized MS/MS fragmentation behavior of phytosterols, diagnostic product ions were chosen for the development of profiling

methods for over 20 naturally-occurring phytosterols. Precursor ion scan-triggered enhanced product ion scan (PIS-EPI) methods were established. Due to enhanced signal intensity, multiple ion monitoring-triggered enhanced product ion scan (MIM-EPI) was employed for confirmation. The screening approach was applied successfully to identify blinded samples obtained from standard mixtures as well as sesame and olive oils. The oil samples contain other phytosterols and their successful identification indicates that, the generalized MS/MS fragmentation behavior is applicable to various structures of phytosterols. Similar approach was attempted for tocopherols and was only hindered by the low concentration of these bioactive metabolites within oil samples.

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AC	Alternating current
ACN	Acetonitrile
APCI	Atmospheric pressure chemical ionization
BHT	Butylated hydroxytoluene
CE	Collision energy
CID	Collision induced dissociation
DC	Direct current
DD	Deodorization distillate
EI	Electron ionization
ESI	Electrospray ionization
FTICR	Fourier transform ion cyclotron resonance
HPLC	High-performance liquid chromatography
HRMS	High resolution mass spectrometry
LC-MS	Liquid chromatography–mass spectrometry
LOD	Lower limit of detection
LIT	Linear ion trap
m/z	Mass to charge ratio
MRM	Multiple reaction monitoring
MIM	Multiple ions monitoring
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry
MS^3	MS/MS/MS
MS^n	Multi-stage mass spectrometry
NLS	Neutral loss scan
PIS	Precursor ion scan
Q	Quadrupole
Qq-LIT	Quadrupole-linear ion trap
QqQ	Triple quadrupole
QqTOF	Quadrupole-time of flight
RF	Radio frequency
TOF	Time of flight

CHAPTER 1

INTRODUCTION

1.1. Background

Canola is one of the most important crops in Canada generating one quarter of all farm-related revenues [1]. A report released in 2017 shows that canola contributes $26.7 billion to the Canadian economy each year, including more than 250,000 Canadian jobs and $11.2 billion in wages [1]. The major production provinces include the western provinces of Alberta, Saskatchewan, and Manitoba. British Columbia, Ontario, and Quebec also grow a large amount of canola.

The main use of canola is the extraction of edible oil. Every year, almost 10 million tons of canola seed is crushed and refined in Canada, producing 3 million tons of canola oil and 4 million tons of canola meal [1]. The deodorization distillate (DD) is a major by-product produced during crude oil refining. Canola oil DD contains large amounts of bioactive metabolites like tocopherols (i.e. vitamin E) and phytosterols; other constituents include free fatty acid glycerides, aldehydes, and ketones that result from the decomposition of acidic compounds[2].

The most notable bioactives in canola oil DD are phytosterols and tocopherols. They are essential for plants and beneficial to human health. Most notably, phytosterols are being used for their cholesterol lowering abilities [3, 4] while tocopherols are natural anti-oxidants [5-7]. Several strategies have been developed to recover and purify these compounds from the DD of different vegetable oils [8-10]. Both bioactivities have been incorporated in functional foods, cosmetics, and pharmaceutical products [11, 12].

Due to the complex nature of DDs and the similar physicochemical property among phytosterols and tocopherols, it is difficult to separate and identify these compounds [13]. Dumont & Narine [14] presented the characterization of soybean oil DD through gas chromatography (GC)-flame ionization detector (FID). Several components of deodorizer distillate samples were identified, including 2 tocopherols (γ-tocopherol and δ-tocopherol) and 3 phytosterols (campesterol, stigmasterol, and sitosterol). Similarly, Naz1 et al. [14] investigated the chemical characterization of the deodorizer distillate of canola and sunflower oils, reporting phytosterol percentages at 21.27-25.53% while tocopherols were within the ranges of 1.29-5.81%.

In recent years, natural products have gained more acceptance among global consumers than synthetic counterparts. Therefore, natural source of phytosterols and tocopherols from the DD may have market desirability and it is, therefore, essential to characterize their composition. As such, analytical strategies should be developed to allow for the development of identification and quantification methods of phytosterols and tocopherols. My M.Sc. project focuses on developing mass spectrometric fingerprints for phytosterols and tocopherols. Specifically, the tandem mass spectrometric (MS/MS) dissociation behavior of these metabolites is established. The common structures are assigned to the various observed product ions. The data is subsequently used for the development of screening and identification methods using high performance liquid chromatography-tandem tandem mass spectrometry (HPLC-MS/MS).

1.2. Edible oil and its main nutrients

Canada has a vast territory and a wide variety of oil crops. Canola, soybeans, sunflowers and flax are the most prevalent oil crops in Canada [15]. However, the evaluation of edible vegetable oils has been mainly focusing on fatty acid composition in the past [16, 17]. Recent studies evaluated other nutrients, such as squalene, phenolic compounds and vitamins [18, 19]. Phytosterols and tocopherols are two important nutrients that exist in edible oils which will be further discussed later in 1.4 and 1.5, respectively. Chimi et al. [20] reported the strong scavenging ability to hydroxyl radicals of polyphenols (eg. hydroxytyrosol and caffeic acid) in olive oil. Squalene is also present in vegetable oils, with the highest content in olive oil [21]. As an active substance that can be produced by the body, squalene has health benefits, such as inhibiting the growth of tumor cells and strengthening the body's immunity [22].

In summary, it is important to study the nutrients within edible vegetable oils and seek the nutritional contents. Research on edible oils helps evaluate their impact on health, increase the value of oil products, and promote the development of specialty oils, such as blended oils [23].

1.3. Canola oil deodorizer distillates (CODDs)

Vegetable oils are rich sources of phytosterols and tocopherols [24, 25]. However, vegetable oils are subjected to a refining process in order to improve their taste and shelf life prior to consumption. Refining such as neutralization, bleaching and deodorization produces many by-products, such as the DD [26, 27]. Deodorization is a crucial step during vegetable oil refining process in which considerable phytosterols and tocopherols are removed along with the odorous compounds which could influence the taste, smell,

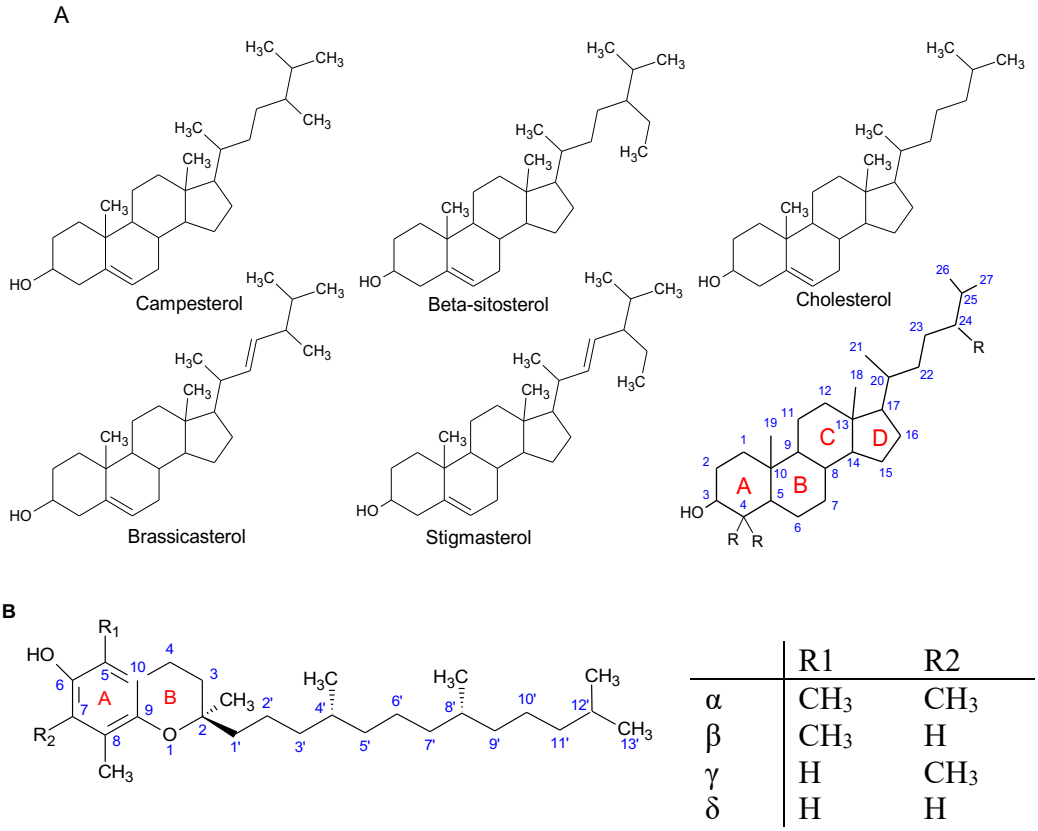
and physical appearance of the oil [28]. The potential value of the DD essentially depends upon its content of bioactive compounds. It is a rich source of phytosterols and tocopherols although the amounts and profiles will differ based on the applied conditions during the oil refining process [29, 30]. The amount of tocopherols present in the DDs is in the ranges of 10–13% [31] while phytosterols are at 5–30% [32]. Different separation methods for these components are reported [33-35]. Solvent extraction and crystallization are commonly applied to recover phytosterols over tocopherols. Recovering sterols over tocopherols usually use solvent extraction and crystallization, as theses method do not require high pressure and do not result in tocopherol oxidation [33]. However, there are some disadvantages for solvent extraction and crystallization strategies. They require large amounts of solvents that is not an environmentally friendly approach.

On the other hand, Supercritical fluids are more commonly employed for tocopherols separation [34]. In the process of supercritical carbon dioxide extraction, carbon dioxide passes through the target mixture under a specific temperature and pressure [36]. The biggest advantage of supercritical fluid extraction is that post-reaction separation of the components is easy to operate by depressurization. Additionally the experimental process does not need high temperatures for the low critical temperature of carbon dioxide [37]. Nonetheless, the requirements of high pressure greatly increase cost, and the separation method is economically viable only if the rate of production is higher than 25% [38]. Only fatty acids can be separated from tocopherol under these specific conditions [31]. However, the above discussed methods for tocopherols separation were mainly applied at a low scale lab-level. In many instances, DDs are added back to the meal to serve as an

energy source; this is a very low value application considering their high content in bioactive compounds.

1.4. Phytosterols

Phytosterols are found in all plants with high concentrations present in unrefined vegetable oils [39, 40]. They are structurally and functionally similar to cholesterol; however, they possess an extra methyl or ethyl group on the side chain and sometimes the side chain bears an additional double bond. More than 250 phytosterols have been identified [41]. The structures of the four most common phytosterols are shown in Scheme 1.1. Phytosterols are responsible for the stabilization of the phospholipid bilayer of the cell membrane which may related to their ability to regulate the fluidity and permeability properties of the membrane [42]. They also control other membrane-associated metabolic processes, such as the activity of membrane-bound enzymes[43].



	R1	R2
α	CH_3	CH_3
β	CH_3	H
γ	H	CH_3
δ	H	H

Scheme 1.1 Schematic representation of the structure and nomenclature of (A) phytosterols, cholesterol, and (B) tocopherols

As cholesterol analogs, they compete with the absorption of cholesterol, when consumed [44]. It is documented that they have the ability to reduce plasma cholesterol concentrations by competitively blocking cholesterol absorption from the intestinal lumen [45, 46]. As early as in the 1950s, studies on the effects of phytosterols on cholesterol levels have showed that both free phytosterols and phytosterol esters have cholesterol-lowering effects [14, 47]. Miettinen et al. [48] reported for the first time that dietary intake of phytosterol esters can reduce total serum cholesterol and low-density lipoprotein (LDL) cholesterol levels in patients with hypercholesterolemia. It is established that a daily intake of 2 grams of phytosterols can effectively reduce LDL-C by 10% [49], which was shown to reduce the incidence of coronary heart disease by 10-20% [50].

Epidemiological data suggest that phytosterols can be related to a reduced risk of multiple cancers. In fact, dietary sterols can reduce the incidence of several common cancers such as colon, breast and prostate cancer[51]. The anticancer mechanism maybe due to phytosterols' potential effect on the host immune system. Phytosterols could promote a stronger anti-tumor response in the host system by inducing enhanced immune recognition of cancer cells [51]. Phytosterols could also influence the growth of endocrine tumors due to their structural similarity with estrogen [52, 53]. In addition, there are many studies, illustrating that phytosterols could directly inhibit tumor growth, due to their effects on cell cycle progression [54], apoptosis [55], and tumor metastasis [54].

In addition, these nutritional compounds have been reported to show anti-inflammatory[56], antibacterial[57], and antitumor activities [58, 59]. Some studies

indicate that individuals with high risk for cardiovascular disease might benefit from consuming dietary supplements or foods rich in phytosterols [60].

1.5. Tocopherols

Tocopherols are natural antioxidants biochemically synthesized by plants for the protection against the oxidation of plant tissues [61]. Tocopherols, also known as vitamin E, are generally a mixture of four isomers, alpha- (□), beta- (□), gamma- (□) and delta- (□) tocopherols (Scheme 1.1B). Alpha-tocopherol is the most widely distributed and most active vitamin E in nature [62, 63].

Tocopherols' main function is to act as an antioxidant in cell membranes, lipoproteins, and foods, preventing free radical formation [64]. The antioxidant activity of tocopherols stems from their ability to donate phenolic hydrogens to lipid radicals. Because of the electron-donating property of methyl groups on the chromanol ring, α-tocopherol with a fully substituted chromanol ring has the highest potency in donating electrons and becomes the most powerful antioxidant [64]. They also showed promising effects as preventative and therapeutic agents against cancer [65], probably attributed to their antioxidant characteristics, scavenging free-radicals [64, 66-68]. Additionally, it has been suggested that tocopherols may enhance the immune response [69] and inhibit the progression of cardiovascular diseases [70]. In fact, the American Heart Association (AHA) indicates that vitamin E in supplements can help prevent heart diseases [70]. Tocopherols are now widely used as additives in different pharmaceuticals, foods, and in cosmetics. In the food industry, vitamin E is mainly used as an antioxidant. It helps

maintain a stable and long-lasting fresh flavor of processed foods and has been widely incorporated in edible oils and dairy products.

1.6. The source and safety of phytosterols and tocopherols

Phytosterols cannot be synthesized by the human body and can only be obtained from food sources. They are mainly found in vegetable oils, cereals, potatoes, vegetables, fruits, and nuts [71-73]. Among them, vegetable oils and cereals have the highest concentrations of phytosterols [74, 75].

The United States Food and Drug Administration (FDA) authorized a health claim in 2000 for conventional foods containing phytosterols, because of the relationship between phytosterol esters and the reduced risk of coronary heart disease (CHD) [76]. In 2010, FDA amended its health claim based on data for esterified and nonesterified phytosterols that expanded the cholesterol lowering effects to include nonesterified phytosterols [77].

To date, phytosterols are approved for use in North American, Asia, Europe, Oceania, and South America [78]. There are no serious side effects with consumption of phytosterols at low doses, as reported in both animal and human experiments [79]. There are several reports in the literature have suggested that phytosterols may possess oestrogenic activity [80, 81]. While phytosterols influence the metabolic profiles of cholesterol in the large intestine. This may affect the level of female sex hormone. In one study, daily doses of 8.6 g phytosterols in margarine were provided to adult humans for 3–4 weeks. The result indicated that phytosterols have no effect in gut microflora and female sex hormone levels [82]. Another study showed that phytosterols would not produce increased concentrations of sterol oxides [83] and was also reported that there is

no evidence for the mutagenicity of phytosterols and phytosterol esters. This was assessed with an independent bacterial mutation assay, chromosome aberration assay in human peripheral blood lymphocytes, and mammalian cell gene mutation assay [84]. In conclusion, phytosterols are safe for consumption at low doses and do not have harmful effects on reproduction or pose cancer risk.

Unlike phytosterols, which have a wide range of sources, the main source of vitamin E is from edible vegetable oils [85]. In the process of oil refining, the content of vitamin E is reduced to about 60-70% of that of crude oil, with most left in the deodorized distillate. A-tocopherol is the most common form of vitamin E used in supplements, while the main dietary sources are sunflower and olive oils [86]. However, due to the higher consumption of soybean and corn oil, gamma-tocopherol is the most common form in the American diet [87]. Table 1 lists natural tocopherol contents of various refined vegetable oils and crude oils [88]. As shown in Table 1, only α-tocopherol is reported after refinement. In fact, Ferrari et al. [89] reported 57%, 14% and 36% loss of total tocopherols by chemical refining in corn, soybean and rapeseed oils, respectively. Alpaslan et al. [90] reported that 24. 6% of total tocopherols were lost at the end of the physical refining process while Medina-Juarez et al. [91] demonstrated that the loss of the total tocopherol was 40.6% after physical refining of soybean oil.

Table 1.1. The content of vitamin E in crude oil and refined oils

	Crude oil/（mg/100g）				Refined oil/（mg/100g）	
	total	α	β＋γ	δ	total	α
Rapeseed oil	52-57	16-22	31-52	0.8-2	34-52	12-18
Sunflower seed oil	51-74	47-72	2-7	1	32-52	29-49
Soybean oil	87-113	5-16	58-76	22-33	72-117	5-12
Corn germ oil	84-148	7-31	74-113	2-6	68-77	14-21
Palm oil	13-19	11-177	1-3	0.2	0.2-0.9	0.1-0.7
Coconut oil	0.3-2.5	0.1-0.5	0.2	0.2	0.20-0.9	0.1-0.7
Cottonseed oil	84-96	41-49	35-50	0.4-0.8	24-69	21-37
Safflower oil	45-54	43-51	2-8	0.8	27-35	24-32

Dietary intake and metabolic data on tocopherols and other forms of vitamin E, as well as their biological activity were studied as early as the 1980s [92]. The lymphatic pathway is the major route for absorption of tocopherols or their derivatives [93]. Tocopherols has functional use in foods as a nutrient, dietary supplements, and antioxidants [94], and is generally admitted as safe chemical preservatives for use in foods [95]. Tocopherols are effective antioxidants for animal fat and other animal products [96] with recommended use concentrations in the range of 100 to 300 ppm. When administered at high doses, tocopherols are reported to induce alterations in several parameters such as metabolism, growth, and development [97, 98]. However, the vast majority of these alterations are reported to be reversible once tocopherols are no longer administered. There are also no safety issues indicating that tocopherols are irritants when used as cosmetic ingredients [99]. It can, thus, be concluded that tocopherols can be safely incorporated into dietary supplements and cosmetic formulations.

Extraction of phytosterols and tocopherols from the CODD has great potential economic impact, turning a low-value byproduct into one with great benefits that can be

potentially incorporated into human food. Therefore, the analysis, both qualitatively and quantitatively, is important to the extraction process. Mass spectrometry coupled with separation techniques is widely used for the analysis of these bioactives, extracted from edible oils and the DDs [100-102].

1.7. Mass Spectrometry

Mass spectrometry (MS) plays an essential role in the qualitative and quantitative analysis of small molecules, including nonpolar ones, such as phytosterols and tocopherols. However, the complexity of DDs makes it difficult for analysis with MS only. Gas chromatography (GC)-tandem mass spectrometry (MS/MS) has been the conventional method for the analysis of phytosterols [103-106], as it provides efficient separation of phytosterol isomers [107]. However, GC-MS analysis is time-consuming due to the need of tedious chemical derivatization [4, 73, 108]. In addition, the use of electron impact or chemical ionization may result in the fragmentation of the molecule instead of producing the desired intact ionized molecules [109-111]. As an alternative to GC-MS, high performance-liquid chromatography (HPLC)-MS has been employed for the analysis of phytosterols and tocopherols. Due to the structural similarity of phytosterols as well as tocopherols, long HPLC run times are often needed to separate these compounds [112, 113], but MS/MS analysis can facilitate the detection/quantification of overlapping peaks.

1.7. 1. Ionization techniques

In mass spectrometry, ionization is the first step in which the analytes enter the gas phase as ions. There are several ion sources with each having its own advantages and disadvantages for different applications. In soft ionization techniques, such as atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI), high performance-liquid chromatography (HPLC) is routinely coupled with mass spectrometry to analyze underivatized phytosterols and tocopherols [114-117].

In general, ESI is particularly suitable for the analysis of polar molecules, while APCI gives better ionization of less polar compounds[118].

1.7.1.1. Electrospray ionization (ESI)

ESI is an ideal choice for compounds with basic or acidic functional groups, namely molecules with multiple charges or molecules with electronegative atoms (e.g., O, N, F) [119]. ESI allows for the ionization of analytes in solution. The ionized analytes are transferred from the solution to the gas phase in the form of single or multiply charged ions [120]. It is an ideal ionization source for molecules with electronegative atoms, or compounds carrying basic or acidic functional groups. However, in the case of low-polarity and nonpolar compounds, ESI is not considered as the most effective ionization source.

Despite their hydrophobicity, phytosterols and related compounds, such as squalene, were analyzed with ESI coupled to quadrupole instruments [121]. For example, the content of free phytosterols in tobacco leaves were determined using HPLC coupled to a triple quadrupole mass spectrometry (QqQ) [104]. The chromatographic separation was

performed on a BEH phenyl column with water and methanol as mobile phase. However, this study is one of the few reports that use ESI as ion source for the quantitative analysis of free phytosterols [104]. Four compounds, namely cholesterol, stigmasterol, β-sitosterol, and ergosterol were quantitatively determined. $[M+H-H_2O]^+$ ions were observed and selected for the quantification in positive ionization. Satisfactory results were obtained with a reported limit of quantification (LOQ) in the range of 4.8−9.7 ng/mL [104]. Another study of phytosterols used nano-LC coupled with nano-ESI-MS operated in the positive ion mode to analyze phytosterols in extra-virgin olive oil [122]. The $[M+H-H_2O]^+$ ion was also observed during qualitative analysis for all tested phytosterols (stigmasterol, campesterol, brassicasterol, β-sitosterol, and cholesterol). However, quantitative analysis was performed using HPLC coupled with an ultraviolet (UV) detector. ESI was sufficient for identification, but it was not used for quantification, as the nano LC-MS/MS platform could not detected a mixture of standards at the LOD of the LC-UV quantitative method. This was attributed to the low ionization efficiency of target phytosterols [122].

Canabate-Diaz et al. [114] evaluated the efficiency of ESI versus APCI to analyze phytosterols in olive oil. The authors reported variations in the ionization of phytosterols using ESI; only fucosterol and cholesterol formed $[M+H-H_2O]^+$ ions while β-sitosterol and fucosterol were observed as protonated ions $[M+H]^+$. On the other hand, cholesterol, erythrodiol, and uvaol formed sodiated species $[M + Na]^+$ while β-sitosterol, erythrodiol, and uvaol also produced $[2M+H]^+$ ions. It was reported that stigmasterol and sitostanol were not detected using ESI [114]. On the other hand, $[M+H-H_2O]^+$ ions are consistently formed with APCI [114, 123]. In addition, unexpected observation of

$[M+H]^+$ ions when ammonium acetate is added to the mobile phase was reported and it was rationalized by losing gaseous ammonia (NH_3) from the ammonium adduct [123]. It is apparent that the ionization behavior in ESI for phytosterols was not consistent despite that various structures sharing the same structural backbone.

Similar to phytosterols, tocopherols are also nonpolar molecules lacking protonation sites. Lanina, S.A. et al. [124] compared the applicability of ESI versus APCI in both positive and negative ionization to analyze four tocopherol homologues, namely, α-, β-, γ-, and δ-tocopherol. The protonated$[M+H]^+$ and molecular $[M^{\cdot}]^+$ ions are formed in both ESI and APCI in the positive ion mode (when acetic acid is added to the mobile phase). In addition, minor sodium adducts $[M+Na]^+$ were also formed in ESI positive ionization. $[M+H-H_2]^+$ ions were also observed for α- and β-,tocopherols which could be due to dehydrogenation of $[M+H]^+$ ions. However, the formation of adduct ions were not observed in positive APCI [124]. Similar ion formation was observed when ESI, APCI, and atmospheric pressure photoionization (APPI) were compared in positive ionization for the development of a quantitative method for tocopherols in soybean oil [125]. In fact, the major observed ion species were $[M-H]^+$, $M^{\cdot+}$, and $[M+H]^+$. The $[M-H]^+$ species was suggested to originate from the dehydrogenation of $[M+H]^+$ ions [124, 126]. Although APPI was chosen for the development of quantification method in that study [125], APCI is clearly the most widely used ionization source for tocopherol analysis. [124, 127-129]

1.7.1.2. Atmospheric pressure chemical ionization (APCI)

APCI is another soft ionization technique that is better suited for hydrophobic or nonpolar low molecular-weight compounds, like phytosterols and tocopherols, and it is widely applied in lipid analysis [130, 131]. Unlike ESI, APCI involves a desolvation process before ionization. The gaseous solvent molecules react with ionized nitrogen gas and subsequently ionized in the source [132, 133]. Depending on proton affinity, analytes are ionized either by charge transfer, addition, or removal of a proton. This technique is ideal for low polarity or nonpolar low molecular-weight compounds, and it is widely applied in lipid analysis [130, 131].

Many studies were published using APCI-MS for the analysis of phytosterols [100, 134-136]. An early report of the use of LC-APCI- MS for steroid analysis evaluated the MS/MS dissociation of 60 steroids [137]. It showed that the ion that was protonated followed by the loss of water was the observed base peak of almost all tested steroids. However, no phytosterols were assessed in this study. The ionization mechanism using APCI for phytosterols was similar to the one reported for steroids, as discussed in the following section.

Reported APCI-MS analysis of phytosterols is primarily quantitative in nature. Abundant $[M+H-H_2O]^+$ and [M+H-acid] $^+$ ions were observed for phytosterols and phytosterol esters, respectively, for the identification of free and esterified sterols in tobacco leaves [138]. MS measurements were performed with a QqQ mass spectrometer equipped with an APCI ion source. Millan et al. reported the use of APCI ion-trap instrument to determine phytosterols in oenological (grape and wine) matrices [139]. The $[M+H-H_2O]^+$ ions were the most dominant observed ions. The most abundant

phytosterol in all samples was β-sitosterol, followed by stigmasterol, fucosterol and campesterol. According to Liao et al. [134], APCI-MS analysis in the positive ion mode was also successfully applied for the identification of phytosterols in the hexane extraction of edible animal fats. APCI positive mass spectra of sterols (campesterol, stigmasterol, β-sitosterol, and β-sitostanol) were observed as $[M+H-H_2O]^+$.

The use of tandem MS increases the sensitivity in the quantification of phytosterols in edible oils. Quantification of phytosterols in vegetable oils using multiple reaction monitor (MRM) mode achieved LOD values in the range of 2−25 ng/mL [102]. In addition to vegetable oils, phytosterols were analyzed in plant samples, such as vegetables, tobacco, and wine using APCI in positive ionization. [140-142]. For example, Millan et al. [142] then established a method for the identification and quantification of phytosterols in oenological matrices employing the MRM mode with an LOQ of 8 ng/mL. This analytical method was then applied for screening and quantifying phytosterols present in various matrices (pulp, skin, seed, and wine). Various product ions are utilized in MRM mode for the monitoring of phytosterols; however, the $[M+H-H_2O]^+$ ions are the selected precursor ions for most phytosterols.

Similar to phytosterols, the identification and quantification of hydrophobic tocopherols were reported in edible oils [143], nuts [144], and fruits [145, 146], using LC-APCI-MS. Identification of tocopherols and tocotrienols in foods, such as peanuts, almonds, spinach and bran, as well as other samples, such as latex and tablets [116, 147], was accomplished using HPLC-APCI-MS by monitoring the molecular ion $[M^{\cdot}]^+$. Similarly, tocopherols and tocotrienols were identified and quantified in human plasma using $^2H_9$-R-tocopherylacetate and $^2H_9$-R-tocopherol as internal standards with a lowest

LOQ of 96 nmol/L. Clare L. F. et al. [143] developed an MS/MS method for the simultaneous quantification of tocopherols and phytosterols in canola oil by monitoring two MRM transitions for tocopherols and one transition for phytosterols. The quantitative method was then applied to determine tocopherols and phytosterols in different oils (canola, palm fruit, sunflower and olive oils) while α-, γ-, δ-tocopherols, and β-sitosterol, campesterol, brassicasterol were identified [129].

According to the above studies, APCI showed better ionization efficiency for phytosterols and tocopherols rather than ESI. In general, free phytosterols formed $[M+H-H_2O]^+$ and phytosterol esters formed [M−Fatty Acid+H] $^+$ ions, respectively. Unlike ESI, the ionization behavior of phytosterols with APCI is consistent. As for tocopherols, no adduct ions were not formed during APCI analysis, whereas sodiated adducts [M+Na] $^+$ were reported when using ESI in positive ion mode. Thus, APCI ionization is widely adapted for the analysis of phytosterols and tocopherols. In fact, APCI was employed for the analysis when ESI gave low ion intensity for phytosterols [114] and tocopherols [124]. These results may indicate the suitability of APCI as the ionization source of choice for the analysis of phytosterols and tocopherols.

1.7.1.3. The Choice between ESI and APCI

The choice of the ionization source is crucial as it determines the overall efficiency of the analytical method. As discussed above, ESI ionization of phytosterols was inconsistent despite structural similarities among the various phytosterols. This could due to the low electronegativity and less stability of the generated [M+H] $^+$ ion of phytosterols. Thus, the application of ESI has limitations, especially when the

concentrations of phytosterols are very low. As for tocopherols, research showed that APCI and ESI resulted in similar behavior during MS analysis [124, 125]. However, there are studies demonstrating that APCI is more sensitive than ESI for tocopherols and tocotrienols quantification [148, 149]. In summary, APCI has been widely employed due to its great and consistent ionization efficiency in primarily forming $[M+H-H_2O]^+$ ions in phytosterols, and $[M^{\cdot}]^+$ ions in tocopherols.

Despite the wide use of APCI for the analysis of phytosterols and tocopherols, little is done to fully characterize the numerous product ions observed during MS/MS analysis. There is a need for a universal MS/MS fingerprint of these compounds. Such comprehensive analysis will contribute to the development of effective profiling methods and the identification of new structures.

In my research, both ESI and APCI were tested for the qualitative analysis of phytosterols and tocopherols.

1.7. 2. Mass analyzers

Various MS instruments were utilized to attain structural elucidation and develop effective screening strategies. The following sections will summarize various MS technologies utilized in my research, including quadrupole, linear ion trap, and orbitrap MS instruments. In addition, MS/MS, multi-stage (MS^n) analysis will be summarized and explained.

A mass analyzer is the component of the mass spectrometer where ions are separated based on their *m/z* values and sent to the detector and later converted to a digital output. There are six general mass analyzers, including three trapping analyzers: linear ion trap

(LIT), orbitrap, and Fourier transform ion cyclotron resonance (FTICR), and three non-trapping analyzers: quadrupole (Q), time of flight (TOF), and sector mass analyzer. In the following sections, I will discuss the MS analyzers used in my study.

1.7.2.1. Quadrupole (Q)

Quadrupole mass analyzer consists of four cylindrical rods that are parallel to each other. One pair of opposing rods is connected together electrically and charged with a voltage (V_{dc}+V_{rf}), while the other pair of rods is charged with another voltage (V'_{dc} +V'_{rf}), where V_{dc} is direct current (DC) and V_{rf} is radio frequency (RF) voltage. The RF and DC vectors differ by 180 degrees, and the ions of one specific mass-to-charge (*m/z*) ratios are passed by adjusting the RF and DC voltages [150]. The ion at a specific *m/z* is oscillated by the high electric field force of the quadrupole. The V_{dc}/V_{rf} value added to the quadrupole ensures that only ions with pre-set *m/z* perform a stable oscillation in the middle, reaching the detector through the quadrupole and forming a mass spectrum signal. Other ions form an unstable oscillating trajectory causing them to eventually hit the quadrupole rods and neutralize [150].

The quadrupole mass spectrometer excels at applications where targeted ions are analyzed. However, the sensitivity is mass-dependent and decreases with the increasing value of *m/z* of the ions. Therefore, its mass range is limited up to 4000 Da [150].

1.7.2.2. Linear ion trap (LIT)

The linear ion trap uses a set of quadrupole rods to confine ions radially and a static electrical potential on end cap electrodes to confine the ions axially. The linear form of the

trap can be either used as a selective mass filter, or as an actual trap by creating a potential well for the ions along the axis of the electrodes [150].

Ions can be captured and stored by the ion trap with a certain alternating applied current (AC) voltages. When the amplitude or frequency of the RF voltage between the quadrupole rods and the exit lens is changed, the ions with different *m/z* values can be expelled out of the ion trap axially. Ions are expelled radially when an appropriate AC voltage on two opposite rods is applied. The ion trap can also be used as a collision cell to perform collision-induced dissociation (CID) of ions in the well. This could be used to analyze the generated product ions, and determine the composition of the ions by the observed fragment ions [150].

1.7.2.3. Orbitrap

Orbitrap is also an ion trap mass analyzer consisting of an outer barrel-like electrode and a co-axial inner spindle-like electrode. It traps ions in an orbital motion around the inner electrode on elliptical trajectories; their electrostatic attraction to the inner electrode is balanced by their inertia. Meanwhile, the ions also move back and forth along the axis of the central electrode. The mass spectrum is converted from the image current of the trapped ions using the Fourier transform of the frequency signal [151, 152]. A mass resolution up to 150,000 for ions produced by laser ablation has been demonstrated [151]. It also provides a high mass accuracy, a high dynamic range, and high sensitivity [153, 154]. Based on these properties, orbitrap-based mass spectrometers are used in life science such as pharmacology [155], food [156], and safety analysis [157].

1.7.2.4. Tandem mass spectrometry

Tandem mass spectrometry, also known as MS/MS, involves multiple steps of mass analyzers [158]. MS/MS is generally conceived in two ways: tandem in space (e.g. QqQ, quadrupole time-of-flight) and tandem in time (e.g. ion trap). Since MS/MS was commercialized, it has been successfully applied to both qualitative and quantitative analysis [159, 160].

QqQ consists of two quadrupole mass analyzers in series, with a non-mass-resolving quadrupole between them acting as a collision cell. The majority of applications of QqQ is the quantification of trace analytes in complex matrices [161]. Quantification of different phytosterols and tocopherols in various biological samples have been performed with LC-MS/MS [104, 143, 162]. Quadrupole time-of-flight (QqTOF) is similar to QqQ but with a TOF as the second mass analyzer instead of quadrupole which allows for accurate mass measurements. Therefore, QqTOF is mostly used for the identification of target compounds in complex matrices [139, 163].

Trapping mass analyzers are tandem-in-time instruments. The various stages of MS analysis are performed in the same analyzer but at different time. The advantage of tandem-in-time mass analyzers is their ability to perform more than two steps of analysis, which is referred as MS^n. However, it does not perform well in quantitative analysis and cannot perform precursor ion scans and neutral loss scans. However, these limitations can be overcome by introducing a hybrid quadrupole-linear ion trap (LIT) (QTRAP®) in which a LIT, replaces the second Q in the QqQ instrument.

1.7.2.5. Triple quadrupole-linear ion trap

Triple quadrupole-linear ion trap is based on a triple quadrupole platform where Q3 can be operated either in the normal RF/DC mode or in the LIT mode [164]. The triple quadrupole-linear ion trap system has no new scan functions; however, scan combinations of triple quadrupole mode and trap mode can be performed in the same LC/MS run, which gives new possibilities for both quantitative and qualitative analysis [165].

1.7. 3. LIT operating modes

Scanning modes that were utilized in my work include, precursor ion scan (PIS), neutral loss scan (NLS), and multiple reaction monitoring (MRM). Below is a list of several modes that have been applied in this study.

MS^3 mode. The triple quadrupole-linear ion trap system has MS^3 capabilities that involve additional fragmentation step of product ions trapped in the linear ion-trap [164]. The precursor ions selected by Q1 to the pressurized collision cell Q2 accomplish the first stage MS/MS fragmentation. The produced product ions and residual precursor ions are transferred to the Q3 linear ion trap for further analysis. The second generation precursor ion is isolated within the linear ion trap, where they are excited by a single frequency auxiliary signal and fragmented to give the MS^3 product ion spectrum [158].

MS^3 could provide further structural information about each compound and aid in the rationalization of the proposed fragmentation pathways [166]. It has been successfully utilized to illustrate the fragmentation pattern of different bioactive compounds, hence providing further structural information of the observed product ions. For example, Tomoko et. al. [167] elucidated the fragmentation behavior of vitamin E homologues using

MS^3 to identify their presence in medicinal plants. However, only base peaks of each vitamin E were assigned structures; no detailed fingerprint of tocopherols as well as fragmentation mechanism was discussed [167]. To the best of my knowledge, no similar research has been reported relating to phytosterols.

Multiple reaction monitoring (MRM): The MRM mode is the most commonly used analysis mode in QqQ, in which ions of particular m/z values are selected in the first analyzers (Q1), dissociated in the collision cell (Q2), and the selected produced ion is analyzed in the second mass analyzer (Q3). When employing MRM methods with a QTRAP, the LIT simply operates as a Q analyzer [104]. A quantitative method for the determination of free phytosterols, namely, cholesterol, ergosterol, stigmasterol and β-sitosterol, in tobacco leaves were successfully developed using a QTRAP instrument in MRM mode [104].

Neutral loss scans (NLS) and **precursor ion scans (PIS)**: NLS are used for screening experiments. Both Q1 and Q3 are scanning simultaneously. Q3 is offset by the neutral loss under investigation. Only compounds that have a specific loss are detected [168]. While using PIS, Q3 is set to allow only a product ion of specific m/z value to pass and Q1 is scanning. PIS are used for screening experiments where a group of compounds produce the same product ion(s) [158].

Enhanced product ion mode: In enhanced product ion (EPI) mode, the selection of the precursor ion is performed in Q1 utilizing RF/DC isolation at any resolution. Collision-

induced dissociation (CID) occurs in the collision cell Q2, and product ions are trapped in Q3 operated in LIT mode. This will allow for ion accumulation, resulting in better MS/MS spectra.

Information-dependent acquisition: an information-dependent acquisition (IDA) method allows for "on-the-fly" MS/MS spectra collection during a single chromatographic process. In IDA mode, a survey scan is performed that acquire the data. If the selection criteria are met, a second scan (data dependent) is then performed [169, 170]. As such, the metabolite detection and MS/MS data acquisition are acquired in the same LC-MS run, allowing for identification. The QTRAP retains the traditional triple quadrupole scan modes such as **NLS**, **PIS** and **MRM**. However, MIM-dependent MS/MS acquisition, a novel scanning method for metabolite identification on a triple quadrupole-linear ion trap instrument was employed in this study. The term MIM is a scanning method based on the MRM mode, described above. Several possible phytosterol *m/z* values were monitored in both Q1 and Q3. The collision energy in Q2 is set to the lowest value to minimize fragmentation. This is aimed at reducing the time needed in the steps to screen for the targeted ions [171]. The use of survey scans, with **EPI** as a dependent scan, is reported to achieve better selectivity than MRM-EPI [172].

1.8. Research hypothesis and objectives

The assignment of the product ion structure is not shown or explained in most of the published work for the analysis of phytosterols and tocopherols. However, Mo et. al. [102] attempted to propose structures of product ions observed during MS/MS analysis of phytosterols. However, only the product ions chosen for selected reaction monitoring

(SRM) were assigned structures. Therefore, there is a need to fully characterize the CID-MS/MS of both phytosterols and tocopherols in which both the structures and genesis of the produced ions is clearly elaborated. Such knowledge is needed for developing screening and quantification methods as well as for metabolite identification and structural determination of related compounds.

In summary, the fragmentation patterns of phytosterols and tocopherols have not yet been fully investigated. There are no reported detailed MS/MS data to establish the fingerprints of either phytosterols or tocopherols. In fact, no work has compared the MS/MS of various structures to create a general MS/MS fingerprint.

The main purpose of my M.Sc. project was to investigate the ionization and tandem mass spectrometric patterns of phytosterols and tocopherols. Subsequently, profiling methods were established.

1.8.1. Establishment of the fragmentation patterns (i.e., fingerprints) of phytosterols and tocopherols using APCI-MS/MS.

1.8.1.1. Hypothesis

Tested phytosterols and tocopherols will show similar fragmentation behavior during MS/MS analysis, which will lead to a common MS/MS fragmentation pattern for each group .

1.8.1.2. Objectives

A. Assessment of the CID-MS/MS fragmentation patterns of selected phytosterols.

a. Confirm the molecular structure of a series of selected phytosterols and tocopherols using single-stage MS, exact mass measurement, MS/MS and MS^3 analysis.

b. Establish a universal MS/MS fragmentation pattern for tested compounds.

- Compare the fragmentation patterns of 4 phytosterols;
- Identify the unique product ions for different phytosterols to be used in targeted analysis.

B. Assessment of CID-MS/MS fragmentation patterns of tocopherols.

a. Confirm the molecular structure of a series of tocopherols using single-stage MS, exact mass measurements, MS/MS and MS^3 analysis.

b. Establish a universal MS/MS fragmentation pattern for tested compounds.

- Compare the fragmentation patterns of tocopherols;
- Identify the unique product ions for different tocopherols to be used in targeted analysis.

1.8.2. Establishment of the phytosterols and tocopherols profiling methods using HPLC-APCI-MS/MS.

1.8.2.1. Hypothesis

Profiling methods will be able to identify phytosterols and tocopherols in CODD, edible oil, and standard mixture.

1.8.2.2. Objectives

Establish HPLC-MS/MS methods for phytosterols and tocopherols profiling using a QTRAP mass spectrometer.

a. Select diagnostic ions for PIS and common neutral loss for NLS.

b. Establish PSI-EPI scan for phytosterols and tocopherols profiling.

c. Establish multiple ion monitoring (MIM)-EPI scanning mode to confirm the PSI-EPI data.

CHAPTER 2

THE ESTABLISHMENT OF TANDEM MASS SPECTROMETRIC FINGERPRINTS OF PHYTOSTEROLS AND TOCOPHEROLS

This chapter describes the multi-stage tandem mass spectrometric behavior of phytosterols and tocopherols with more details than the published paper. The development of targeted profiling strategies of phytosterols and tocopherols in vegetable oils was also discussed in the published research article. However, the profiling method will be discussed later in an individual chapter in the thesis.

2.1. Introduction

The CID-MS/MS behavior of phytosterols and tocopherols are evaluated in this part. Before that, both APCI and ESI were applied to test the ionization behavior of phytosterols and tocopherols. Herein, the MS/MS dissociation behavior of phytosterols and tocopherols is elucidated, to establish generalized MS/MS fingerprints. MS/MS and multistage (MS^3) analysis revealed common fragmentation behavior among the four tested phytosterols, namely β-sitosterol, stigmasterol, campesterol and brassicasterol. Similar analysis was conducted for the evaluated tocopherols (i.e. α-tocopherol, β- tocopherol, γ- tocopherol and δ-tocopherol). Mr. Jiang performed all the above MS experiments. HRMS experiments were also employed for ions structure validation with the help of Mr. Bryn Shurmer in Canadian Food Inspection Agency. As such, a universal MS/MS fragmentation pathway for both phytosterols and tocopherols were successfully established for the first time.

Vegetable oils play an important role in human nutrition since they are a daily food component. As characteristic components of vegetable oils, phytosterols and tocopherols are essential for plant biochemistry [43, 61] and they also possess beneficial health effects to humans [42, 64, 70]. Phytosterols are responsible for the stabilization of the phospholipid bilayer of the cell membrane by regulating the fluidity and permeability properties of the membrane [42]. They also control other membrane-associated metabolic processes, such as the activity of membrane-bound enzymes [43]. Phytosterols are structurally and functionally similar to cholesterol; however, they possess an extra methyl or ethyl group on the side chain and sometimes with an extra double bond on the side chain. As cholesterol analogs, they compete with the absorption of cholesterol [44] if taken orally

and they are currently incorporated in food due to their well-established blood cholesterol-lowering abilities [3, 4, 59]. In addition, they have been reported to show anti-inflammatory [56], antibacterial [57] and antitumor [58] activities. There are many structural forms of phytosterols, such as β-sitosterol, campesterol, brassicasterol and stigmasterol [173] (Scheme 1.1A).

Tocopherols, on the other hand, are a class of organic compounds with vitamin E activity. Structurally, they possess a chromane ring and a hydrophobic side chain (Scheme 1.1B). They exist naturally in four isoforms, namely alpha, beta, gamma and delta that only differ in the number of methyl groups and their position in the chromane ring. Tocopherols possess antioxidant activity [5-7] and they have showed promising effects as a preventative and therapeutic agent against cancer [64-66]. Additionally, it has been suggested that tocopherols may enhance the immune response [69] and inhibit the progression of cardiovascular diseases [70] . In summary, tocopherols and phytosterols have a wide range of health applications and are used as additives in food, pharmaceutical, and cosmetic products [11, 174, 175].

Phytosterols and tocopherols are found in plants, such as seeds, grains, and legumes [24], with high concentrations in unrefined vegetable oils [24, 25]. However, vegetable oils are subjected to a refining process to improve their palatability, quality and shelf life. The deodorization distillate (DD) is a by-product produced during the deodorization stage of crude oil refinement [176]. It contains large amounts of bioactive metabolites including tocopherols (i.e. vitamin E) and phytosterols. Depending on the seed oil, the amount or compositional distribution of phytosterols and tocopherols will vary [24, 25, 177]. Several

strategies have been developed to recover and purify these compounds from plant sources as well as from DD of different vegetable oils [8-10].

To effectively analyze phytosterols and tocopherols, analytical strategies need to be developed to allow for their identification and quantification. Liquid chromatography-tandem mass spectrometry (LC-MS/MS), either with an electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI), is commonly used for the identification and quantification of phytosterols and tocopherols in different biological samples [104, 143, 162]. For example, Tan and coworkers [104] developed a quantitative method for the determination of cholesterol and free phytosterols, namely, ergosterol, stigmasterol and β-sitosterol in tobacco leaves using multiple reaction monitoring (MRM) mode. Phytosterols ionized as protonated species that instantly lost a water molecule forming an abundant $[M+H-H_2O]^+$ ion, used for MS/MS analysis. Despite that several MRM transitions were used for quantification, the structural assignment of the product ions was not shown or explained [29]; which is the case in most published methods [104, 143, 162]. However, Mo S. et al. [102] proposed structures of the product ions observed during MS/MS analysis of phytosterols in the saponified extracts of edible oils. However, only the structures of the product ions chosen for selected reaction monitoring (SRM) were reported by showing the cleavage site. The exact structures and possible fragmentation mechanisms such as the formation of double bond or cyclization were not discussed or elucidated. As for tocopherols, Tomoko I. et al. [167] performed MS/MS analysis on the protonated species $[M+H]^+$. However, the reported MS/MS data did not show sufficient dissociation and only two major product ions for each of the tocopherols were structurally assigned.

Therefore, there is a need to fully characterize the MS/MS behavior of phytosterols and tocopherols to allow for the development of selective qualitative and quantitative analytical methods. For example, the MS/MS fingerprints of the novel drug delivery agents, gemini surfactants, were established, showing unique collision induced dissociation (CID)-MS/MS behavior among various structural families [178-180]. The data were subsequently utilized to develop targeted MS methods to selectively quantify these compounds in cellular matrix [155, 181, 182]. Similarly, MS/MS can be used to develop screening methods, based on the PIS and NLS [183, 184]

To the best of our knowledge, no detailed MS/MS analysis of either phytosterols or tocopherols were reported to establish their MS/MS fingerprints. In fact, no work has compared the MS/MS of various structures of different phytosterols and tocopherols to create a generalized MS/MS pattern. Therefore, we evaluated the CID-MS/MS of four major phytosterols and tocopherols and the data is further utilized to develop LC-MS-based screening strategies.

2.2. Materials and Methods

2.2.1. Samples and Reagents

All solvents were of LC-MS grade and all chemicals were of analytical reagent grade, purchased from Fisher Scientific (Pittsburg, PA, USA) .

β-Sitosterol, campesterol, stigmasterol, and brassicasterol each at 98% purity were purchased from Toronto Research Chemicals (Ontario, Canada) while α-tocopherol (99.9%), γ-tocopherol (96.8%), and δ-tocopherol (94%), were purchased from Sigma Aldrich (Canada).

2.2.2. Sample preparation

Stock solutions of phytosterols and tocopherols standards were prepared at 1 mg/mL in chloroform and stored at –20°C. For MS, MS/MS, and second-generation MS (MS^3) analysis, each stock solution was further diluted to 5 µg/mL with acetonitrile containing 0.01% acetic acid.

2.2.3. MS analysis

MS analysis of four standard phytosterols (β-sitosterol, stigmasterol, campesterol and brassicasterol) and tocopherols was performed using the AB SCIEX 6500 QTRAP® quadrupole-linear ion trap mass spectrometer (Qq-LIT-MS), equipped with an alterable APCI/ESI source (AB Sciex, Concord, ON, CA). Reference standards were directly infused at a flow rate of 10 µL/min with an integrated syringe pump. The instrument was operated in the positive ion mode with a declustering potential (DP) of 40V and vaporization temperature of 400 ℃ when APCI was equipped. While positive ESI was performed using a5.0 kV spray voltage and a capillary temperature 250 ℃.

For high resolution MS and MS/MS analysis, a Thermo Scientific Q Exactive™ Quadrupole-Orbitrap equipped with APCI source (Thermo Fischer Scientific, Waltham, MA, USA) was utilized. The reference standards were introduced via flow injection analysis (FIA) using an Ultimate 3000 UHPLC system. The flow rate was 0.4 mL/min under isocratic elution (99% acetonitrile and 1% methanol). The injection volume was 10 µL and the overall run time was 0.5 minutes. To provide back-pressure for the pump, a CSH C18 pre-column (130Å, 1.7 µm, 2.1 mm×5 mm, Waters, Milford, MA, USA) was used. Full scan data were acquired from *m*/*z* 50 to 500 with a resolution of 98995 (at *m*/*z*

400). Targeted MS/MS spectra were acquired using the ions appended in an inclusion list. The selected ions were subjected to high-energy C-trap dissociation (HCD) with normalized collision energy of 35% and an activation time of 100 ms.

2.2.4. Structure assignment

2-D structures of all phytosterols, tocopherols, and product ions generated during MS/MS analysis were developed using ACD/chemsketch free version software (ACD Labs, Toronto, Ontario, Canada). The software was used to draw molecules, ions, schematic diagrams, and calculate their properties, e.g. monoisotopic mass.

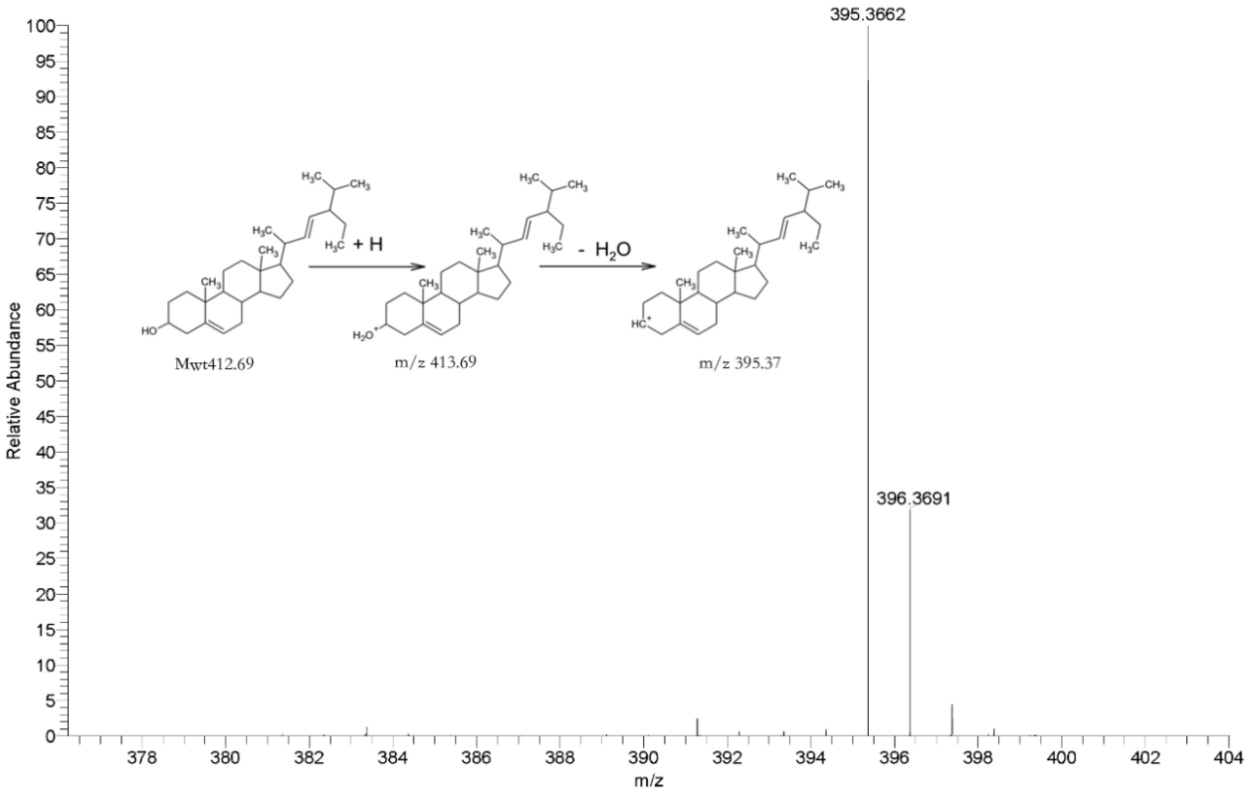
2.3. Results and discussion

2.3.1. Single-stage MS analysis

Two different ionization techniques, APCI and ESI in positive ionization, were evaluated for the analysis of four phytosterols and four tocopherols (Scheme 1.1). 4 phytosterol standards, namely stigmasterol, brassicasterol, campesterol, and □-sitosterol are selected to represent the major sterols generally found in edible oils. APCI showed better ionization than ESI particularly for phytosterols during direct infusion (Appendix A-Figure 1). Tocopherols, however, showed similar performance for both APCI and ESI.

Full scan MS analysis of phytosterols showed abundant $[M+H\text{-}H_2O]^+$ ions, resulting from the loss of a water molecule from the protonated species. For example, β-sitosterol's (molar mass: 414.71 $g \cdot mol^{-1}$) mass spectrometric analysis showed a base peak at *m/z* 397.37 corresponding to $[414.71 + H - H_2O]^+$ (Scheme 2.1). However, $[414.71 + H - 2H_2]^+$ was also observed as a minor ion during phytosterol ionization (Appendix A-Figure 1).

Similar analysis was performed with the other 3 phytosterols and similar ionization pattern was observed. The $[M + H - H_2O]^+$ was then selected for MS/MS and MS^3 analysis. The structures were assigned and justified. Most product ions were formed by cleavage of the C-ring or penta cycle of the phytosterols although the C_{17} side chain also contributed to the formation of minor fragment ions.



Scheme 2.1. Full scan MS analysis of stigmasterol showing the predominant ionization pathway of stigmasterol via loss of a water molecule (precursor ion, $[M+H-H_2O]^+$ at *m*/*z* 395.4).

On the other hand, tocopherols ionized primarily as the molecular ion, $M^{\cdot+}$. Exact mass measurements using high-resolution mass spectrometry (HRMS, Thermo Scientific Q Exactive™ Quadrupole-Orbitrap), were conducted for both phytosterols and tocopherols. Observed mass accuracies were less than 3 ppm (Table 2.1). Exact mass measurements confirmed the molecular structures of the tested compounds, as well as the proposed ionization mechanism (Scheme 2.2).

Table 2.1. Full scan MS analysis of phytosterols (a) and tocopherols (b) using Quadrupole-Orbitrap instrument.

a. Compound	Molecular Weight	*m/z* (*theoretical*) $[M+H-H2O]^+$	*m/z (measured)*	ppm
Stigmasterol	412.7	395.3672	395.3662	-2.60
Brassicasterol	398.7	381.3516	381.3510	-1.52
β-sitosterol	414.7	397.3829	397.3818	-2.71
Campersterol	400.7	383.3672	383.3664	-2.16

b. Compound	Molecular weight	*m/z* (*theoretical*) $[M]^{\cdot+}$	*m/z* (*measured*)	ppm
α-tocopherol	430.7	430.3805	430.3792	-3.02
β- tocopherol	416.7	416.3649	416.3639	-2.40
γ- tocopherol	416.7	416.3649	416.3637	-2.88
δ- tocopherol	402.7	402.3492	402.3490	-0.50

2.3.2. Multi-stage MS analysis

2.3.2.1. MS/MS analysis of phytosterols

The $[M+H-H_2O]^+$ was selected for MS/MS and MS^3 analysis of phytosterols. Structural identification of each phytosterol was established based on CID-MS/MS and MS^3 data from QqQ-LIT-MS and confirmed by exact mass measurements using Quadrupole-Orbitrap-MS/MS. During MS/MS analysis, most product ions were formed by cleavage of the C-ring and/or the penta cycle of the phytosterols, while dissociation at the C_{17} side chain contributed to the formation of minor product ions. Schemes 2.1 and 2.2A show the full scan MS and MS/MS spectra for stigmasterol from HRMS as representative structure, respectively. All tested phytosterols share the same core structure; it is, therefore, highly expected that common dissociation behavior will be shared among the various phytosterols.

Stigmasterol is used as a representative model for phytosterols. The MS/MS analysis of stigmasterol showed a complex spectrum, and the structure of the major product ions were rationalized and confirmed via MS^3 analysis. The fragmentation process starts with three unique pathways that results in the formation of three singly charged product ions observed at *m/z* 311.27, 297.26 and 285.26 (Scheme 2.2B), designated as ions S1, S2 and S3 (S indicates a cleavage on the side chain). Each of these ions undergoes further fragmentation as explained below, where three unique dissociation pathways are identified. It was observed that the sites 1, 2, and 3 will first break when the cyclic structures dissociate (Table 2.2). This can be attributed to the favorable initial dissociation at the bond closed to the side chain (i.e. bond 1, in table 2.2). The observed relative abundance of related ions confirms this speculation. For example, the order of relative abundance of D1, D2 and D3 is D1> D3 >D2. Since the fragmentation involves the formation of radical intermediaries

[185], radical reaction mechanism will affect how the MS/MS dissociation will be initiated. While the tertiary carbon radical ion is more stable, the relative abundance of the product ions will differ [186]. However, subsequent to S1, S2 and S3 formation, there are many identical product ions that are shared among the three main fragmentation pathways. Table 2.2 shows the common product ions and fragment site during the CID MS/MS analysis of phytosterols.

Product ion S1 dissociates via five different pathways forming five product ions, designated as S4, S5, C1 (C indicates a cleavage in the C ring), D2 and D3 (D indicates a cleavage in the D ring) (Scheme 2.2B). Product ion S4 at *m/z* 269.22 is formed via the dual loss of an ethyne moiety and CH_4 on the side chain (Scheme 2.2B). On the other hand, product ion S5 at *m/z* 255.21 is generated by the complete elimination of the side chain. Inner-ring dissociation of S1 occurs at ring D yielding product ions D2 and D3 at *m/z* 229.19 and 215.18, respectively, and at ring C yielding C1 at *m/z* 201.16 (Table2.2).

The product ion S4 at *m/z* 269.22 can undergo two main fragmentation processes. The first one involves rearrangement of ring B to a penta-carbon cyclic structure yielding the ion at *m/z* 135.12 (B1). The second fragmentation process involves cleavage of ring D yielding the product ion D1 at *m/z* 241.20. D1 is further dissociated to three product ions from the cleavage of ring B or ring C, forming ions B3 at *m/z* 109.10 and C2 at *m/z* 187.15, respectively (Table 2.2). Product ion C3 at *m/z* 173.13 is formed via cleavage within ring C through the neutral loss of C_5H_8. Furthermore, product ion, C5, is formed through a loss of ethyne moiety as shown in Scheme 2.2B.

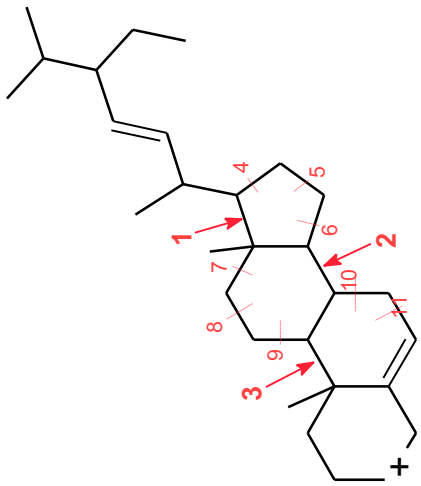
The second major pathway during CID-MS/MS dissociation involves a C=C double bond cleavage at the side chain. Cleavage at the C22-C23 bond yields the product ions S2

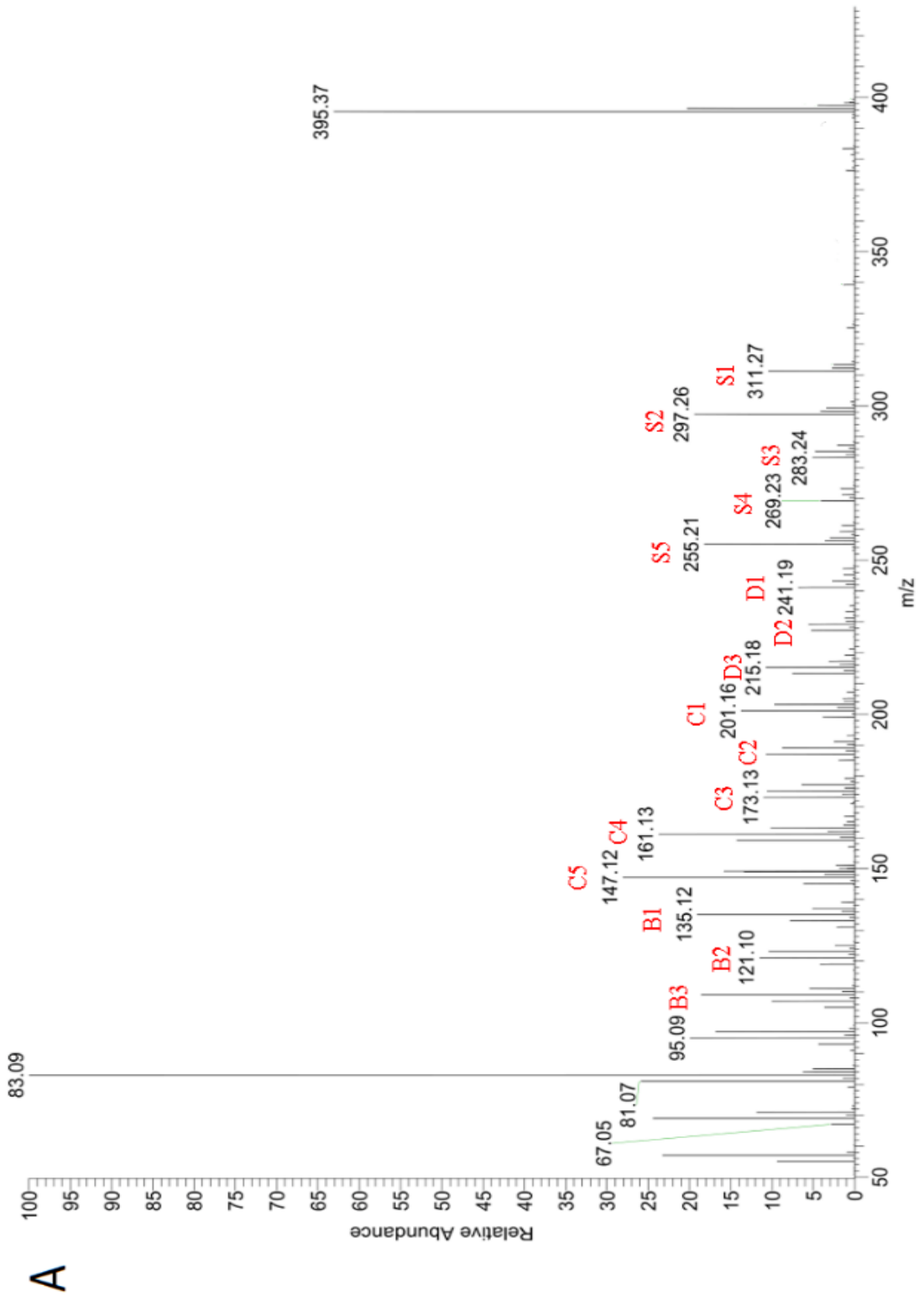
at *m/z* 297.26 (Scheme 2.2A) due to the neutral loss of C_7H_{14}. This product ion can only be formed from phytosterols whose side chain have a double bond at positions C22-C23, i.e. stigmasterol and brassicasterol (Scheme. 1.1A). The subsequent partial loss of the remaining part of the side chain at position C17 leads to the formation of S4 at *m/z* 269.23. Ion S2 loses the whole side chain to form the product ion S5 at *m/z* 255.21, a dominant product ion of the tested phytosterols bearing double bond at position C22-C23 (Scheme 1.1A). Subsequently, cleavages within ring C of S5 produces two product ions, designated as C3, and C4 as shown in Scheme 2.2B. Ions C3, C4 and C5 are common ions observed in all tested phytosterols, due to common structural features (Scheme 1.1A). C4 can further yield C5 at *m/z* 147.12 by losing a methyl group on ring B. Further dissociation within ring B of C5 leads to formation of B2. The genesis of all observed ions was confirmed by MS^3 analysis (Appendix-A, Table 1).

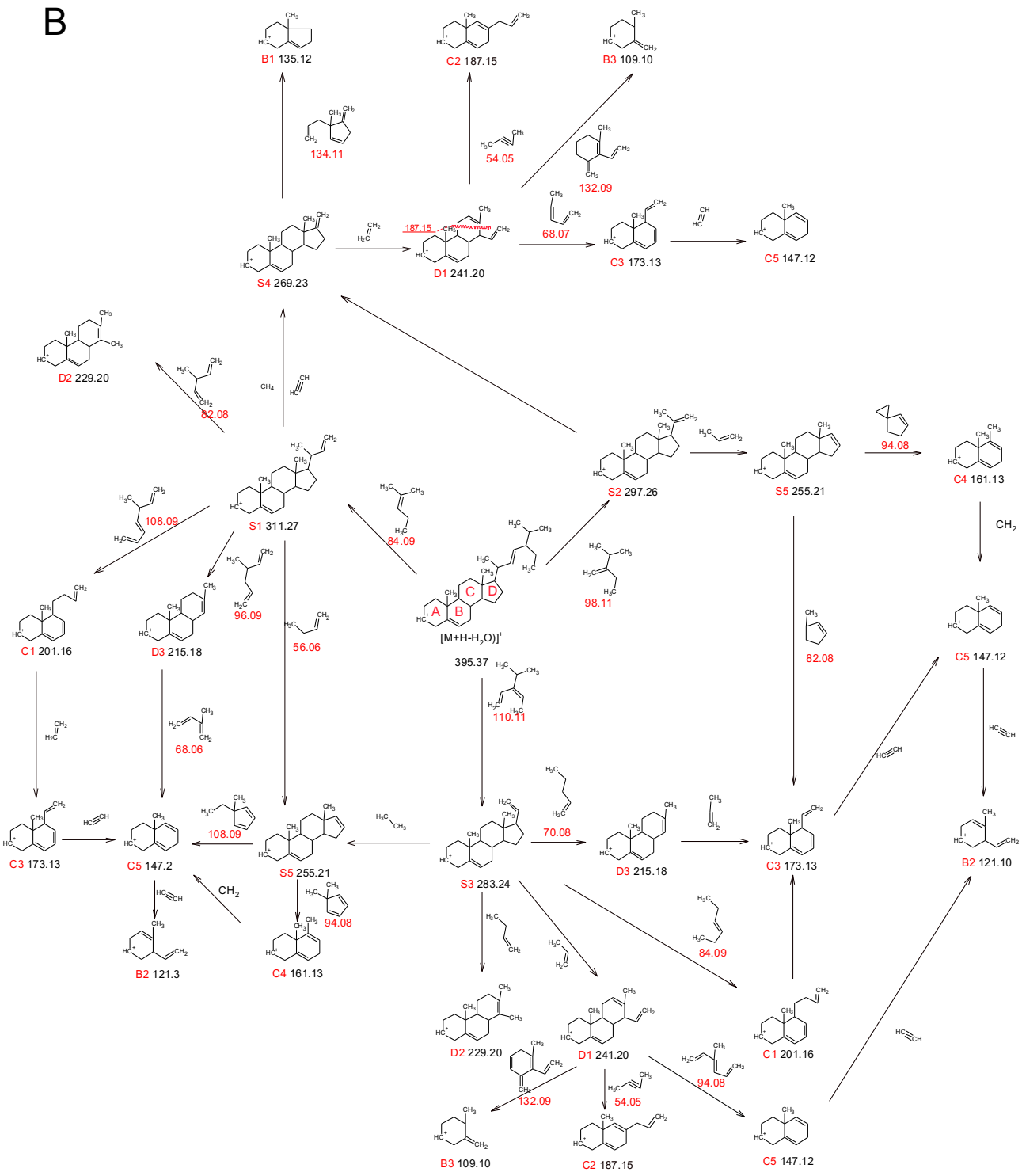
The third dissociation mechanism involves the formation of ion S3 at *m/z* 285.26 that is generated from the cleavages of bonds C20-C22 of the side chain. Subsequently, S3 yields five different product ions (S5, C1, D1, D2 and D3, Scheme 2.2B), which is supported by MS^3 analysis (Appendix A-Table 1). D1 at *m/z* 241.20 is produced by inner-ring cleavage of ring D at the C-17 position. The subsequent dissociation within ring C of ion D1 produces two ions at *m/z* 187.15 (C2) and 147.12 (C5). The former is formed via the loss of two ethyne moieties while the latter is formed due to a retro-Diels–Alder reaction which is the typical mechanism that is responsible for the cleavage of the cyclohexene rings. Furthermore, inner cleavage of ring B yields the ion designated as B3 at *m/z* 109.10. All the dissociation pathways and ion structures proposed in Scheme 2.2B

were supported by MS^3 analysis (Appendix A-Table 1) and exact mass MS analysis (Appendix A-Table 2).

Table 2.2. The common product ions and corresponding fragmentation sites related to the ring structure (Fragmentation on side chain is not included).

Precursor ion $[M+H-H_2O]^+$	Product ions	Dissociation sites
	B1	2, 3 & 12
	B2	3 & 10
	B3	3 & 11
	C1	1, 2 & 13
	C2	4, 9 & 13
	C3	2 & 7
	C4	2 & 8
	C5	2 & 9
	D1	1 & 4
	D2	1 & 5
	D3	1 & 6


A
Relative Abundance
m/z
83.09
81.07
67.05
95.09
B3
121.10
B2
135.12
B1
147.12
C5
161.13
C4
173.13
C3
201.16
C2
215.18
C1
D3
241.19
D2
255.21
D1
269.23
S5
283.24
S4
297.26
S3
311.27
S2
395.37
S1

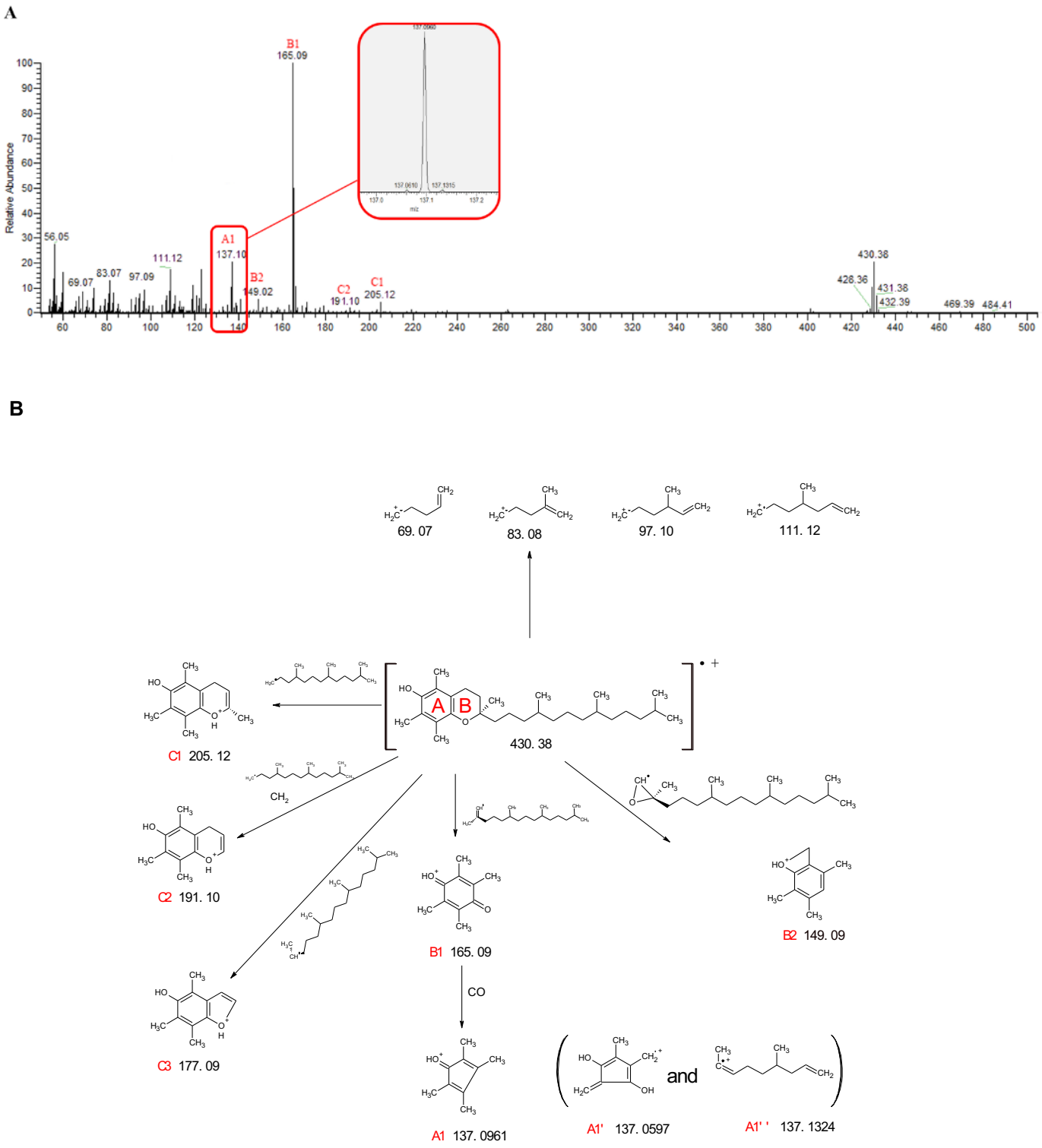


Scheme 2.2. A. MS/MS spectra of stigmasterol; B. the proposed fragmentation pathway showing the genesis of the various product ions as confirmed by MS^3 analysis.

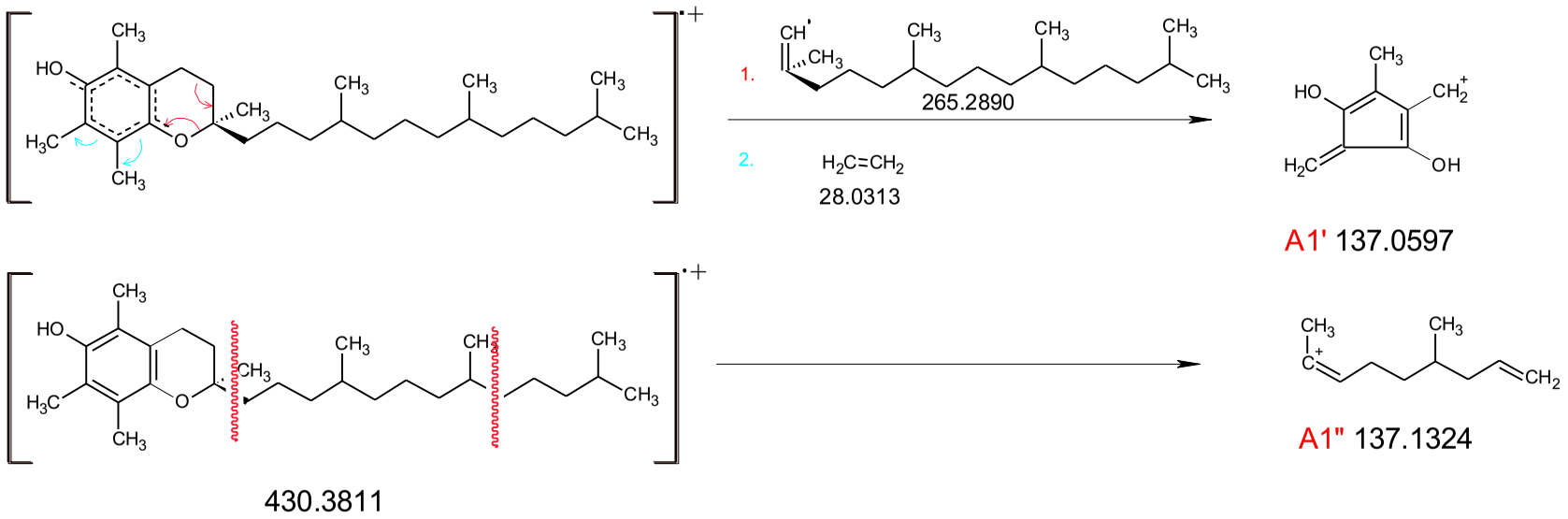
The detailed MS/MS fragmentation pattern of each tested phytosterols, along with the spectrum of all tested phytosterols can be found in Appendix B.

2.3.2.2. MS/MS analysis of tocopherols

α-tocopherol has been used as a representative example to illustrate the CID-MS/MS behavior of tocopherols (Scheme 2.3A). The fragmentation pathway for the tocopherol molecular ion $[M]^{\cdot+}$ at *m/z* 430.38 is shown in Scheme 2.3B. It should be noted that both the protonated and the molecular ions are observed during MS analysis of tocopherols. The protonated ion was dominant during direct infusion (10 µL/min), while the molecular ion was dominant when HPLC-MS conditions (800 µL/min) either through FIA or when tee-split flow was employed. This observation was consistent and it was linked to the flow rate of the mobile phase. Varying flow rates i.e. 0.05, 0.1, 0.15, 0.2, 0.5 and 0.8 mL/min were investigated on their influence on the two competing ionization mechanisms for tocopherols. It was found that, with an increase of mobile phase flow rate, the molecular ion became the dominant ion. Both the protonated and molecular ions were present at equal intensities at 100 µL/min, while the protonated ion was favored at lower flow rates and the molecular ion at higher than 100 µL/min flow rates. Both the protonated and molecular ions were reported for MS/MS analysis in different studies [101, 126]. However, both ions observed at *m/z* 430 and 431 showed identical spectra during MS/MS analysis. Currently, we have no explanation for the observed ionization phenomenon.



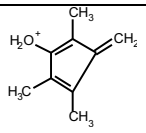
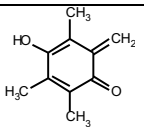
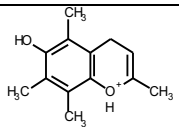
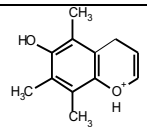
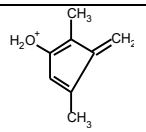
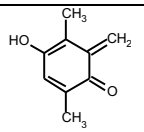
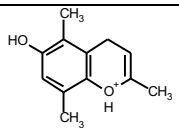
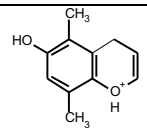
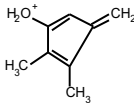
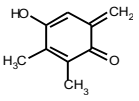
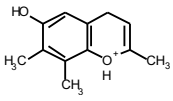
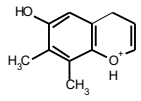
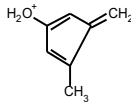
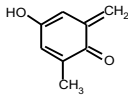
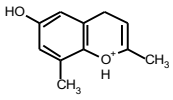
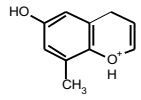
Scheme 2.3. A. MS/MS of α-tocopherol; B. the proposed dissociation behavior, showing the genesis of the various product ions as confirmed by MS^3 analysis.



Scheme 2.4. The proposed fragmentation mechanism of the ions designated as A1' and A1".

In the present work, the molecular ion was chosen for MS/MS analysis since the PIS/MIM profiling (discussed in Chapter 3) will be performed using a mobile phase flow rate at 800 μL/min. The CID-MS/MS showed an abundant ion B1 at *m/z* 165 due to a retro-Diels−Alder reaction [187] and a minor product ion C1 at *m/z* 205 due to an α-cleavage at site 2-1'(The numbers represent the IUPAC numbering as shown in Scheme 1.1B). The molecular ion of tocopherol dissociates via inner ring fragmentation at the sites 1-2 and 3-4 (Scheme 1.1) with rearrangements to form the highly conjugated stable ion B1 observed at *m/z* 165. Further dissociation of B1 at *m/z* 165 was verified by MS^3 analysis (data not shown), resulting in the formation of an abundant ion A1 at *m/z* 137 through the loss of a CO. However, several structures can be assigned to A1 ion based on the Qq-LIT-MS/MS data. To assign the appropriate structure, accurate mass measurement was employed. High resolution MS/MS analysis showed the product ion at *m/z* 137.0960 confirming its ring structure as shown in Scheme 2.3B. Interestingly, careful examination of the MS/MS high resolution spectra showed additional minor ions at *m/z* 137.1315 (A1', $C_8H_9O_2^+$) and 137.0605(A1'', $C_{10}H_{17}^+$) (Scheme 2.3A). These product ions are different from the abundant ion at *m/z* 137.0961 (A1, $C_9H_{13}O^+$), an observation which indicates that additional product ions were also formed but in a low abundance (Scheme 2.4). A1 formation is probably favored to its highly conjugated structure in which the charge is eventually localized at the electronegative oxygen (Scheme 2.3). The same mechanism drives the formation of the ion observed at m/z 177, which appears in beta-, gamma-, and delta-tocopherol and the ions at m/z 123, 150, and 191, which appear in beta- and gamma-tocopherols. These ions represent similar ions, generated from various precursor ions (Table 2.2).

Table 2.3. The proposed structures of the product ions of observed during MS/MS analysis of tocopherols

	A1	B1	C1	C2
α-tocopherol	 *m/z* 137.20	 *m/z* 164.20	 *m/z* 205.27	 *m/z* 191.25
β-tocopherol	 *m/z* 123.17	 *m/z* 150.17	 *m/z* 191.25	 *m/z* 177.22
γ-tocopherol	 *m/z* 123.17	 *m/z* 150.17	 *m/z* 191.25	 *m/z* 177.22
δ-tocopherol	 *m/z* 109.15	 *m/z* 136.15	 *m/z* 177.22	 *m/z* 163.19

The need for high resolution MS/MS data was also evident when rationalizing the structures of the product ions observed at *m/z* values below 100. Product ions below *m/z* 100 can theoretically be formed either from the dissociation of chroman ring or the side chain. For example, $C_5H_6O^+$ (ring) and $C_6H_{11}^+$ (side chain) have the same *m/z* value at 83. However, high-resolution MS/MS analysis showed that the ions with exact *m/z* values of 69.0705, 83.0860 and 97.1015 are subsequent losses of CH2 moieties. Such observation confirmed that these product ions are generated from the carbon side chain with a $C_xH_y^+$ formula.

As a final note, β- and γ- isomers cannot be distinguished from their MS/MS data since the only difference between the two isomers is the position of the methyl groups on the chroman ring (Scheme 2.1B) and as such, they share the same MS/MS fragmentation pattern. The detailed MS/MS fragmentation patterns of other tocopherols and their associated spectra can be found in Appendix B.

2.4. Conclusion

In this study, the MS/MS dissociation behavior of four phytosterols and four tocopherols were discussed. To accomplish this task, the ionization behavior of phytosterols and tocopherols were studied using both ESI and APCI ionization techniques. APCI was chosen for further analysis as it gave better ionization for these compounds. Significant $[M+H-H_2O]^+$ peaks were detected for phytosterols with APCI. However, both protonated and molecular ions were detected when dealing with tocopherols and this observation was linked to the flow rate of the mobile phase. This ionization behavior of

tocopherols should be taken into account during the development of MS-based qualitative or quantitative analytical methods.

The fragmentation patterns of phytosterols and tocopherols were established and confirmed via MS^3 analysis and HRMS that verified the chemical structures of product ions and the fragmentation pathway. The established MS/MS fingerprints were employed to predict the dissociation behavior of other naturally-existing phytosterols due to their similar structural features and fragmentation behavior among all tested phytosterols and tocopherols, respectively.

The MS/MS behavior of phytosterols and tocopherols is currently being used to develop a targeted LC-MRM-MS/MS quantification method which will be reported upon completion. With the use of diagnostic product ions, the selectivity of quantification can be ensured especially when dealing with complex matrices.

CHAPTER 3

THE DEVELOPMENT OF TARGETED PROFILING STRATEGIES IN VEGETABLE OILS

This chapter focus on the development of targeted profiling strategies of phytosterols and tocopherols in vegetable oils. The work presented in this chapter is also included in the peer-review research article cited in the disclaimer above.

3.1. Introduction

Based on the generalized MS/MS fragmentation behavior of phytosterols, diagnostic product ions were chosen for the development of profiling methods for over 20 naturally-occurring phytosterols. Precursor ion scan-triggered enhanced product ion scan (PIS-EPI) methods were established. Due to enhanced single intensity, multiple ion monitoring-triggered enhanced product ion scan (MIM-EPI) was employed for confirmation.

Vegetable oils are one of the major sources of nutrients in the human diets since they are a daily food component. However, characterization of vegetable oils has been focused only on the principal components, for example, triacylglycerol[188, 189]. In recent years, the recognition of minor components that generally constitute the unsaponifiable matter has received more attention. These components include important bioactives that affect the nutritional quality of individual oils.

Phytosterols and tocopherols are two important building blocks of the unsaponifiable matter. Phytosterols often exist as a mixture of different sterol compounds sharing similar skeletal structure. There are several phytosterols reported existing in different vegetable oils, such as β-sitosterol, campesterol, brassicasterol and stigmasterol [173] (Scheme 1.1A). The composition of phytosterols varies among different plant species, which could be used for testing adulteration. For example, the phytosterol composition of hazelnut oil has been successfully used as a detection tool for adulteration of olive oil with hazelnut oil [190]. While tocopherols are natural antioxidants frequently occurring in plants and comprise of four homologues (Scheme 1.1B). The composition and content of tocopherols also vary among different plant species. Hence, it's crucial to include profiling of phytosterols and tocopherols content in the detailed characterization of vegetable oils.

To effectively analyze phytosterols and tocopherols in vegetable oils, comprehensive analytical strategies need to be developed. Liquid chromatography-tandem mass spectrometry (LC-MS/MS), either with an electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI), is commonly used for the identification and quantification of phytosterols and tocopherols in different biological samples [104, 143, 162]. Here we report the profiling methods for the phytosterol and tocopherol content using LC-MS/MS. This part of work is based on the MS/MS behavior analysis of major phytosterols and tocopherols.

The screening approach was applied successfully to identify blinded samples obtained from standard mixtures as well as sesame and olive oils. The oil samples contain other phytosterols and their successful identification indicates that, the generalized MS/MS fragmentation behavior is applicable to various structures of phytosterols. Similar approach was attempted for tocopherols and was only hindered by the low concentration of these bioactive metabolites within some oil samples.

3.2. Materials and Methods

3.2.1. Samples and Reagents

All solvents were of LC-MS grade and all chemicals were of analytical reagent grade, purchased from Fisher Scientific (Pittsburg, PA, USA).

Olive oil (Organic Extra Virgin, Terra Delyssa®) and sesame oil (Baraka®) were obtained from a local store while canola oil deodorizer distillate (CODD) was a gift from LDM foods (Yorkton, Saskatchewan, Canada). β-Sitosterol, campesterol, stigmasterol, and brassicasterol each at 98% purity were purchased from Toronto Research Chemicals

(Ontario, Canada) while α-tocopherol (99.9%), γ-tocopherol (96.8%), and δ-tocopherol (94%), were purchased from Sigma Aldrich (Canada).

3.2.2. Sample preparation

Stock solutions of phytosterols and tocopherols standards were prepared at 1 mg/mL in chloroform and stored at –20°C. For MS, MS/MS, and second-generation MS (MS^3) analysis, each stock solution was further diluted to 5 µg/mL with acetonitrile containing 0.01% acetic acid.

For olive and sesame oil, the unsaponifiable matter was analyzed. The extraction was done as reported with some modifications [108, 191, 192]. Briefly, 5 g of each oil was saponified with 1M Potassium hydroxide (KOH) prepared in 95% ethanol for 1 h at 65 °C. This was followed by the addition of 50 mL water and the unsaponifiable matter was extracted three times with 50 mL hexane. The combined organic phase was washed with water until the washings were neutral to phenolphthalein and dried under anhydrous sodium sulfate. The solvent was then evaporated on a rotovap and the residue further dried under high vacuum.

For CODD, the phytosterols were isolated from the unsaponifiable matter as follows: CODD (5 g) was saponified as described above after which water was added to precipitate phytosterols. Vacuum filtration was performed and the residue was washed before drying under vacuum. For MS analysis, approximately 5 mg of each sample (extracts from olive, sesame, and CODD) were dissolved in chloroform and further diluted to the required concentration with acetonitrile containing 0.01% acetic acid.

3.2.3. Precursor ion scan (PIS)- or multiple ion monitoring (MIM)- triggered enhanced product ion scan (EPI)

IDA methods were performed using AB SCIEX 6500 QTRAP® quadrupole-linear ion trap mass spectrometer (Qq-LIT-MS), equipped with an APCI source (AB Sciex, Concord, ON, CA). The instrument was operated in the positive ion mode with a declustering potential (DP) of 40V and vaporization temperature of 400 ℃. The various MS parameters are shown in Table 3.1.

Table 3.1. MS parameters during ionization

MS Parameter	Value
Nebulizer current	2.5 µA
Curtain gas	30 psi
Source temperature	400 °C
Declustering potential	40V

PIS was employed for screening phytosterols and tocopherols using the data gathered from MS/MS analysis while MIM was employed for confirmation due to better MS/MS signal in the EPI mode.

PIS- and MIM-EPI were carried out after HPLC separation of the compounds on an Agilent 1290 UHPLC system (Agilent, Santa Clara, CA, USA). The analytical column was an Agilent Poroshell C18 column (2.1 mm × 150 mm, 5µm) in series with a guard column (2.1 mm × 4.7 mm, 2.7 µm) of the same packing material. An isocratic elution consisting of acetonitrile: methanol (99:1 v/v) with 0.1% acetic acid was used at a flow rate of 800 µL/min. The column temperature was set at 30^{o}C and the injection volume was 3 µL.

The parameters used for PIS were similar to those already applied in MS/MS analysis. The product ions observed for the tested compounds were used as the product ions for PIS while the scan range was set from *m/z* 350 to 450. The DP was set at 80 V, and the collision energy (CE) was at 25 eV. The threshold for information-dependent acquisition (IDA) triggered for the EPI was set at 50,000 ion counts. For EPI, DP was set at 80 V, and the CE was set at 30 eV to induce detailed MS/MS spectrum.

A MIM-EPI scan was adopted to confirm and acquire better MS/MS signal for target compounds. The MIM scan is based on a multiple reaction monitoring (MRM) mode in

triple-quadruple MS instruments. Unlike in MRM, the MIM scan was carried out targeting the same ions in Q1 and Q3, respectively, with the minimal CE (5 eV) in the collision cell [169]. The threshold for IDA triggered for the EPI acquisition was set at 100,000 ion counts for phytosterols. Each MIM transition was monitored with a 50 ms dwell time, DP of 80 V, and a CE of 30 eV. For tocopherols, the threshold for IDA trigger for the EPI acquisition was set at 50,000 ion counts and the CE was 40 eV. Other scanning conditions are summarized in table 3.2.

Table 3.2 MIM-EPI scanning conditions for the analysis of phytosterols and tocopherols

Compound		m/z range		CE (CES)
		Q1	Q3	
β-sitosterol	MIM	397	397	5
	EPI		50-450	25(10)
campesterol	MIM	383	383	5
	EPI		50-450	25(10)
brassicasterol	MIM	381	381	5
	EPI		50-450	25(10)
stigmasterol	MIM	395	395	5
	EPI		50-450	25(10)
α-tocopherol	MIM	430	430	5
	EPI		50-450	30(10)
β-tocopherol	MIM	416	416	5
	EPI		50-450	35(10)
γ-tocopherol	MIM	416	416	5
	EPI		50-450	35(10)
δ-tocopherol	MIM	402	402	5
	EPI		50-450	40(10)

PIS-EPI and MIM-EPI methods were then applied to blinded samples composed of a mixture of reference phytosterols standards and unsaponifiable matter extracted from olive and sesame oils to confirm the capability and reliability of the method.

3.3. Results and discussion

3.3.1. Identification of phytosterols in CODD extraction

Among the numerous product ions observed, four common product ions, B3, C5, C4 and D3 at *m/z* 109, 147, 161 and 215, were chosen for PIS, for the following reasons: (1) the product ion showed abundant signal during MS/MS analysis; (2) they were common in the four tested phytosterols (Table 3.3); and (3) the structures make them characteristic to phytosterols, allowing better selectivity in screening. Two other product ions, S2 and S2' at *m/z* 297 and 301 (Appendix A-table 3) were also selected as an indicator of the presence or absence of double bond on the site C22-C23 of the side chain.

Table 3.3. Summary of product ions of tested phytosterols

	Stigmasterol	Brassicasterol	β-Sitosterol	Campesterol
S1	√	√	×	×
S2	√	√	×	×
S3	√	√	×	×
S4	√	×	×	×
S5	√	√	×	×
D1	√	√	×	×
D2	√	√	√	√
D3	√	√	√	√
C1	√	√	×	×
C2	√	√	×	×
C3	√	√	×	×
C4	√	√	√	√
C5	√	√	√	√
B1	√	√	√	√
B2	√	√	×	×
B3	√	√	√	√

The various phytosterols in tested CODD should yield common product ions (listed in Appendix A-table 4). Therefore, these compounds can be detected through PIS method that is designed based on the fragmentation pattern of the four tested phytosterol standards. Information about the reported phytosterols content in sesame and olive oils is summarized in Appendix A-table 5 [24, 102, 114]. All metabolites yielding common product ions at *m/z* 109, 147, 161 and 215 in MS/MS were considered as potential phytosterols. Information about the relevant compounds, such as retention time, precursor ions *m/z* value, and major fragment ions, is summarized in Table 3.4. In fact, all phytosterols could share the common fragmentation pattern with the tested phytosterols during CID-MS/MS, due to their similarity in structure. Furthermore, the specific structures of these compounds can be deduced via the analysis of their MS/MS spectra.

Table 3.4. Phytosterols detected and Structural Characterization in CODD via PIS using HPLC-MS/MS

RT*(min)	Identification	MW (Da)	Precursor ion (m/z)	Major product ions (m/z)
4.1	brassicasterol	398.68	381.4	69, 147, 161, 255, 297
5.0	campesterol	400.69	383.4	135, 147, 161, 301
5.8	β-sitosterol	414.72	397.4	135, 147, 161, 297

* Retention time

A PIS-EPI scan was also employed, showing three major chromatographic peaks whose signal strength varied substantially (Figure 3.1A). The variation may be due to the fact that the collision energy was not optimized for the various phytosterols existing in oils. Therefore, a multiple ion monitoring-triggered enhanced product ion scan (MIM-EPI)

method was adopted to profile and acquire MS/MS for the targeted compounds. Compared to PIS-EPI, MIM-EPI displayed a better profiling performance with stronger signal for each possible phytosterol and a more detailed MS/MS spectrum (Figure 3.1B). This is due to MIM narrowed ion scan range in Q1, which helps acquire more data points. MIM-EPI is reported to be a powerful tool for metabolite profiling, especially useful for identifying metabolites with low concentrations [193, 194].

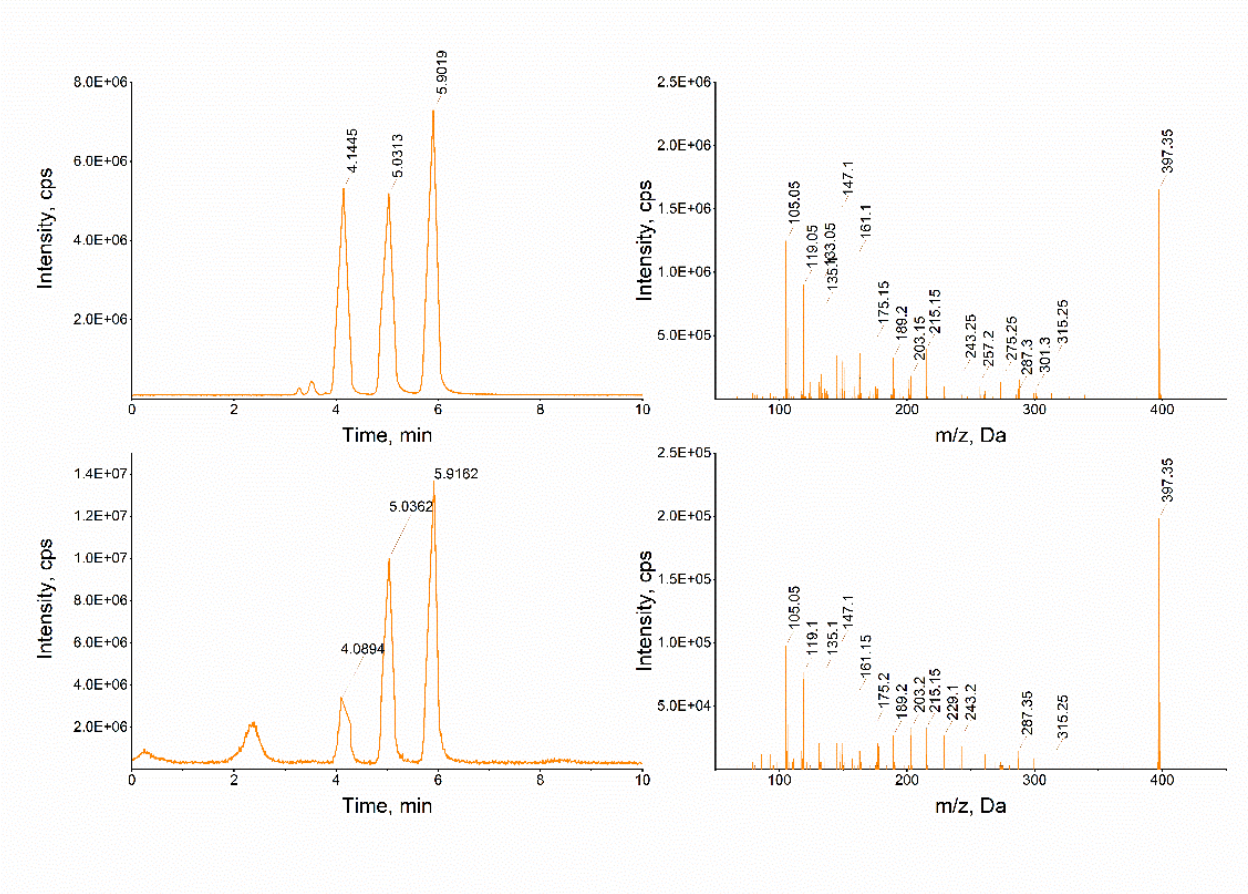


Figure 3.1. Comparison of PIS-EPI and MIM-EPI in profiling of phytosterols and MS/MS data for stigmasterol. Profiling data obtained by PIS-EPI (A1) and MIM-EPI(A2); MS/MS spectrum of stigmasterol from PIS-EPI (B1) and MIM-EPI (B2).

For tocopherols, only MIM-EPI scan was conducted, since only 4 forms exist in nature. Therefore, tocopherols can be detected through MIM scan method easily as only four transitions are needed to be monitored.

The profiling method was then applied to the unsaponifiable matter of CODD. At least three tocopherols were detected in the sample (Figure 3.2). However, the retention time and *m/z* value of β and γ-tocopherols are identical and cannot be differentiated (co-elute at 3.48 minute).

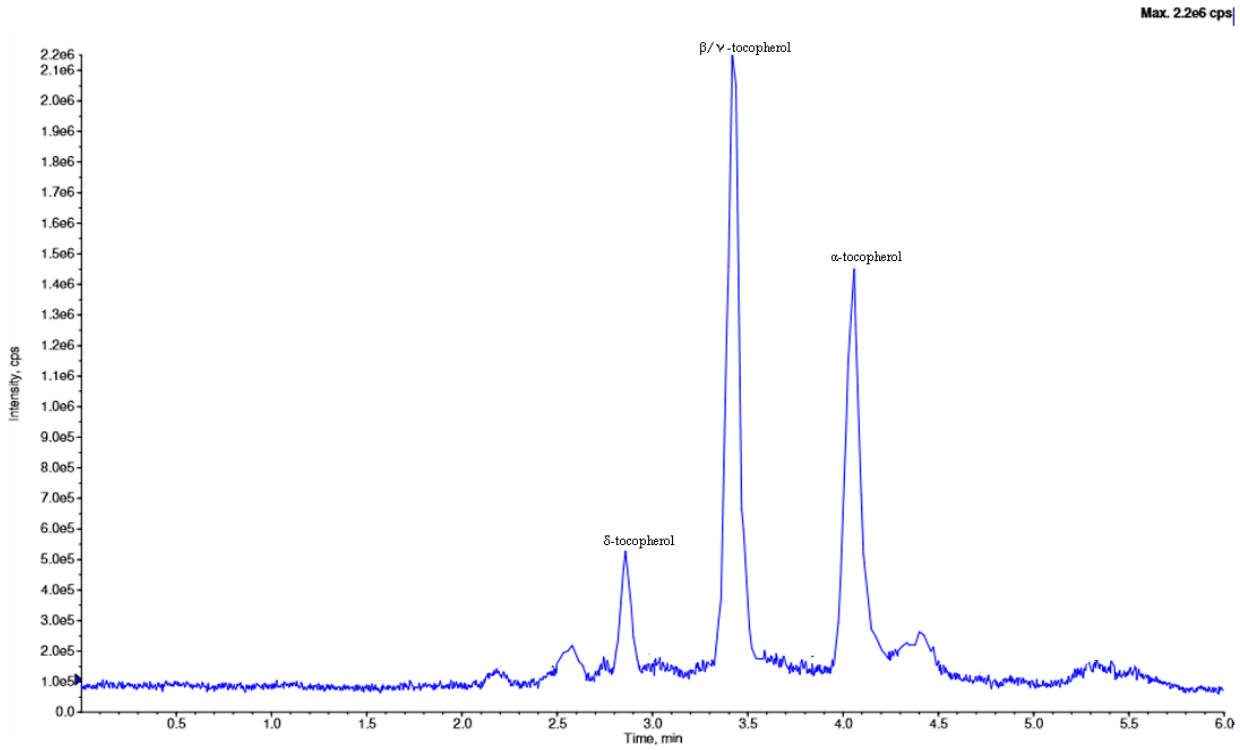


Figure 3.2. Total ion current (TIC) chromatogram of MIM-EPI scan for tocopherols in CODD.

A combined MIM-EPI method detecting both phytosterols and tocopherols on the same run was also conducted. The phytosterols are dominant due to their high concentrations (Figure 3.3A). However, tocopherols can still be identified (Figure 3.3B). This experiment has shown the utility of the developed method to simultaneously screening for both phytosterols and tocopherols within one sample.

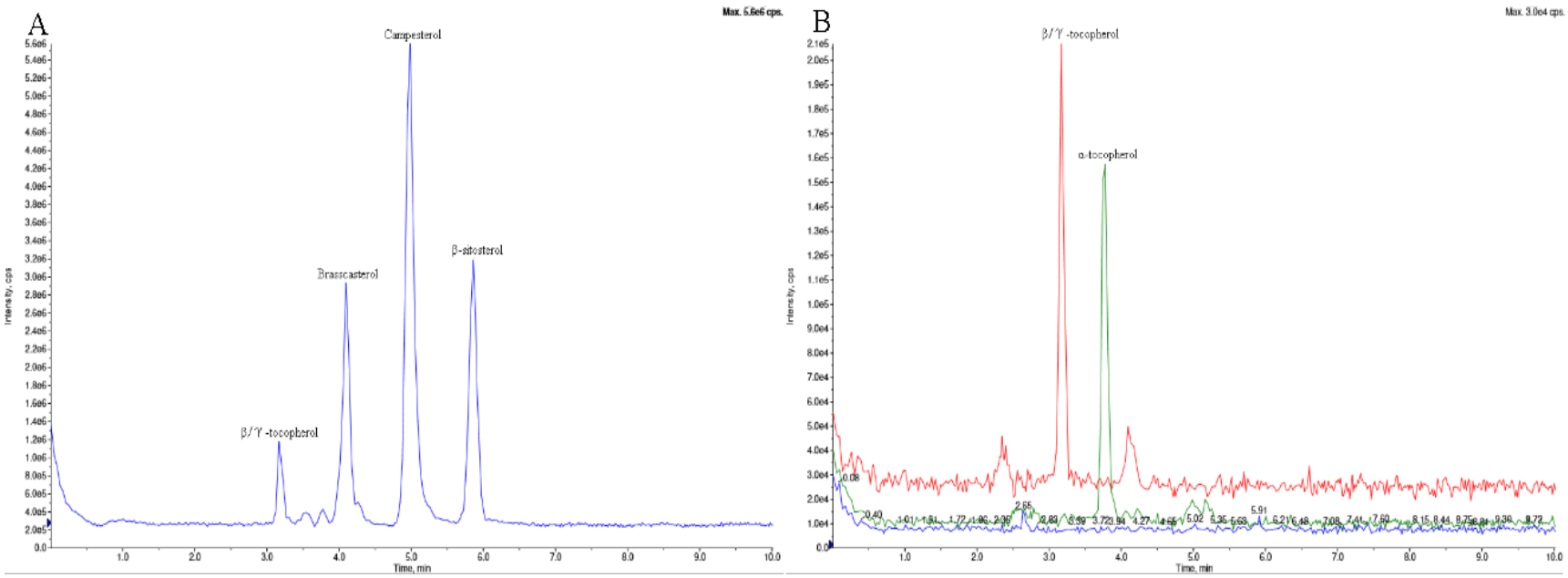


Figure 3.3 A. Tatogram of MIM-EPI scan for phytosterols and tocopherols in CODD; B. Extracted-ion chromatogram (XIC) of tocopherols (Red: transition 416→416, green: transition 430→430, blue: transition 403→403)

3.3.2. Identification of phytosterols and tocopherols in the unsaponifiable matter of vegetable oils

Various phytosterols, reaching up to 16, have been reported as component of vegetable oils [114, 195-198]. The developed generalized MS/MS fragmentation behavior (Table 3.3, Scheme 2.3B) can theoretically be applied to other naturally-existing phytosterol structures.

To test the suitability of the scanning method, a blinded experiment was conducted. The operator (First author, Jiang, K.) was not aware of the content of the five samples prepared by co-author, Gachumi, G. Upon analysis, three samples which are mixtures of different standard compounds were successfully identified. The other two are phytosterols extracted from sesame and olive oils (Figure 3.4). The identification was based on the presence and intensity of the ions as well as the retention time of the observed peaks. Specifically, the relative intensity of campesterol is much higher than avenasterol in sesame oil according to published reports (Appendix A-table 5). While the relative intensity of campesterol, and Avenasterol is similar in olive oil. On the other hand, both δ5 and δ7-avenasterol are reported to be present in sesame oil, while only δ5-avenasterol in olive oil [199-203]. Thus, sample 4 was successfully identified as an extract from olive oil (Figure 3.4A), while sample 5 was from sesame oil (Figure 3.4B). It is necessary to indicate that the precursor ion in one specific *m/z* may be generated from different isomers, yielding the same MS/MS spectra. For example, avenasterol share the same *m/z* at 395 with stigmasterol, because the only difference of these two phytosterols is the position of the double bond on the side chain. However, the chromatographic retention times in the reverse-phase column

vary among these structures according to published studies [204, 205]. Therefore, both compounds were identified using retention time along with *m/z* values. Tocopherols were, however, not detected in these oil samples (data not shown). This could be due to their reported low concentrations in edible oils [200, 202].

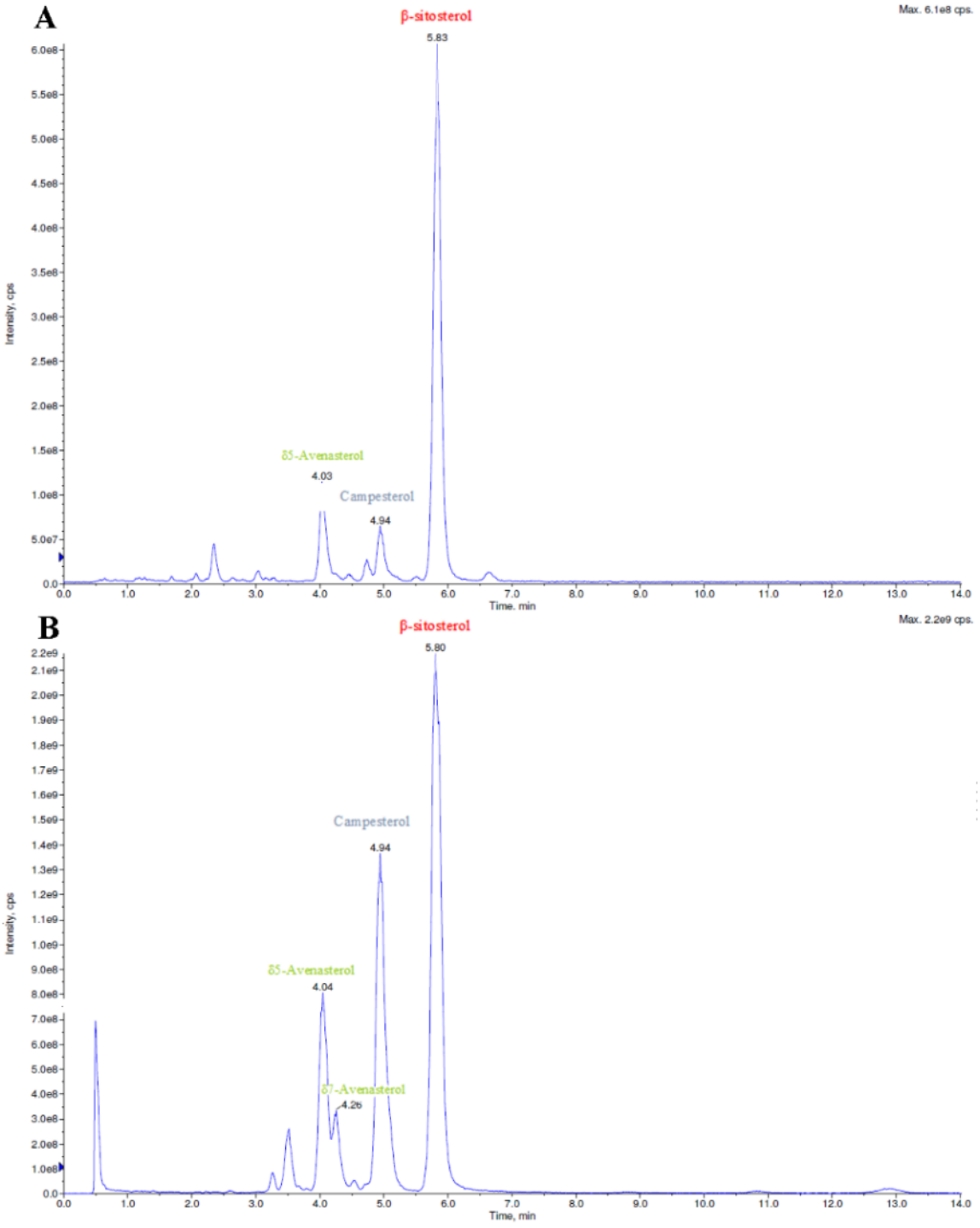


Figure 3.4. Comparison of precursor ion scan spectra of phytosterols profiling in olive oil (A) and sesame oil (B).

3.4. Conclusion

This study has clearly demonstrated how the analysis of two main constituents of the unsaponifiable matter, namely, phytosterols and tocopherols, is an important part of detailed characterization of vegetable oils. Qualitative profiling MS-based methods were developed based on the MS/MS dissociation behaviors. The qualitative approach combined PIS and MIM-EPI which enhances the identification capability of quadrupole-linear ion trap instrument in metabolite analysis. Both olive and sesame oils have been shown to be a possible source of β-sitosterol, campesterol, and avenasterol. However, this study was only able to qualitatively characterize phytosterols that constitute the bulk of the unsaponifiable matter in the tested vegetable oils. As being characteristic of the oil content, quantitative analysis of phytosterols is still needed.

The utility of the new profiling method was demonstrated in the identification of the phytosterols and tocopherols present in CODD extraction. Three phytosterols, namely brasscasterol, campesterol and β-sitosterol, and at least three tocopherols were detected in the samples. The result has revealed the CODD's potential as a rich source of phytosterols and tocopherols.

CHAPTER 4

OVERALL DISCUSSION AND FUTURE DIRECTION

4.1. Discussion

Qualitative analysis is an integral part of this thesis. Both phytosterols and tocopherols produced complex MS/MS spectra, and little has been done to fully characterize the numerous product ions observed during MS/MS analysis. In this work, the ionization behavior of phytosterols and tocopherols are tested. The universal CID-MS/MS behavior of phytosterol (as $[M+H-H_2O]^+$) and tocopherol (as $[M]^{\cdot+}$) are established, generating generalized MS/MS fingerprints. Such comprehensive analysis allowed for the efficient profiling of new structures of these compounds as well as developing targeted analysis methods. The qualitative approach combined PIS and MIM-EPI was employed for MS-based profiling in both oil extract and CODD samples. Meanwhile, a quantitative method was developed by other research members [out the scope of the thesis], utilizing the MS/MS data generated in this thesis.

4.1.1. Mass spectrometric analysis of phytosterols and tocopherols

High resolution MS single stage analysis is initially performed for the identification of phytosterols and tocopherols. $[M+H]^+$ was either not observed or observed in relatively low abundance. On the other hand, the $[M+H-H_2O]^+$ and $[M]^{\cdot+}$ species were dominantly observed in the single stage positive ion mode of APCI-MS analysis. Observed mass accuracies were less than 3 ppm for all tested compounds (Table 2.1). Other species, such as $[M+H-H_2]^+$ and $[M+H-2H_2]^+$ for phytosterols and $[M+H]^+$ for tocopherols were also observed, albeit at lower abundance.

MS/MS dissociation behaviors were subsequently elaborated by investigating the fragmentation behavior employing MS/MS and MS^3 analysis. Diagnostic product ions were successfully identified, and their proposed structures were confirmed via MS^3 analysis and high-resolution MS. These diagnostic ions can be used for qualitative and quantitative analysis of the tested as well as related compounds. Similar CID-MS/MS dissociation behavior was observed with all tested phytosterols and tocopherols. Thus, a general fragmentation pattern of phytosterols (Scheme 2.2) and tocopherols (Scheme 2.3) were, for the first time, established. Despite the existence of published work with structural assignments for some of the observed product ions [102, 206], only few structures of the product ions were reported. The exact structures and possible fragmentation mechanisms, such as the formation of double bond or cyclization were not discussed or elucidated before, to the best of my knowledge.

Despite the generation of a generalized MS/MS fragmentation pattern for phytosterols, changes of product ions were observed within individual phytosterols that are related to the double bond within the side chain. However, side chain fragmentation cannot generate characteristic product ions but might affect the observed ion intensity of product ions with low *m/z* values (*m/z* < 100). For example, the relative abundance of product ion with *m/z* 83.09 is much higher in the MS/MS spectrum of phytosterols with double bond on the side chain (e.g. stigmasterol) in comparison to phytosterols with saturated side chain. The majority of the observed product ions are presented in Table 2.2.

In the case of tocopherols, the same mechanism drives the formation of all observed product ions. For example, the most abundant product ions at *m/z* 165, 151, 151 and 123 for the α-, β-, γ-, and δ-tocopherols were observed (Scheme 2.3, Table 2.3). These ions are

generated through the same fragmentation mechanism on the chromane ring. The different *m/z* values is due to different number and positions of methyl groups on the chromane ring. It is worth emphasizing that all tocopherols share the same side chain. The product ions generated from the alkyl side chain effected the analysis of fragmentation in the chromane part. HRMS allowed for the confirmation of the proposed structures of the observed product ions (for example, product ions A1, A1' and A", Scheme 2.3A, Scheme 2.4).

The established MS/MS pathways of the group of compounds tested were further confirmed via MS^3 analysis and the molecular formula of the reported product ions was also confirmed with MS/MS conducted with HRMS.

In summary, universal MS/MS dissociation behaviors of phytosterols and tocopherols were established. The data will serve as a foundation for metabolite profiling of phytosterols and tocopherols and as a base for quantitative methods that employ the MRM mode.

4.1.2. Metabolites profiling of phytosterols and tocopherols

The established MS/MS dissociation data of phytosterols and tocopherols were then utilized to develop LC-MS-based screening strategies. Profiling of phytosterols and tocopherols is commonly conducted using GC-MS. Mitei et al. [25] have reported the use of GC–MS and HPLC for the profiling of phytosterols and tocopherols content of selected seed oils from Botswana. However, the phytosterol profiling part was still conducted by GC-MS [25]. Millan et al. [139] reported the use of LC-APCI-HRMS for a targeted metabolomics study of grapes based on their phytosterol contents. The authors achieved accuracy in discriminating between grape varieties based on phytosterol contents.

However, with HRMS, some unknown phytosterols with similar structures can be overlooked.

In my research, a qualitative approach combining PIS and MIM-EPI enhanced the identification capability of quadrupole-linear ion trap instrument. Utilizing the data gathered from MS/MS, PIS-MS method was successfully developed for screening phytosterols and tocopherols. MIM was then employed for confirmation due to better MS/MS signal in the EPI mode. The developed strategy proved efficient for conducting profiling experiments and can aid in identifying new structures. As such, the phytosterol profiles for olive and sesame oils were clearly distinguish (Figure 3.4). A combined MIM-EPI method detecting both phytosterols and tocopherols on the same run was also conducted. This experiment has shown the possibility and utility of the developed method to simultaneously screen both phytosterols and tocopherols within an oil sample. However, tocopherols were detected in CODD extract sample but not in oil samples. This is due to the low concentrations of tocopherols within edible oils. In addition, the extraction method is optimized for phytosterols, so the signal of tocopherols is possibly suppressed. As such, additional work is needed to improve the sensitivity of the profiling strategy.

4.2. Future direction

4.2.1. Quantitative analysis

The identified diagnostic product ions from the established MS/MS pattern of each phytosterols and tocopherols can be used for the quantification of these compounds in the MRM mode. Quantitative methods can be optimized using the established MS/MS fingerprints. Using unique transitions during the MRM-quantification ensures selectivity

and sensitivity of the method especially when dealing with complex matrices. In fact, information from the MS/MS data acquired in this study has been successfully used to quantify target analytes within CODD as well as pharmaceutical formulations intended for functional foods [207].

4.2.2. Qualitative analysis

As shown in my thesis, MS/MS fingerprints are successfully used for the identification of target compounds in oils and DDs. The qualitative profiling method established in the study has the potential to identify other structures of phytosterols. For example, avenasterol was detected in the profiling method as shown in Figure 3.4. The phytosterol profiles for olive and sesame oils clearly distinguish between the two oils. As such, the profiling strategies, possibly combined with quantitative analysis, can be applied for the analysis of high value oils, such as extra virgin olive oil to detect adulteration with less expensive oils. To achieve that, proper internal standards are needed to establish relative quantification methods.

4.3. Final conclusions

A universal MS/MS dissociation behavior of four phytosterols and four tocopherols were established and confirmed. The data would be very useful in the development of MS-based analytical methods.

The established MS/MS fingerprints were employed to predict the dissociation behavior of other naturally-existing phytosterols due to their similar structural features. The qualitative approach combined PIS and MIM-EPI which enhances the identification

capability of quadrupole-linear ion trap instrument in metabolite analysis. The utility of the new method was then successfully applied for the identification of phytosterols and tocopherols in oil samples (sesame and olive) as well as CODD. The MS/MS behavior was successfully used to develop a targeted LC-MRM-MS/MS quantification method.

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Appendix A

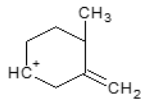
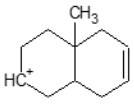
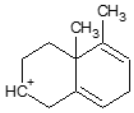
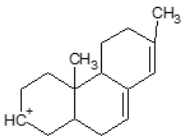
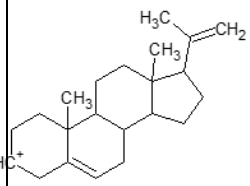
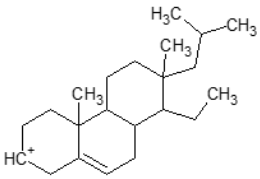
Summary of MS/MS/MS experiment for stigmasterol, using QqQ-LIT.

MS/MS fragment ions of stigmasterol	MS/MS/MS fragment ions
α-tocopherol	⟶ 269.2
	⟶ 255.3 ⟶ 187.2
	⟶ 173.3
	⟶ 161.3
	⟶ 147.2
	⟶ 135.2
	⟶ 121.3
	⟶ 109.3
	⟶ 241.2 ⟶ 187.2
	⟶ 173.3
	⟶ 147.2
	⟶ 135.2
	⟶ 109.3
	⟶ 229.3
	⟶ 215.4 ⟶ 187.2
	⟶ 173.3
	⟶ 147.2
	⟶ 135.2
	⟶ 121.3
	⟶ 109.3
	⟶ 201.2 ⟶ 173.3
	⟶ 121.3
	⟶ 109.3
	⟶ 187.2
	⟶ 161.3
297.3	⟶ 269.2
	⟶ 255.3
	⟶ 241.2
	⟶ 215.4
	⟶ 201.2
	⟶ 187.2
	⟶ 173.3
	⟶ 161.3
	⟶ 147.2
	⟶ 135.2
285.3	⟶ 229.3
	⟶ 215.4
	⟶ 201.2
	⟶ 187.2
	⟶ 173.3
97.2	⟶ 69.1

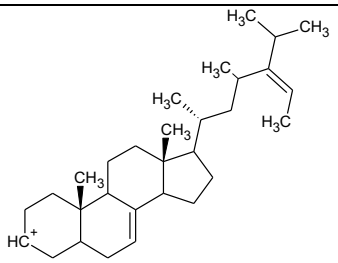
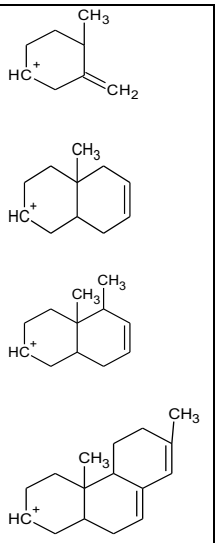
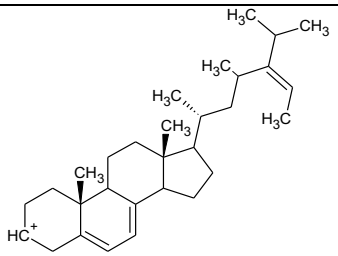
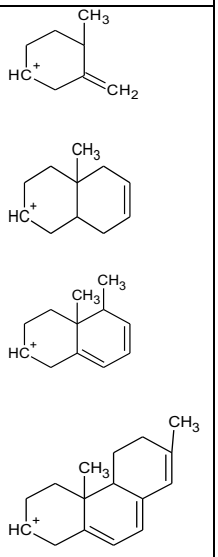
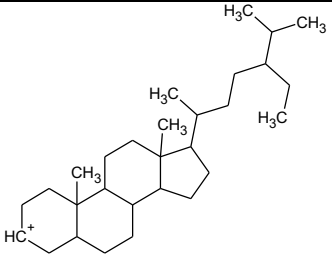
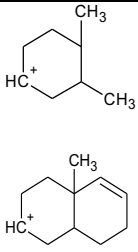
Summary of product ions accuracy for stigmasterol, using Q-exactive.

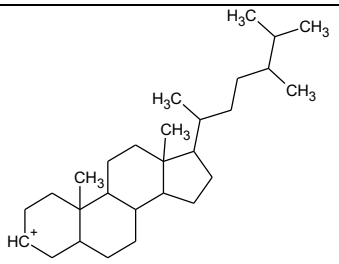
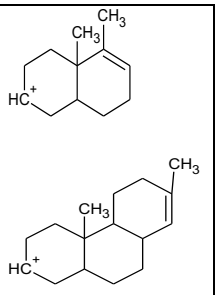
	m/z observed	*m/z* theoretical	Mass accuracy (ppm)
S1	311.2723	311.2733	3.3026
S2	297.2570	297.2577	2.2775
S3	285.2572	285.2577	1.6722
S4	269.2255	269.2264	3.2575
S5	255.2100	255.2107	2.8486
D1	241.1944	241.1951	2.8069
D2	229.1946	229.1951	2.0812
D3	215.1790	215.1794	1.9844
C1	201.1634	201.1638	1.8741
C2	187.1478	187.1481	1.7473
C3	173.1321	173.1324	2.1775
C4	161.1321	161.1325	2.3397
C5	147.1166	147.1168	1.5430
B1	135.1166	135.1168	1.6800
B2	121.1011	121.1012	0.6358
B3	109.1013	109.1012	1.1274

Structures of tested phytosterols abundant product ions.

B2	C4	C5	D3	S2	S2’
109 	149 	161 	215 	297 	301 

Structures of phytosterols present in vegetable oils and the expected structure of the abundant product ions.

Avenasterol		109 149 163 215	
δ7-Stigmasterol		109 149 161 213	
Sitostanol		111 149	

Campestanol		163 217	

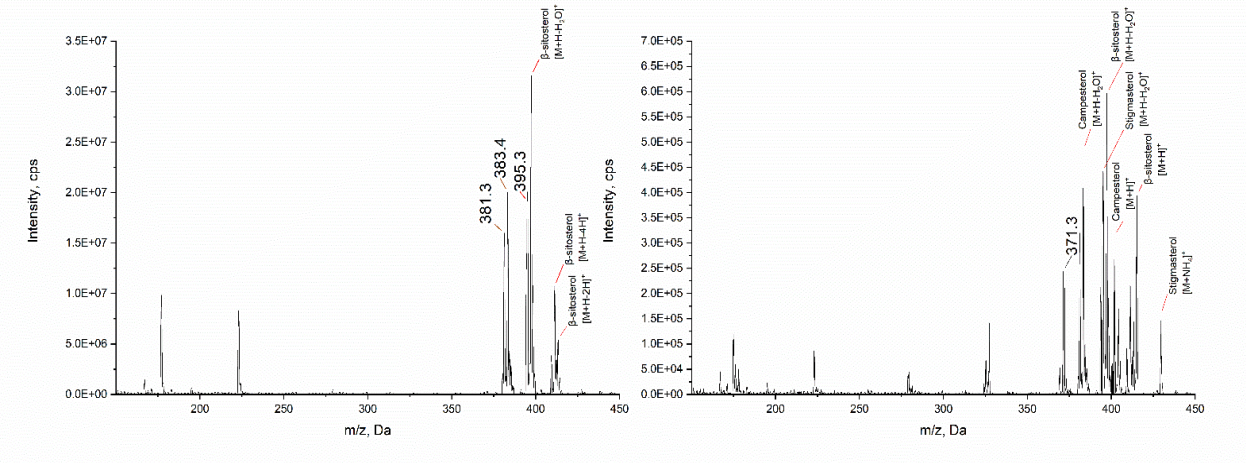
Phytosterols content information from literatures (mg/100g)

*nd=not detected; -- = not reported; LLOQ = Lower Limit of Quantification

		Sitosterol	Campesterol	δ7-Campesterol	Stigmasterol	δ7-stigmasterol	Brassicasterol
Sesame, extra virgin[208]	Free	217.3 ±1.18	36.3 ±0.59	——	24.1 ±0.33	——	nd*
	Esterified	113.9 ±2.23	38.2 ±1.16	——	8.9 ±0.11	——	nd*
Sesame, toasted[208]	Free	221.0 ±1.18	36.3 ±0.59	——	26.9 ±0.33	——	nd*
	Esterified	114.4 ±2.23	40.1 ±1.16	——	9.8 ±0.11	——	nd*
Roasted Sesame Oil[199]		260.5 ± 18.6A	——	——	25.1 ± 2.2A	5.0 ± 0.2A	9.4 ± 0.5A
Crude Sesame Oil[200]		467.7 ± 0.5	13.0 ± 0.2	——	48.1 ± 0.3	6.4 ± 0.1	——
Sesame Oil[201]		337.67±12.89	97.34±12.23	2.93±1.71	32.99±9.71	——	nd*
	toasted	227.41±12.86	96.63±12.81	2.46±1.22	36.42±4.97	4.40±1.79	0.86±0.22
Sesame Oil[202]		310	100	——	36	——	nd*
Sesame Oil[203]		173.78±7.20	45.29±1.45	——	18.49±1.30	——	——
Sesame Oil[209]		263	135	——	47	——	——
Sesame Oil[210]		321.5 ± 46.3	88.0 ± 3.8	——	41.8 ± 2.7	——	<LLOQ
Olive 1[208]	Free	70.3 ±1.18	2.2 ±0.59	——	1.6 ±0.33	——	nd*
	Esterified	52.0 ±2.23	2.1 ±1.16	——	1.1 ±0.11	——	nd*
Olive 2[208]	Free	74.0 ±1.18	2.3 ±0.59	——	1.4 ±0.33	——	nd*
	Esterified	55.2 ±2.23	2.0 ±1.16	——	0.9 ±0.11	——	nd*
Olive, extra virgin[208]	Free	105.5 ±1.18	3.4 ±0.59	——	0.9 ±0.33	——	nd*
	Esterified	27.1 ±2.23	1.1 ±1.16	——	nd*	——	nd*
Olive oil refined[202]		133	5.6	——	2.7	——	<1
Olive Oil extra virgin[203]		120	5.9	——	1.5	——	<1
Olive Oil[122]		120.9 ± 3.0	5.42 ± 0.16	——	2.36 ± 0.07	——	<LLOQ

Table continued

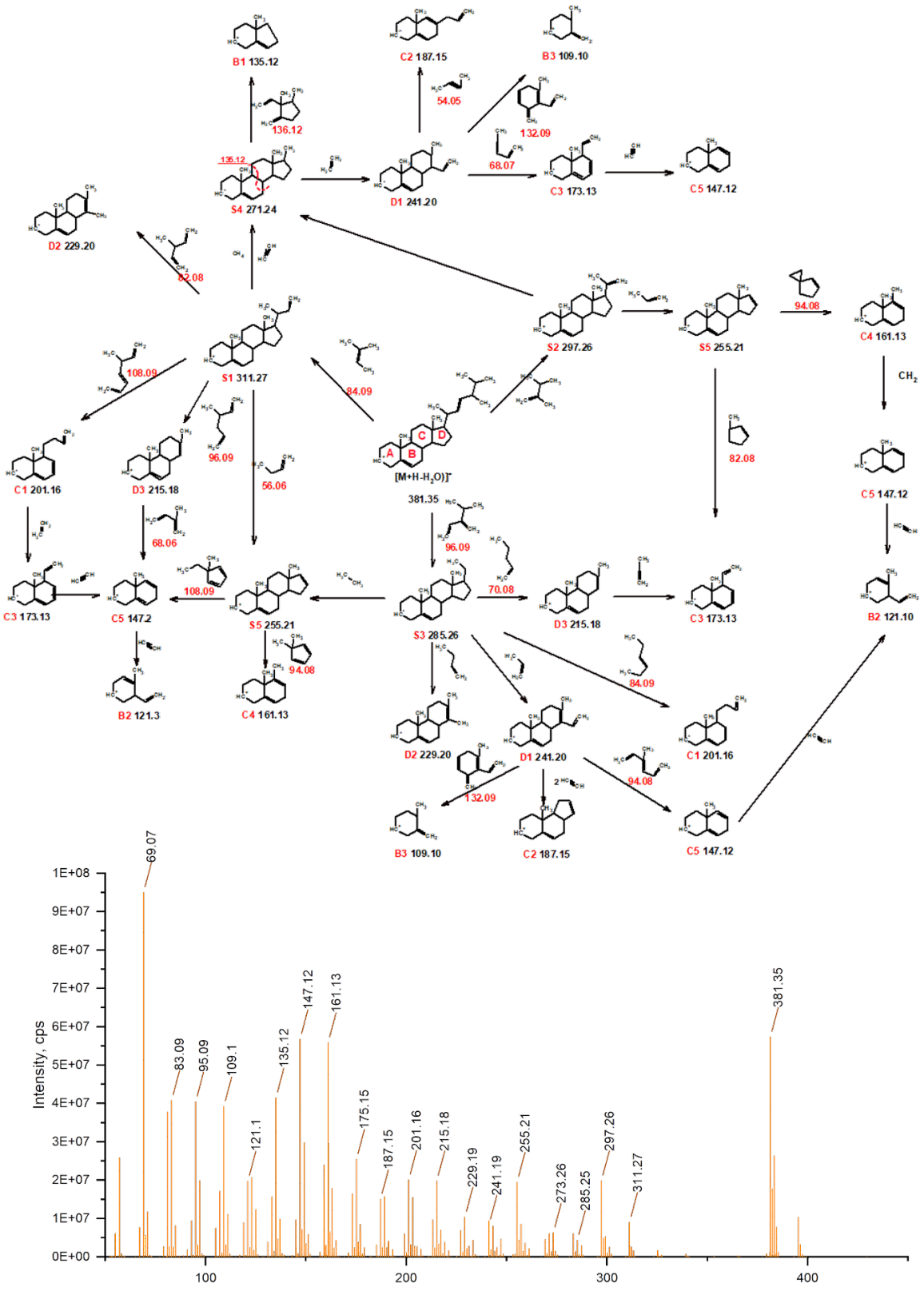
	δ5-Avenasterol	δ7-Avenasterol	Sitostanol	Campestanol	Cholesterol	24-Metylencholesterol
Sesame, extra virgin[208]	24.5 ±0.28	——	1.1 ±0.09	1.6 ±0.28	nd*	92.24±13.58
	26.9 ±1.16	——	0.7 ±0.13	nd*	nd*	——
Sesame, toasted[208]	22.8 ±0.28	——	1.1 ±0.09	1.1 ±0.28	nd*	——
	30.6 ±1.16	——	0.9 ±0.13	nd* ±0.18	nd*	——
Roasted Sesame Oil[199]	20.3 ± 1.6A	9.3 ± 1.1A	——	65.6 ± 5.4A	——	——
Crude Sesame Oil[200]	70.6 ± 0.3	8.4 ± 0.2	——	——	0.0 ± 0.0	——
Sesame Oil[201]	50.76±12.86	5.09±1.99	——	7.19±3.26	0.40±0.01	
	51.86±2.89	4.98±4.98	——	7.03±0.33	0.57±0.30	9.89±1.41
Sesame Oil[202]	47	6.8	——	3.1	5.1	12
Sesame Oil[203]	——	——	——	——	——	——
Sesame Oil[209]	82	——	4	2	——	——
Sesame Oil[210]	——	——	——	——	——	——
Olive 1[208]	6.7 ±0.28		1.8 ±0.09	0.7 ±0.28	<0.5 ±0.25	——
	9.4 ±1.16		1.6 ±0.13	nd*	<0.5 ±0.08	——
Olive 2[208]	7.7 ±0.28		1.5 ±0.09	0.7 ±0.28	<0.5 ±0.25	——
	9.5 ±1.16		1.3 ±0.13	nd*	<0.5 ±0.08	——
Olive, extra virgin[208]	15.2 ±0.28		0.9 ±0.09	0.7 ±0.28	<0.5 ±0.25	——
	6.6 ±1.16		0.9 ±0.13	nd*	<0.5 ±0.08	——
Olive oil refined[202]	19	1.2	5	<1	nd*	60
Olive Oil extra virgin[203]	23	<1	3.9	<1	nd*	54
Olive Oil[122]	——	——	——	——	——	——



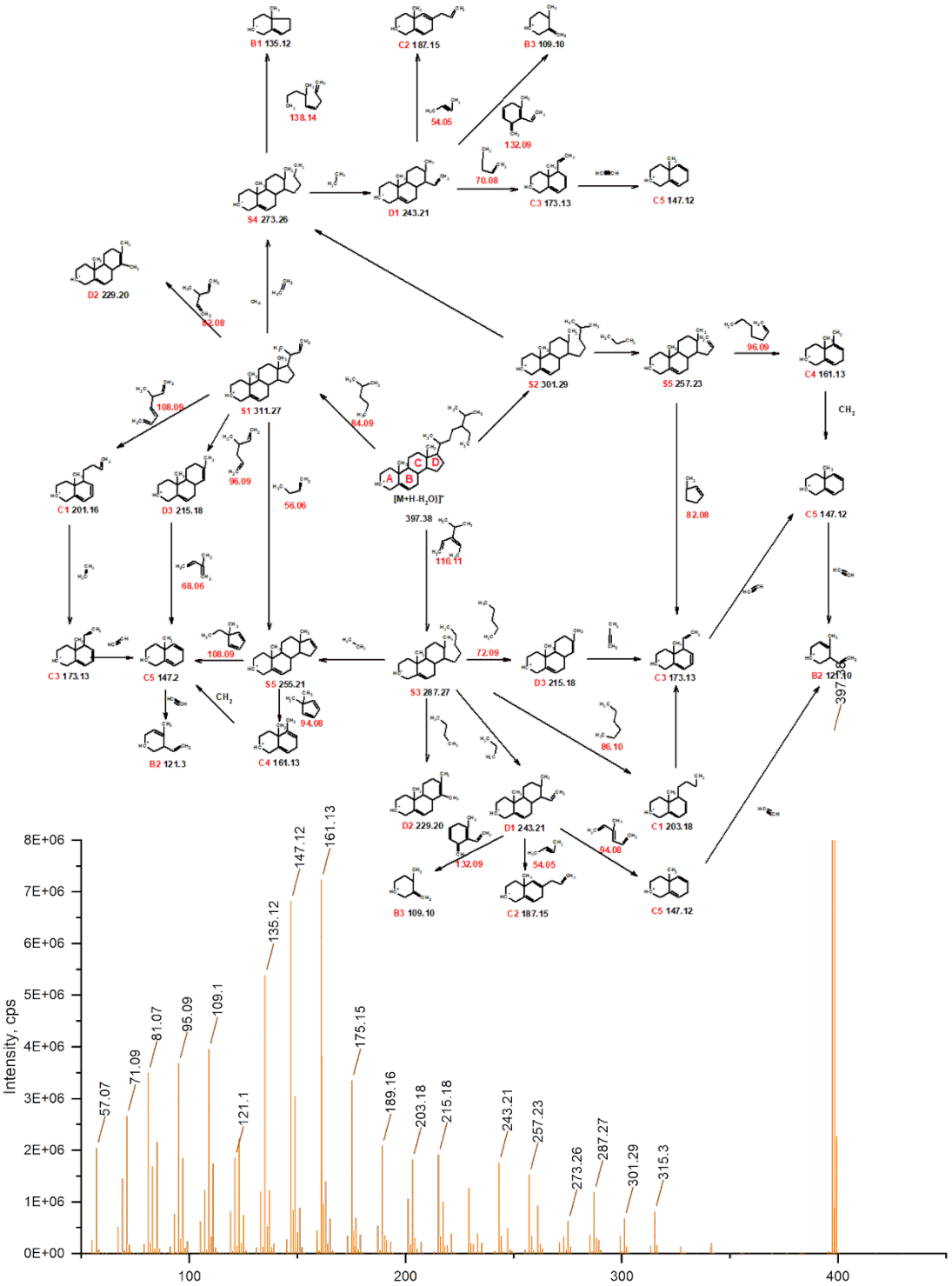
The comparison of APCI (A) and ESI (B) ionization for tested phytosterols during direct infusion.

Appendix B

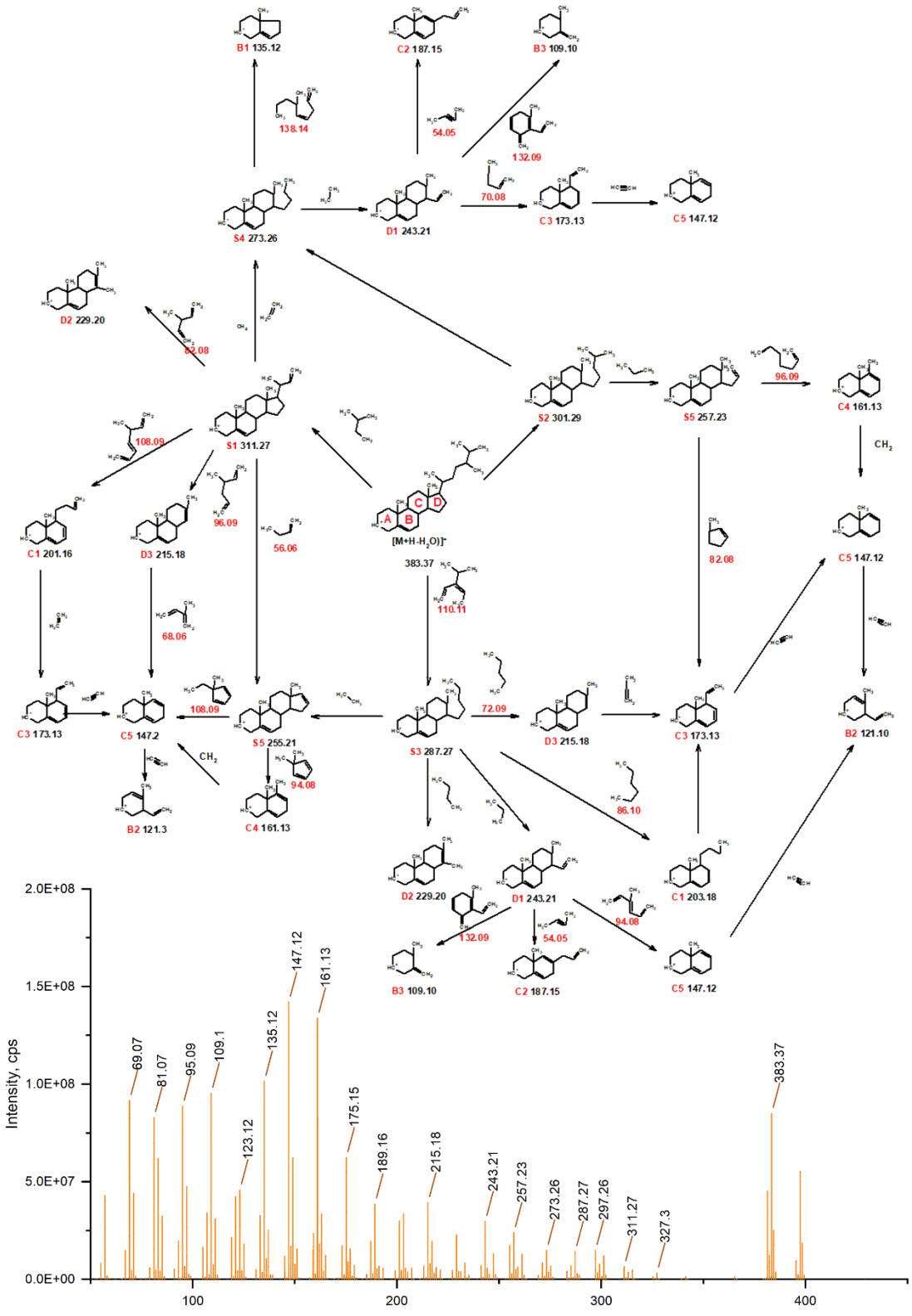
THE FRAGMENTATION PATTERNS AND SPECTRA OF TESTED PHYTOSTEROLS AND TOCOPHEROLS IN APCI-MS/MS ANALYSIS



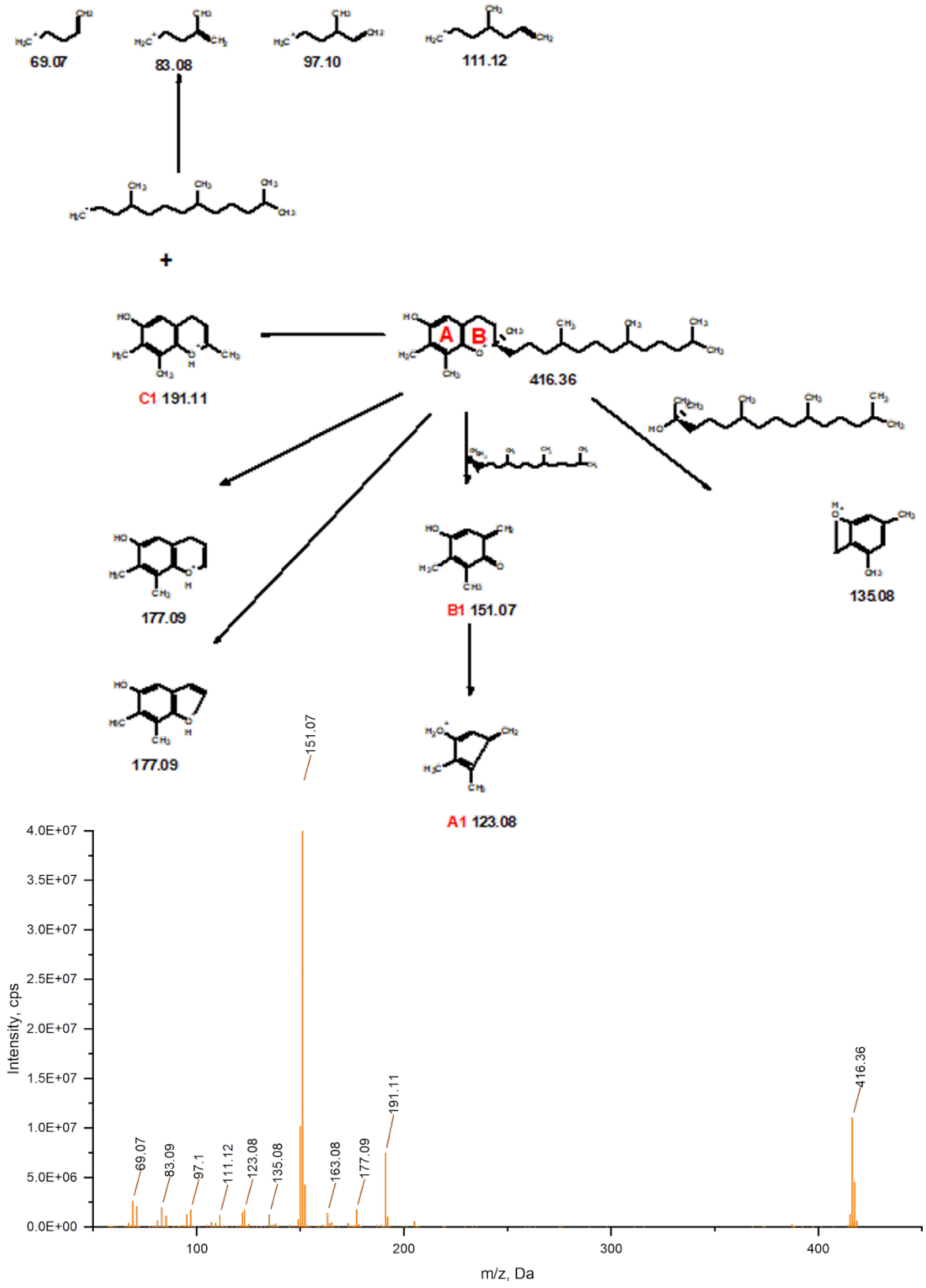
Scheme 1. APCI-MS/MS fragmentation pattern of $[M+H-H_2O]^+$ of brassicasterol



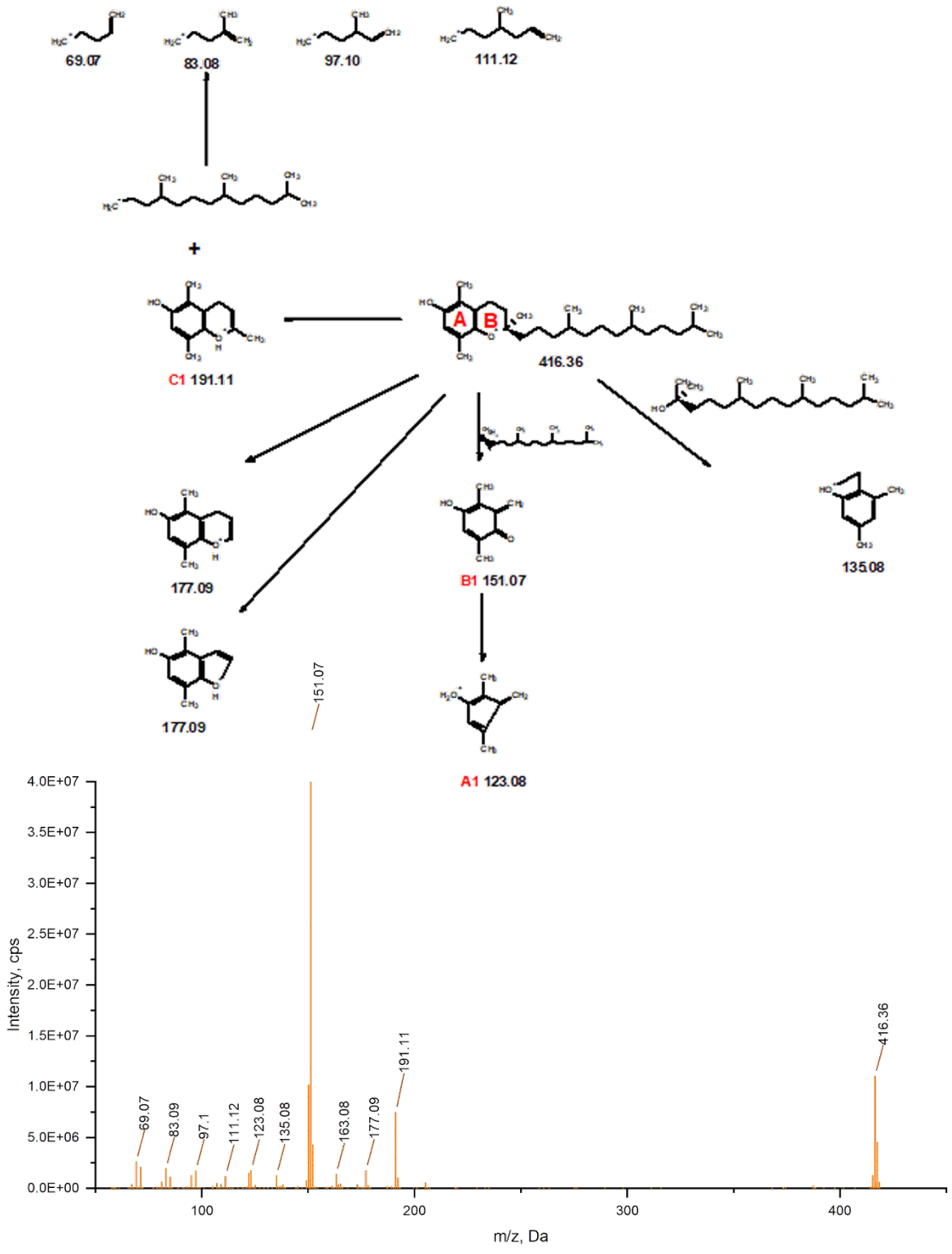
Scheme 2. APCI-MS/MS fragmentation pattern of $[M+H-H_2O]^+$ of β-sitosterol



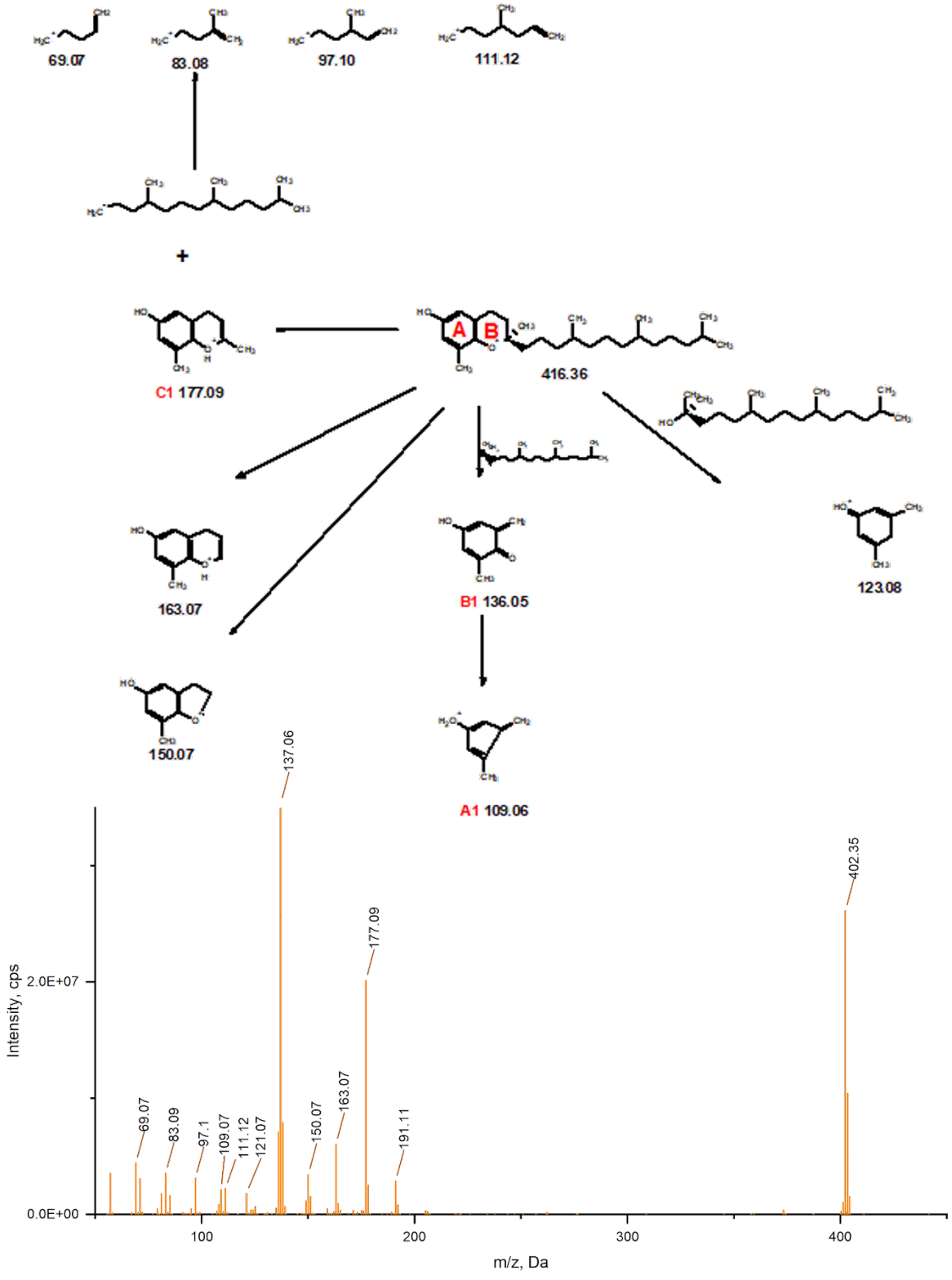
Scheme 3. APCI-MS/MS fragmentation pattern of $[M+H-H_2O]^+$ of campesterol



Scheme 4. APCI-MS/MS fragmentation pattern of $[M]^{\cdot+}$ of β-tocopherol



Scheme 5. APCI-MS/MS fragmentation pattern of $[M]^{\cdot+}$ of γ-tocopherol



Scheme 6. APCI-MS/MS fragmentation pattern of $[M]^{\cdot+}$ of δ-tocopherol

Additional Information

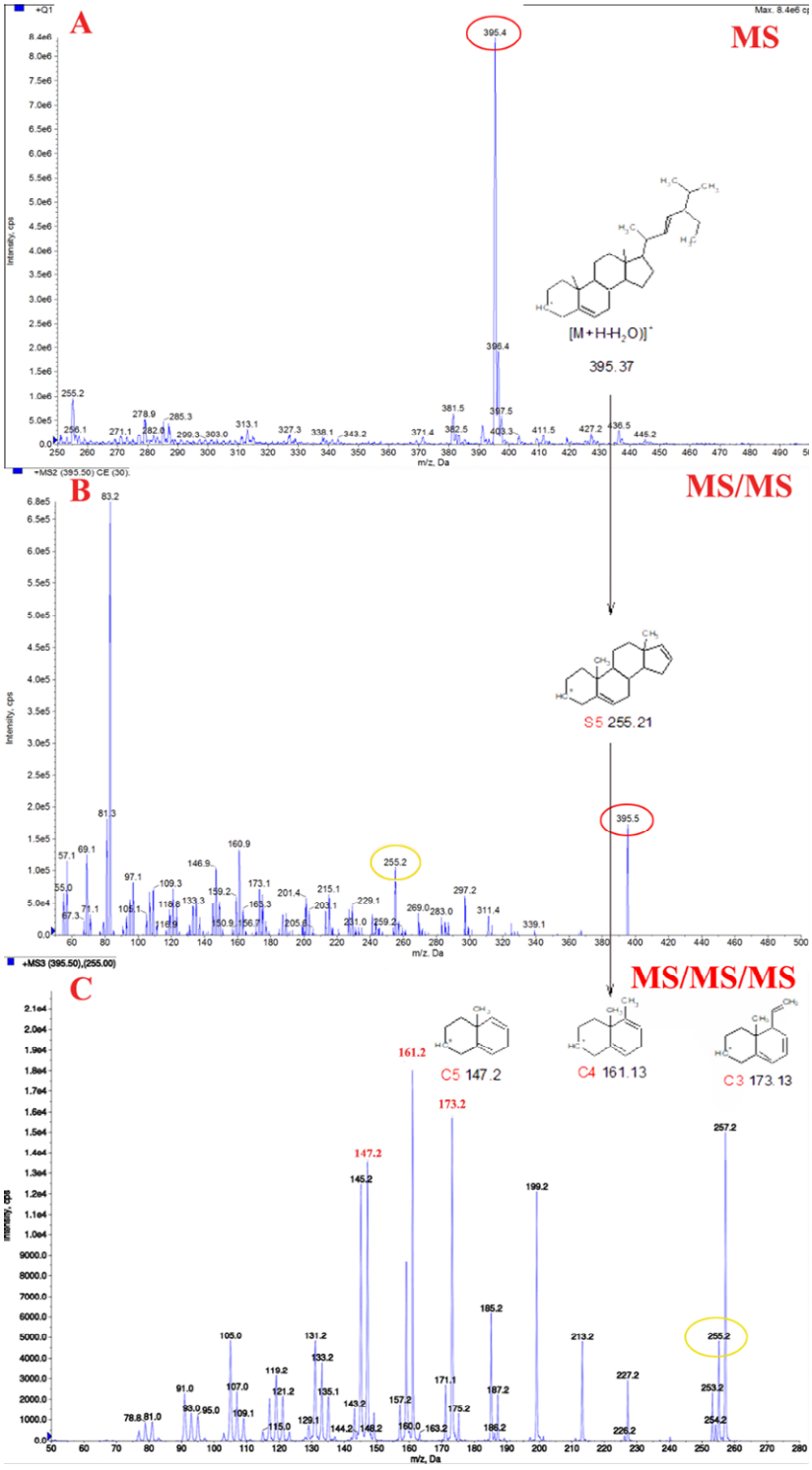
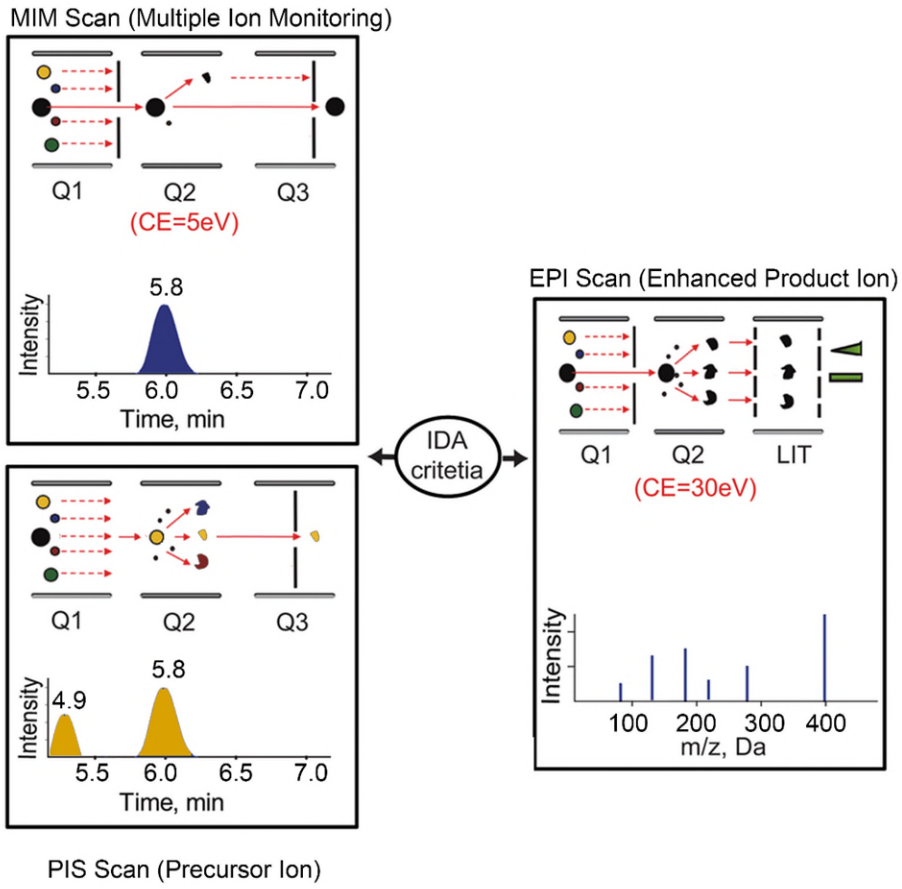


Figure 1. An example of MS^3 analysis of stigmasterol. A. MS spectra of $[M+H-H_2O]^+$; B. MS/MS spectra of $[M+H-H_2O]^+$; C. MS/MS/MS spectra of product ion *m/z* 255.2.



Scheme 1. The Main Procedures for PIS- and MIM-EPI -Based phytosterols and tocopherols profiling.